KIDNEY TRANSPLANT REJECTION

Diagnosis and Treatment
Third Edition, Revised and Expanded

edited by

Lorraine C. Racusen
Kim Solez
James F. Burdick

Kidney Transplant Rejection

M. Dekker 1998

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Lorraine C. Racusen

The Johns Hopkins University School of Medicine
Baltimore, Maryland

Kim Solez

University of Alberta
Edmonton, Alberta, Canada

James F. Burdick

The Johns Hopkins University School of Medicine
Baltimore, Maryland

MARCEL DEKKER, INC. NEW YORK · BASEL

Library of Congress Cataloging-in-Publication Data

Kidney transplant rejection: diagnosis and treatment / edited by Lorraine C. Racusen, Kim Solez, James F. Burdick. 3rd ed., rev. and expanded.
p. cm.
Includes bibliographical references and index.
ISBN 0-8247-0139-9 (alk. paper)
1. KidneysTransplantationComplications. 2. Graft rejectionPrevention. 3. Immunosuppressive agents. 4. Transplantion immunology. I. Racusen, Lorraine C. II. Solez, Kim. III. Burdick, James F.
[DNLM: 1. Kidney Transplantation. 2. Graft Rejection. WJ 368 K455[DNLM: 1. Kidney Transplantation. 2. Graft Rejection. WJ 368 K455 1998]
RD575.K5 1998
617.4'610592dc21
DNLM/DLC
for Library of Congress 98-15516
CIP

This book is printed on acid-free paper.

Headquarters
Marcel Dekker, Inc.
270 Madison Avenue, New York, NY 10016

tel: 212-696-9000 ; fax: 212-685-4540

Eastern Hemisphere Distribution
Marcel Dekker AG
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
tel: 44-61-261-8482; fax: 44-61-261-8896

World Wide Web
http://www.dekker.com

The publisher offers discounts on this book when ordered in bulk quantities. For more information, write to Special Sales/Professional Marketing at the headquarters address above.

Current printing (last digit):
10 9 8 7 6 5 4 3 2

PRINTED IN THE UNITED STATES OF AMERICA

PREFACE

In recognition of recent rapid advances in the field of transplantation, we have undertaken a new and updated third edition of *Kidney Transplant Rejection.* Both previous editions of the book have been very well received by reviewers and colleagues alike. In this, as in previous editions, the text is focused on basic immunological principles, mechanisms of rejection, diagnostic modalities, infections in the transplant setting, and clinical treatment of renal allograft rejection. All chapters have been contributed by recognized experts in the field of renal transplantation.

The introductory part, covering the biology of the allograft response, has been expanded from previous editions. Basic chapters on the molecular basis of transplantation immunity, cell-mediated and antibody-mediated rejection, cytokines and the immune response, anti-idiotype responses, and immunological tolerance have been replaced by completely new or extensively updated text. New chapters include discussions of renal injury and preservation, mechanisms of chronic rejection, and chimerism and xenografting, all important new areas in which knowledge is expanding rapidly.

In the area of rejection diagnosis, the most up-to-date version of the Banff schema, widely used for the pathological assessment and grading of renal allograft rejection, is provided, and new molecular markers which may enhance diagnostic accuracy are discussed. Fine-needle aspiration cytology and radiologic modalities in the monitoring of the renal allograft are discussed in new and revised chapters.

An important component of the book is a series of updated chapters on immunosuppressive therapy. In this part are included chapters on basic biology, mechanisms of action, and results of clinical testing of antilymphocyte antibody, other agents currently in use, including cyclosporine, FK506, and mycophenolate mofetil, and those being developed and in early clinical testing, including brequinar, rapamycin, and 15-deoxy-spergualin. Discussions include clinical dosing and use of these agents individually and in combination. Immune monitoring of renal allograft recipients is also considered.

In the final part, chapter authors address the systemic complications of immunotherapy. Included are up-to-date discussions of cancer, cytomegalovirus, Epstein-Barr virus, and hepatitis in the renal allograft population.

We feel that this book presents a very contemporary discussion of these important

issues and will provide a useful reference for the many professionals involved in experimental and clinical aspects of renal allografting.

LORRAINE C. RACUSEN
KIM SOLEZ
JAMES F. BURDICK

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CONTRIBUTORS

Khalid Al-Fakeeh, M.D. Department of Pediatrics, University of Toronto, and Hospital for Sick Children, Toronto, Ontario, Canada

William M. Baldwin III, M.D., Ph.D. Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Hallgrimur Benediktsson, M.D. Department of Pathology, University of Calgary, and Foothills Hospital, Calgary, Alberta, Canada

William E. Beschorner, M.D. Ximerex, Inc., and Transplantation Section, Department of Surgery, University of Nebraska Medical Center, Omaha, Nebraska

Jeffrey Bluestone Ben May Institute, University of Chicago, Chicago, Illinois

James F. Burdick, M.D. Division of Transplantation and Vascular Surgery, Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, Maryland

George W. Burke, M.D. Division of Transplantation, Department of Surgery, University of Miami School of Medicine, Miami, Florida

Gaetano Ciancio, M.D. Division of Transplantation, Department of Surgery, and Department of Urology, University of Miami School of Medicine, Miami, Florida

Robert B. Colvin, M.D. Harvard Medical School and Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts

A. Benedict Cosimi, M.D. Harvard Medical School and Transplantation Unit, Department of Surgery, Massachusetts General Hospital, Boston, Massachusetts

Francis L. Delmonico, M.D. Harvard Medical School and Transplantation Unit, Department of Surgery, Massachusetts General Hospital, Boston, Massachusetts

Violet Esquenazi, Ph.D. Division of Transplantation, Department of Surgery, University of Miami School of Medicine, Miami, Florida

Neil Ferguson, M.D. Department of Medicine, University of Toronto at St. Michael's Hospital, Toronto, Ontario, Canada

Edward K. Geissler, Ph.D. Department of Medical Technology, University of South Alabama, Mobile, Alabama

Nelson Goes, M.D. Department of Medicine, Allegheny University Hospitals, MCP, Philadelphia, Pennsylvania

Jens Goebel, M.D. Section of Pediatric Nephrology, Department of Pediatrics, Tulane University Medical Center, New Orleans, Louisiana

Philip F. Halloran, M.D., Ph.D., F.R.C.P.(C) Division of Nephrology and Immunology, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

Douglas W. Hanto, M.D., Ph.D. Division of Transplantation, Department of Surgery, University of Cincinnati Medical Center, Cincinnati, Ohio

James Hildreth, M.D. The Johns Hopkins University School of Medicine, Baltimore, Maryland

Barry D. Kahan, M.D., Ph.D. Division of Immunology and Organ Transplantation, Department of Surgery, University of Texas Medical School at Houston, Houston, Texas

Bertram L. Kasiske, M.D., F.A.C.P. University of Minnesota School of Medicine and Division of Nephrology, Hennepin County Medical Center, Minneapolis, Minnesota

Thomas J. Lomis, M.D. The John Wayne Cancer Institute, Santa Monica, California

Sing Leung Lui, M.B.B.S., M.R.C.P., F.H.K.C.P. University Department of Medicine, Queen Mary Hospital, Hong Kong, China

Robin P. Lowry, M.D. Division of Nephrology, Department of Medicine, University of South Alabama, Mobile, Alabama

Joshua Miller, M.D. Division of Transplantation, Department of Surgery, University of Miami School of Medicine, Miami, Florida

Cynthia C. Nast, M.D. Department of Pathology, UCLA School of Medicine, UCLA Medical Center, and Cedars-Sinai Medical Center, Los Angeles, California

Steen Olsen, M.D. Department of Pathology, University Institute of Pathology, Municipal Hospital, and Kommune Hospital, Aarhus, Denmark

Leendert C. Paul, M.D., Ph.D. Director, Renal Division, Department of Medicine, University of Toronto at St. Michael's Hospital, Toronto, Ontario, Canada

Lorraine C. Racusen, M.D., F.C.A.P. Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Anne Rosen, R.N. Division of Transplantation, Department of Surgery, University of Miami School of Medicine, Miami, Florida

David Roth, M.D. Division of Nephrology and Hypertension, Department of Medicine, University of Miami School of Medicine, Miami, Florida

Robert H. Rubin, M.D., F.A.C., F.C.C.P. Harvard-Massachusetts Institute of Technology Center for Experimental Pharmacology and Therapeutics, and Transplantation Unit, Massachusetts General Hospital, Boston, Massachusetts

Bernhard Ryffel, M.D. Transplantation Unit, Department of Immunology, University of Cape Town, Cape Town, South Africa

David H. Sachs, M.D. Harvard Medical School and Transplantation Biology Research Center, Department of Surgery, Massachusetts General Hospital, Boston, Massachusetts

Daniel R. Salomon, M.D. Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California

Marcus N. Scherer, M.D. Department of Medical Technology, University of South Alabama, Mobile, Alabama

Ferda Senel, M.D. Division of Immunology and Organ Transplantation, Department of Surgery, University of Texas Medical School at Houston, Houston, Texas

Sheila Sheth, M.D. Central Radiology, The Johns Hopkins Hospital, Baltimore, Maryland

Nozumi Shinozuka, M.D. Saitama Medical School, Saitama, Japan

Douglas P. Slakey, M.D., M.P.H. Section of Transplant Surgery, Department of Surgery, Tulane University Medical Center, New Orleans, Louisiana

Douglas M. Smith, Ph.D. Aastrom Biosciences, Inc., Ann Arbor, Michigan

Kim Solez, M.D. Department of Laboratory Medicine and Pathology, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada

Judith M. Thomas, Ph.D. Division of Immunobiology, Department of Surgery, University of Alabama at Birmingham, Birmingham, Alabama

John S. Thompson, M.D. Department of Internal Medicine, University of Kentucky, Lexington, Kentucky

James F. Whiting, M.D. Division of Transplantation, Department of Surgery, University of Cincinnati Medical Center, Cincinnati, Ohio

G. Melville Williams Vascular Surgery Service, The Johns Hopkins Hospital, Baltimore, Maryland

Charles G. Winans, M.D. Section of Transplantation, Department of Surgery, The University of Chicago, Chicago, Illinois

Keith Zucker, M.D. Division of Transplantation, Department of Surgery, University of Miami School of Medicine, Miami, Florida

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A Molecular Basis for Transplantation Immunity

DANIEL R. SALOMON

The Scripps Research Institute, La Jolla, California

In the past decade, there has been remarkable progress in the understanding of molecular mechanisms of transplantation immunology. The current view of the immune response begins in the extracellular space with more than 2 dozen cytokines, chemokines, and growth factors and a complex matching set of receptors. The release and levels of these soluble signaling molecules are tightly regulated, as are the expression and function of their receptors. At the cell surface is another array of costimulatory molecules and receptors that further shapes the nature and outcome of cell–cell communications. Across the cell membrane is an equally complex intracellular signal cascade that must determine the agenda of the cell in the face of many simultaneous and often conflicting messages. This intracellular signal cascade can activate cell surface receptors such as adhesion molecules and growth factor receptors, induce the release of cytokines, trigger progression of the cell through the cell cycle to drive proliferation, or signal programmed cell death (e.g., apoptosis). Thus, intracellular signaling influences the evolution of extracellular events in a form of feedback communication. Perhaps most exciting is the growing understanding of the connections between extracellular and intracellular signaling, which allows exploration of the biological regulation of gene transcription and, thus, the central molecular basis of the response of the cell to states of health and disease.

At the bedside, clinical trials in transplantation have been undertaken using a new generation of molecules. These include novel, genetically engineered proteins and monoclonal antibodies, small molecular weight receptor antagonists, and unique peptide analogues. In

parallel, ground-breaking work is moving forward to induce transplantation tolerance and make xenotransplantation successful. The design and interpretation of such clinical trials will require a working understanding of the immune response at the molecular level.

In this chapter, the current knowledge of the complexity of immunity is translated into seven immunological paradigms to enhance a working understanding of clinical transplantation. In turn, such an understanding can help define the clinical potential of new reagents and direct the development of strategies for transplantation immunosuppression and tolerance induction. The paradigms to be discussed are as follows:

1. Antigen presentation, direct and indirect: implications of major histocompatibility complex (MHC) structure
2. Costimulation and the two-signal hypothesis for T-cell activation
3. Programmed cell death (apoptosis)
4. Immune deviation, cytokines, and the Th1/Th2 hypothesis
5. Adhesion molecules and the physics of the immune response
6. Intercellular and intracellular signal cascades
7. A three-dimensional view of immunity

Many aspects of these paradigms are still unknown or disputed. The connections made in this chapter between these paradigms and clinical events are often speculative. Thus, the process of describing the paradigms and creating a set of unifying concepts is, at best, only work in progress. The authors of the subsequent chapters add the rich details of their expertise and bring these concepts into sharper focus.

I. ANTIGEN PRESENTATION, DIRECT AND INDIRECT: IMPLICATIONS OF MHC STRUCTURE

The first step in the initiation of the immune response is the recognition of antigen. Two mechanisms of antigen presentation, direct and indirect, are directly relevant to transplantation (Figure 1). The relative contribution of each mechanism is a matter of great interest and importance (1–3).

Indirect antigen recognition involves antigen presenting cells (APCs) such as macrophages and dendritic cells. This is one of the central mechanisms of immunity. A recent Nobel prize in Medicine was given to two pioneers of indirect antigen recognition, Drs. Zinkernagal and Doherty, for their work in demonstrating how viral antigens are processed and presented to killer T cells (4). The designation of indirect refers to the fact that a patient's APC serves as an intermediary between the reacting T-cell clones and the transplanted donor cell. Indirect antigen presentation by the APC involves at least five distinct steps:

1. Uptake of the foreign antigen into intracellular proteolysosomes
2. Digestion of the whole antigen into peptide fragments
3. Selection of only a subset of immunogenic peptides
4. Placement of these peptides into the antigen presenting groove of the MHC molecules (i.e., the human leukocyte antigens [HLAs])
5. Transport of the MHC–peptide complex to the cell surface for presentation to the T cells

A sixth step to consider is how the patient's APCs make contact with the antigen in the transplant. This could be by physical contact with the antigen expressed on the trans-

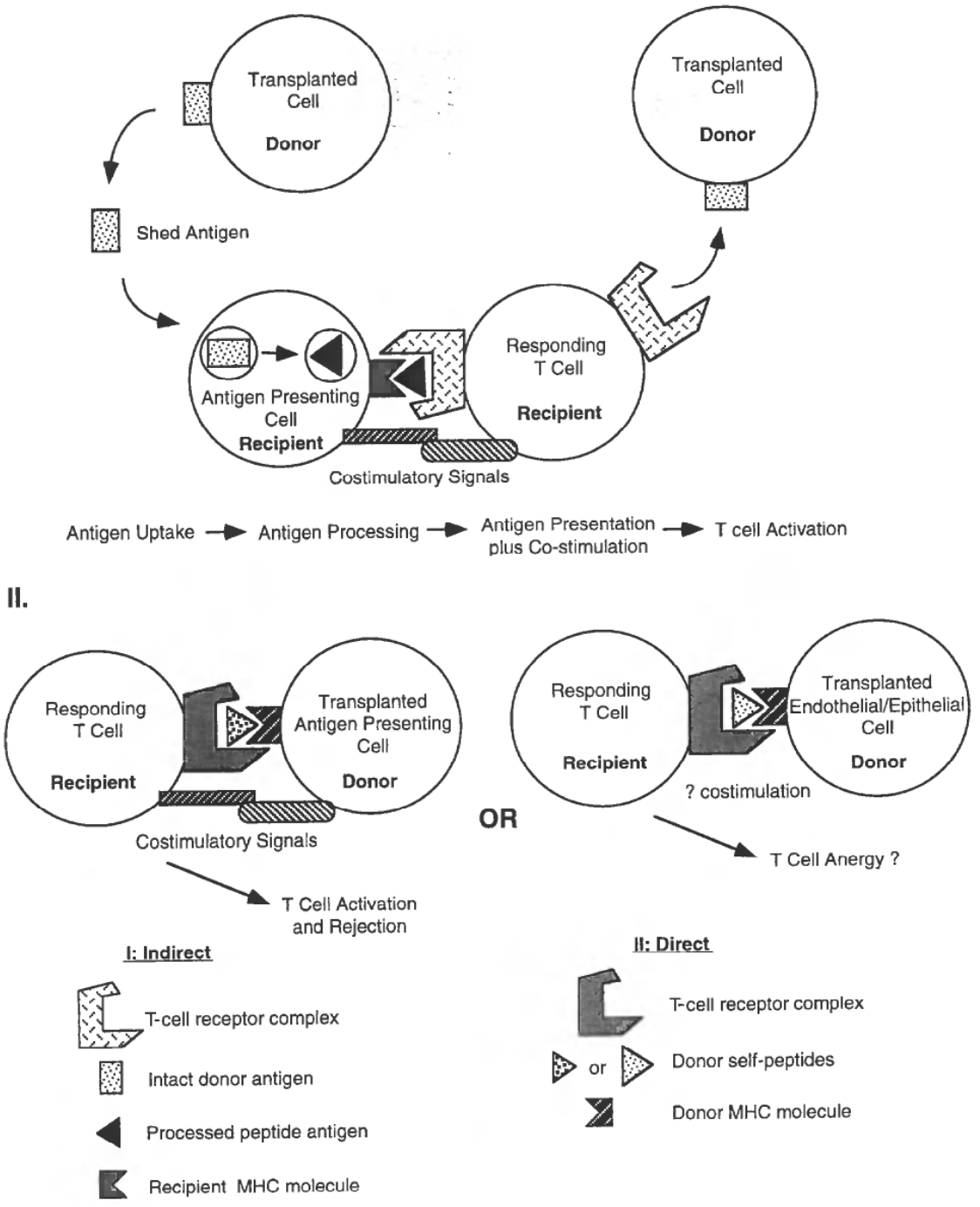

Figure 1 I. Indirect antigen presentation in which processing by host antigen presenting cells leads to presentation of antigenic peptides in the context of self-MHC. II. Direct antigen presentation in which host T cells respond directly to donor antigenic peptides presented by donor-MHC molecules.

planted cells. For example, the APC could traffic to the transplant and physically bind donor antigen on the vascular surface or within the tissue. Donor cells could also traffic out of the transplant and interact with the patient's APCs in the lymph nodes or spleen. This type of donor antigen exposure is one basis of the "passenger leukocyte" theory. Alternatively, the patient's APC can obtain donor antigen by binding soluble antigenic proteins released into

the circulation by donor cell injury, death, or local inflammatory responses. This mechanism could be involved in the setting of acute vascular rejection or could be a consequence of ischemic injury during procurement. The implications of this sixth step are considered further in the adhesion molecule paradigm (see Sec. V).

Where does indirect antigen presentation occur? Indirect antigen presentation can occur in the lymph nodes or spleen (peripheral) or possibly in the thymus (central). It is also likely that indirect antigen presentation occurs in the transplant (local). The cold preservation and warm ischemic injury that occur in transplantation, particularly with cadaver donors, also sets into motion a number of local proinflammatory mechanisms that enhance adhesion and accumulation of APCs in the transplant itself. Under certain circumstances, B cells (5) and the endothelium (6) can also present antigen. Thus, some immunologists distinguish dendritic cells and macrophages as professional antigen presenting cells while implying that B cells and endothelial cells are the amateurs (7) and are less efficient at antigen presentation. This important distinction and its shortcomings are discussed in the next section in the context of the two-signal model of cell activation (see Sec. II).

The elucidation of the basic structure of the MHC molecule was a major scientific advance (8–10). This has allowed visualization of a three-dimensional structure at the top of the MHC molecule called the antigen presenting groove, into which the immunogenic peptide is bound. The structure or sequence of each MHC molecule creates a groove that binds only certain peptides efficiently. Thus, every MHC molecule has a different groove and each can theoretically present a different set of peptides. This correlation between MHC structure and the presentation of specific peptides helps explain how certain diseases such as diabetes mellitus may be linked to specific MHC molecules. It may also explain why certain families have a much higher incidence of autoimmune diseases such as systemic lupus erythematosus or glomerulonephritis; these genetic links to disease may be explained by the inheritance of MHC molecules that favor the presentation of peptides that trigger an autoimmune response. If this thinking is extended to transplantation, it provides one theory for the clinical experience that some patients never reject their grafts whereas others reject their grafts no matter how intense the immunosuppression.

Intracellular proteolysosomes are organelles in which protein antigens ingested by APCs are lysed and processed for presentation. Another feature of antigen processing is that a number of structural or transport proteins in the proteolysosome are encoded by genes located in the same chromosomal region as the histocompatibility genes. Thus, inherited differences in these antigen processing genes are linked to MHC alleles and may also regulate which antigenic peptides are selected for presentation in the groove of MHC molecules. The final result of antigen processing is that the APC breaks down a very large foreign protein into many peptide fragments in the proteolysosome, yet apparently selects only a relatively small subset of immunogenic peptides for presentation to T cells. Although this is not proven for transplantation, it is likely to be true. Via this process, a specific set of peptides are selected that the activated T cells can efficiently recognize when they subsequently encounter the intact foreign protein.

How can our current understanding of indirect antigen presentation suggest a strategy to create tolerance? Starting with a central approach via manipulating the thymus, note that T-cell development in the thymus has two objectives: (1) eliminate T-cell clones that recognize self antigen, and (2) select T-cell clones that efficiently recognize foreign antigens (nonself) (11,12).

Each person inherits a given set of MHC molecules whose antigen presenting grooves are designed to present a finite set of potential immunogenic peptides. The same

MHC molecules present self-MHC in the thymus and foreign antigenic peptides in the periphery. Thus, there should be a common theme or structural motif for each person which governs the types of self and foreign peptides that can be presented. Over the past 10 years, the gene and protein sequences of most of the MHC molecules have been determined, so that the structures of their antigen presenting grooves can be predicted. This information can be used to construct three-dimensional models of MHC–peptide complexes using advanced computer programs. Work is being done to understand the rules imposed by MHC groove structure on peptide presentation. For example, a negatively charged amino acid in one end of the antigen presenting groove favors binding of a peptide with a positively charged residue. Other scientists are synthesizing large libraries of peptides and screening these peptides for MHC binding. The goal of all of this work is to elucidate the rules linking MHC structure to peptide presentation.

When this "code" is broken, it will be possible to choose a set of peptides for a specific set of MHC molecules (13) and take the next step toward rational tolerance induction. Theoretically, if the peptides presented in the thymus can be manipulated, the outcome of T-cell selection could be selectively manipulated. Thus, if the MHC of the recipient and the donor is known, the patient could be given peptides chosen to fool developing T cells into thinking that the donor transplantation antigens are self. There is potential for such a strategy in treating autoimmune disease if the self-reactive antigen can be identified and sequenced. Alternatively, a similar peptide strategy could be used to selectively inhibit antigen presentation in the peripheral lymphoid system or locally in the transplant. That would be accomplished by either (1) using immunogenic peptides matched to donor HLA sequences to tie up or inhibit free T-cell receptors or (2) using peptides that bind the patient's own HLA molecules with such high affinity that they will compete with or even displace the foreign donor HLA peptides before an immune response can be triggered by presentation.

The second type of antigen presentation in transplantation is direct antigen recognition, which bypasses the requirement for processing donor antigen by host APC; the T-cell antigen receptor of the patient responds directly to the alloantigen peptide–MHC complex expressed on the transplanted donor cell surface (14,15). An antigenic peptide is still involved, but that peptide is chosen by the donor cell and is presented in the groove of the donor's MHC molecule. In other words, the peptide is actually a donor self peptide. An example of direct antigen recognition in transplantation is the interaction of the patient's T cells with the endothelial cells of the transplant, leading to acute vascular rejection. Alternatively, the patient's T cells may be confronted with alloantigen "presented" by the transplant donor APCs that are known to come with the organ as "passenger leukocytes." Experimental data in rodent transplant models show that removal of donor leukocytes from the transplanted organ can reduce and even eliminate the risk of rejection. One interesting feature of this interaction with donor APCs is the implication that the population of peptides carried in the groove of the MHC molecule of an APC are likely to be different than the peptides carried in the groove of the same MHC molecules expressed by endothelial or epithelial cells. Furthermore, the process of ingesting, processing, and presenting the alloantigen by the host APC is not a passive process and affects the activation state and cell surface molecule expression of the APC. In contrast, the donor APC expresses its normal MHC molecule, which has suddenly become alloantigen by virtue of transplantation. Thus these different routes of direct versus indirect alloantigen presentation may have important implications for the immune responses that evolve subsequently (see Sec. II). Many researchers believe that direct antigen recognition is the predominant form of transplant antigen presentation. This is supported by in vitro evidence that direct antigen recognition

is functional. Moreover, the prediction that direct antigen presentation would result in activation of a much larger number of different T-cell clones than indirect antigen presentation has been confirmed with T cells cloned from rejection infiltrates (16).

Direct recognition assumes that the patient's T-cell antigen receptor is flexible enough to recognize the donor's self antigenic peptides in the groove of the donor's allogeneic MHC molecules. The immune system has evolved to deal with self and foreign antigens presented by its own body's cells, not to deal with transplanted cells. Thus, the portions of the structure of the T-cell antigen receptor that mediate MHC molecule binding are similar comparing various individuals in the population. The big differences are in the highly variable regions of the T-cell antigen receptor that interact with the antigenic peptide and probably only a few key amino acids flanking the peptide on the presenting MHC molecule. These conclusions regarding structure and function are directly supported by the recent elucidation of the x-ray crystallographic structure of the T-cell antigen receptor, demonstrating its orientation to the MHC molecule and the peptide binding groove (17).

Direct recognition implies that the donor's self peptides can be a key component of the "antigen" being recognized. This is a fascinating molecular insight into the unique nature of the transplant immune response. For example, before organ harvesting, a heart cell in the donor would have a specific set of self-derived peptides in the grooves of its MHC molecules. Because of thymic selection, these self peptides do not induce an immune response by the donor's T cells. However, immediately after transplantation, this same heart cell is suddenly the target for the recipient's T cells. One key question is whether the self peptides in the groove of the donor's heart cell are different than the self peptides that were in the groove of the recipient's own heart cells. If every MHC molecule tends to present a different set of peptides based on the structure of its binding groove, then the transplanted heart cell should present a different set of self peptides than the recipient's heart cell, providing the MHC matching is not perfect. If so, the patient's T cells should readily recognize the allogeneic target cell and its self-peptide–MHC complex as nonself. Many of the peptides eluted from MHC molecules are derived from intracellular proteins (18,19). Therefore, at least simplistically, two structurally identical heart cells could have many of the same self-peptides if these peptides are derived from mitochondrial proteins or intracytoplasmic enzymes characteristic of a heart cell. However, there are many different alleles of enzymes in the population, and these allelic differences may actually serve to delineate individuals for the transplant immune response as effectively as MHC molecule differences. Such non-MHC determined differences may be one explanation for the fact that transplantation across racial barriers despite good MHC matching is still associated with higher rejection rates and poorer long-term graft survival. It would also explain why the effect of MHC matching on rejection and long-term graft survival is not as great as would be expected if the only difference recognized was simply MHC structure.

The next important molecular insight is that direct antigen recognition may not be as efficient as indirect recognition. The definition of efficiency in the context of antigen presentation and immunity is important to consider for transplantation. The arguments develop as follows. The job of the thymus is to select only those T-cell receptors that have the best match for efficient peptide recognition by testing them with self MHC. "Efficient" in this context means that mature immune responses to foreign or altered self antigens will be productive and controlled to achieve rapid clearance of any challenge, yet protect the organism from the risk of an autoimmune backlash. In other words, the system uses efficiency to balance the immune response. The structure of a T-cell antigen receptor determines its avidity or antigen-binding capability. During early T-cell development in the thymus, the immature T cells rearrange the germ line sequences of the two chains of the antigen receptor to cre-

ate millions of combinations. In the next step, called thymic selection, all of these T-cell receptor combinations are sorted and tested for recognition of self-peptides presented by self-MHC molecules. These self-MHC–peptide complexes are presented by thymic epithelial cells in the cortex and bone marrow–derived dendritic cells in the medulla; the role of medullary epithelial cells in selection is less clear. The objective of thymic selection is to kill any T cells whose antigen receptors have either too low an affinity or too high an affinity for self-MHC. If T-cell antigen receptor affinity is too low, then antigen recognition is too inefficient to protect against infection. On the other hand, if affinity is too high, it would be difficult to regulate the immune response, possibly resulting in overreactions or autoimmunity.

More than 90% of all T cells produced in the thymus die there. Thus, the T-cell receptors of circulating T cells have been carefully selected to work optimally with the body's own MHC molecules. Therefore, this argument would suggest that the direct antigen recognition of nonself donor MHC–peptide molecules that occurs after transplantation should not be as efficient as antigen recognition via the indirect pathway in the context of self-MHC. These mechanisms of thymic T-cell receptor selection help explain why the T-cell immune response to donor antigen in transplantation can involve a much larger number of T-cell clones than any reaction to foreign or altered self-antigens presented indirectly by self-MHC. Many investigators have called attention to the fact that the transplant immune response is actually stronger than any normal immune response for this reason.

However, this definition of efficiency can work two ways in transplantation: as an advantage and as a disadvantage. HLA-matching patients and donors is difficult for most transplantations and current practice of matching is far from true matching at the molecular level. Thus, if the patient's T cells are inefficient at binding donor MHC–peptide complexes, the immune response may be weak, thus avoiding rejection and enabling long-term graft survival. Are these the patients who never have rejection and seem to need little or no long-term immunosuppression? Alternatively, other donors would present foreign MHC molecules to which the patient has a significant number of T-cell clones that have a very high affinity. If these clones are activated by direct recognition, it might be hard to suppress or autoregulate this too efficient response. Therefore, would these patients be at higher risk for rejection? Does this explain why some patients do not respond to antirejection therapies? Are these the patients prone to development of chronic rejection?

II. COSTIMULATION AND THE TWO-SIGNAL HYPOTHESIS FOR T-CELL ACTIVATION

A remarkable feature of the immune response is that the recognition of antigen is not sufficient to trigger the full activation of the T cell. In other words, the T-cell antigen receptor may determine the exquisite antigen specificity of a T-cell clone but it cannot produce T-cell activation without the participation of additional signaling mechanisms. Thus, a second or costimulatory signal must be delivered in collaboration with successful antigen engagement (Figure 2).

To understand this concept of costimulation in the context of T-cell activation, two phenomena must be explained:

1. First, the physical strength of the bond between the actual T-cell antigen receptor (TCR) heterodimer and the MHC molecule presenting an antigenic peptide is relatively weak, too weak to stabilize the binding of the T cell to the antigen presenting cell surface. This discovery led to the realization that the CD4 and CD8 molecules are associated with the TCR complex and also bind to the MHC molecule of the APC to create a bridge that

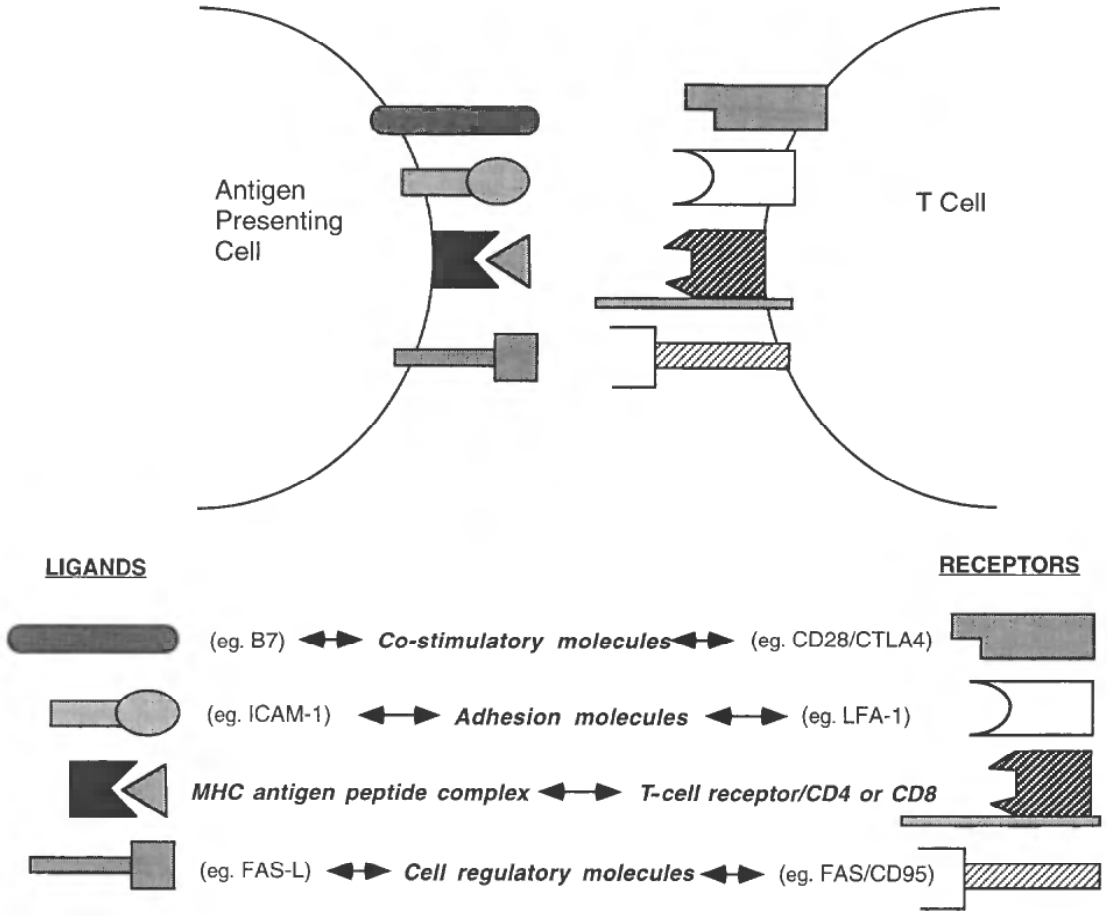

Figure 2 The nature of costimulation is that the T-cell antigen receptor is flanked by a set of receptor molecules with corresponding ligands on the surface of the antigen presenting cell. Engagement of one or more of these molecules is necessary for full activation of the T cell. Moreover, the engagement of these molecules literally shapes the outcome of T-cell interactions with antigen.

stabilizes the T cell–APC interaction. However, it quickly became clear that a large number of adhesion molecules are expressed by T cells and bind to various adhesive ligands expressed by APCs to stabilize cell–cell adhesion (Table 1). One key feature is that the expression of these ligands by APCs is also regulated by inflammatory cytokines such as interleukin (IL) 1, IL6, tumor necrosis factor (TNF), and the interferons. That explains why a cytokine-activated APC is much more efficient at T-cell activation. This is also relevant to cytokine-activated endothelial cells presenting antigen. Perhaps cytokine activation and increased adhesion sites may "even the playing field" for the amateur APCs.

2. Second, even though stable cell–cell adhesion is a critical step in T-cell activation, it is also not sufficient. These adhesion molecules trigger the activation of a complex series of cytoplasmic signaling pathways, and intracellular signals linked to cell surface adhesion events are required for expression of the full program of activation of the T cell. The signaling role of adhesion molecules was a major discovery and promises a whole new set of potential drug targets for immunosuppression.

In considering the terminology, are these adhesion molecules or costimulatory molecules? In one sense, these molecules are legitimately adhesion molecules. They function to physically stabilize the interaction of the T cell with APCs. However, these molecules are also an integral part of the cell signaling pathways, allowing these receptors to also be

Table 1 Adhesion/Costimulatory Molecules with Cellular Ligands

Receptor	Ligand
CD4	MHC class II
CD8	MHC class I
LFA-1 (CD11a)	ICAM-1 (CD54)
LFA-1 (CD11a)	ICAM-2 (CD102), ICAM-3
CD2	LFA-3 (CD58)
CD28, CTLA4	B7-1 (CD80)
CD28, CTLA4	B7-2 (CD86)
VLA4 ($\alpha 4\beta 1$)	VCAM-1 (CD106)
L-selectin (CD62L)	MadCam-1
P-selectin (CD62P)	Sialyl-LewisX
$\alpha 4\beta 7$	MadCam-1, VCAM-1
PECAM (CD31)	CD31
CD31	$\alpha v\beta 3$ (VNR)
CD45	Glycolipids
CD40	CD40 Ligand
CD44	Hyaluronate PLNad

termed costimulatory molecules. They mediate the active, signal-dependent stimulation of the T cell.

The concept of costimulation has several direct applications to clinical transplantation. If a T cell requires costimulatory signals in addition to the engagement of antigen by the TCR, it follows that one level of regulating the immune response is to control the setting in which the T cell encounters antigen.

A. Concept 1

What happens if the T cell encounters an APC expressing a low level of the costimulatory ligands? Antigen recognition (signal one) in the absence of costimulation (signal two) actually makes the T cell anergic (e.g., unresponsive) to further antigen exposure (20); the T cell does not die (see Sec. III). Even though the precise mechanism of T-cell anergy remains unknown, it is an exciting target for developing a novel immunosuppressive strategy. If all the T-cell clones capable of responding to donor HLA antigens are rendered anergic, the danger of rejection is eliminated.

The exposure of APCs to inflammatory cytokines increases the expression of costimulatory ligands and minimizes the possibility that any T cell will encounter antigen *without* an abundance of "second signal" present. This is exactly what the immune system is designed to do. Thus, when rejection or infection is present, the efficiency of antigen presentation and T-cell activation is substantially enhanced. This also occurs in the immediate postsurgical period, when wound healing demands a rich mileu of inflammatory cytokines. The primary biological function of the immune system is to protect the host in states of trauma, and an increased antigen presenting efficiency in such a situation is very useful. However, this may explain why rejection is most common in the first months after transplantation and why stable transplant patients with active viral infections may suddenly have acute rejection. Acute tubular necrosis (ATN) may also be considered a proinflammatory

state. Moreover, a brain-dead donor is often stressed by serious trauma, hypothermia, hemodynamic instability, or subclinical bacteremia resulting from multiple intravenous lines before organ retrieval. Thus, the association of increased rejection with delayed graft function may be due to cytokine-induced increases in costimulatory ligand expression in the graft. Support for this concept is found in the recent data that living unrelated donor transplantation results in significantly better graft survival and less rejection than that observed with cadaver donor organs, despite there being no differences in the degree of HLA matching between these two groups.

B. Concept 2

Antigen presenting cells are not created equal. APCs, such as dendritic cells and some macrophages, normally express higher levels of costimulatory ligands. These are often called "professional" APCs. Next in efficiency are the localized tissue macrophages and some circulating populations that have relatively lower levels of both MHC class II molecules and certain costimulatory ligands than dendritic cells; nonetheless, they are present in large numbers and can traffic rapidly to a site of inflammation. Finally, at the bottom of the APC ladder are B cells, endothelial cells, and even epithelial cells that can also be shown to present antigen under certain circumstances; these cells are normally relatively deficient in costimulatory ligands, may not process antigen readily, and have lower levels of MHC molecules.

In considering the implications of indirect and direct antigen presentation for costimulation, it is clear that direct antigen recognition, in which recipient T cells bind directly to donor antigen on transplanted endothelial or epithelial cells, triggers a different immune response than T-cell activation mediated by indirect antigen presentation via professional APCs. A strict application of the two-signal hypothesis to direct antigen recognition predicts that T-cell anergy, not activation, should result, because a donor endothelial cell, for example, has a very low level of costimulatory ligands (second signal). The explanation for why this is not the case in clinical transplantation may be the expression of inflammatory cytokines in the transplanted organ; perhaps in this circumstance the ability of nonprofessional APCs such as endothelial and epithelial cells to present antigen is enhanced by increased expression of costimulatory molecules.

Nonetheless, it is paradoxical that current understanding of this critical two-signal paradigm does not suggest that direct antigen recognition should be the primary mechanism for transplantation immune responses. This is a good example of why immunological theories must be applied to understanding clinical transplantation with care. However, any good theory has a way to explain an apparent paradox. What if direct antigen presentation involves the alloantigenic MHC–peptide complex carried on donor APCs rather than the donor's endothelial or epithelial cells? In this case, theoretically all the costimulatory molecules would be present and the immune response should result in T-cell activation and not anergy. Following this reasoning, it is possible that there is a dynamic balance in play during donor alloantigen recognition. T-cell activation and transplant rejection would be favored by T-cell interactions with either donor APCs (passenger leukocytes) or host APCs, which have ingested and processed donor alloantigen. On the other hand, T-cell anergy may occur by direct interactions with donor endothelium and epithelial cells in the absence of necessary costimulatory signals. The overwhelming majority of alloantigen in a transplanted organ is expressed on the surface of endothelial and epithelial cells, not passenger leukocytes. The effects of cytokines and other proinflammatory molecules released in the donor patient before cadaver organ retrieval may increase the risk of rejection by upregu-

lating the levels of donor MHC and costimulatory molecule expression. Because this proinflammatory state increases the traffic of circulating and activated leukocytes to the donor organ before harvesting, it is possible that the resulting increase in the donor APC population also plays a role in changing the balance in favor of T-cell activation and transplant rejection. A final twist is that a T cell could engage a foreign antigen directly on a donor epithelial cell but receive the necessary second signal from an adjacent APC, a process called "trans" signaling. A better understanding of these mechanisms of antigen presentation could lead to strategies for reducing the risk of rejection, including approaches to management of the donor patient before harvest.

C. Concept 3

Costimulatory ligands and their T-cell counterreceptors are not created equal. In the previous discussions, costimulatory ligands have been referred to in generic terms. In reality, the different ligands are each likely to have different roles in T-cell activation. This is equally true for the T cell receptors for these ligands.

For example, the costimulatory ligand intracellular adhesion molecule-1 (ICAM-1) is widely expressed on APC cell surfaces, including endothelium, even without cytokine activation. This is also true for the counterreceptor of the T cell, leukocyte function molecule-1 (LFA-1). In contrast, the CD28 and CTLA4 molecules on T cells and their APC ligands, B7-1 and B7-2, are differentially expressed and dependent on activation. One current theory is that APC activation may increase signaling through the CD28 pathway and favor T-cell activation and proliferation. In contrast, signaling through CTLA4 may favor T-cell anergy or at least provide a negative signal blocking IL-2 production and T-cell proliferation (21). Thus, the relative expression of these two T-cell receptors or their two ligands could be regulated as one means of controlling the immune response. Fully activated T cells express high levels of both CTLA4 and CD28. These two receptors may even compete for binding the two known ligands, B7-1 and B7-2, on the APC. In fact, a lymphoproliferative disorder develops in mice in which the CTLA4 gene has been "knocked out" to remove the "negative" signal that this receptor may provide. This result supports the concept that these two receptors may regulate the results of signaling each other (22).

A molecule that has not received the same level of attention is CD45. The CD45 molecule pathway is complicated because the molecule is large and subject to a complex series of alternative splices, creating several different molecules on T cells and leukocytes. It is known, however, that CD45 molecules associate with the TCR–CD3 complex, may cluster with cytokine receptors such as the IL-2 receptor, and are adhesion molecules. The role of CD45 in T-cell activation may be a unique example of a "counter-costimulatory signal" molecule, a role suggested by its structure. CD45 has a phosphatase sequence contained in its cytoplasmic tail. Most of the other costimulatory molecules directly or indirectly trigger tyrosine kinases, which activate their target proteins by phosphorylation of tyrosines. In contrast, the phosphatase activity of CD45 may inhibit this process by removing the activating tyrosine phosphates.

Another theory is that only certain costimulatory signals are involved in the development of T-cell anergy. Blocking the CD28 pathway appears to produce T-cell anergy but not tolerance (21,23,24). In contrast, blocking the LFA-1/ICAM pathway may prevent T-cell activation but does not produce anergy. Thus, there appears to be a hierarchy of costimulatory signals for T cells; not all costimulatory molecules or signals are created equal. Another concept is that engagement of one costimulatory signal can cause upregulation of another ligand. Thus, costimulation may evolve in a series of regulated steps in which the

responding and presenting cells essentially signal back and forth. The next challenge for transplantation immunology is to chart the expression and role of each costimulatory–receptor pair in various clinical transplant situations until the underlying logic for this complex pathway can be elucidated. The ultimate goal of this effort is the selection of the optimal targets for new immunosuppressive strategies. Until this process is further developed, it is reasonable to be cautious in developing expectations for the utility of blocking any given costimulatory pathway in clinical transplantation.

D. Summary

It is both exciting and a significant challenge that clinical investigation will play a major part in advancing the understanding of T-cell activation. Several strategies to selectively inhibit the delivery of costimulatory signals in transplantation have been created. Molecular engineering has been used to create soluble fusion proteins of costimulatory ligands or their receptors (e.g., CD2 and CTLA4/CD28). These can be delivered intravenously, and they effectively block or compete for endogenous receptor–ligand binding. A second approach has been to create humanized monoclonal antibodies against these molecules. Humanized antibodies have the advantage over mouse monoclonals in that they can remain functional in the circulation for up to 3 weeks, allowing for the real possibility of blocking these signals in a clinical situation. A third approach has been to create novel peptide analogues or small molecular inhibitors of costimulatory signaling. Clinical trials with these new agents will offer a number of unique opportunities for immunologists to study the different effects of signal blockade. Overall, this complex pathway of T-cell stimulation is likely to yield potent immunosuppressive strategies. On the other hand, so much is unknown about the primary logic of this system that the current designs and rationales for clinical trials must still be considered complex experiments.

III. PROGRAMMED CELL DEATH (APOPTOSIS)

The recognition that cell death is a highly regulated and complex phenomenon is both a most rational concept and an amazing insight into cell biology (25–27). Apoptosis, or programmed cell death, specifically refers to the complex mechanism that regulates cell destruction by enzymatic pathways contained within the individual cell (Figure 3).

One way of thinking about apoptosis is to consider that, at the most fundamental level, it involves the simple decision of a cell to live or die. Although living involves a strong drive to continue to live, the reality is that death, death of an organism, and death of a cell, are inevitable. To understand the role of death in normal cell physiology, the concept that nothing in cell biology is static must first be considered. Thus, a cell is continually confronted with a changing environment that it must monitor and respond to in order to maintain the health of the organism. This is equally true for epithelial cells, endothelial cells, and all immune cells (e.g., APCs, T and B cells). Cells monitor these environmental changes or stresses by signaling via cell surface receptors. For example, in the case of epithelial cells, growth factor receptors such as epidermal growth factor, nerve growth factor, and various steroids have been linked to the regulation of apoptosis. This is one explanation for the fact that the loss of growth factors are associated with loss of organ function and structure in many pathological states, and also clearly has implications for normal aging mechanisms.

For T lymphocytes, the major signal from the environment relevant to immune-mediated rejection is confrontation with a foreign antigen detected via the T-cell antigen receptor complex. As shown in Fig. 3, however, the complete signaling consequences for the T cell in apoptosis are determined by the interactions of multiple cell surface receptors and

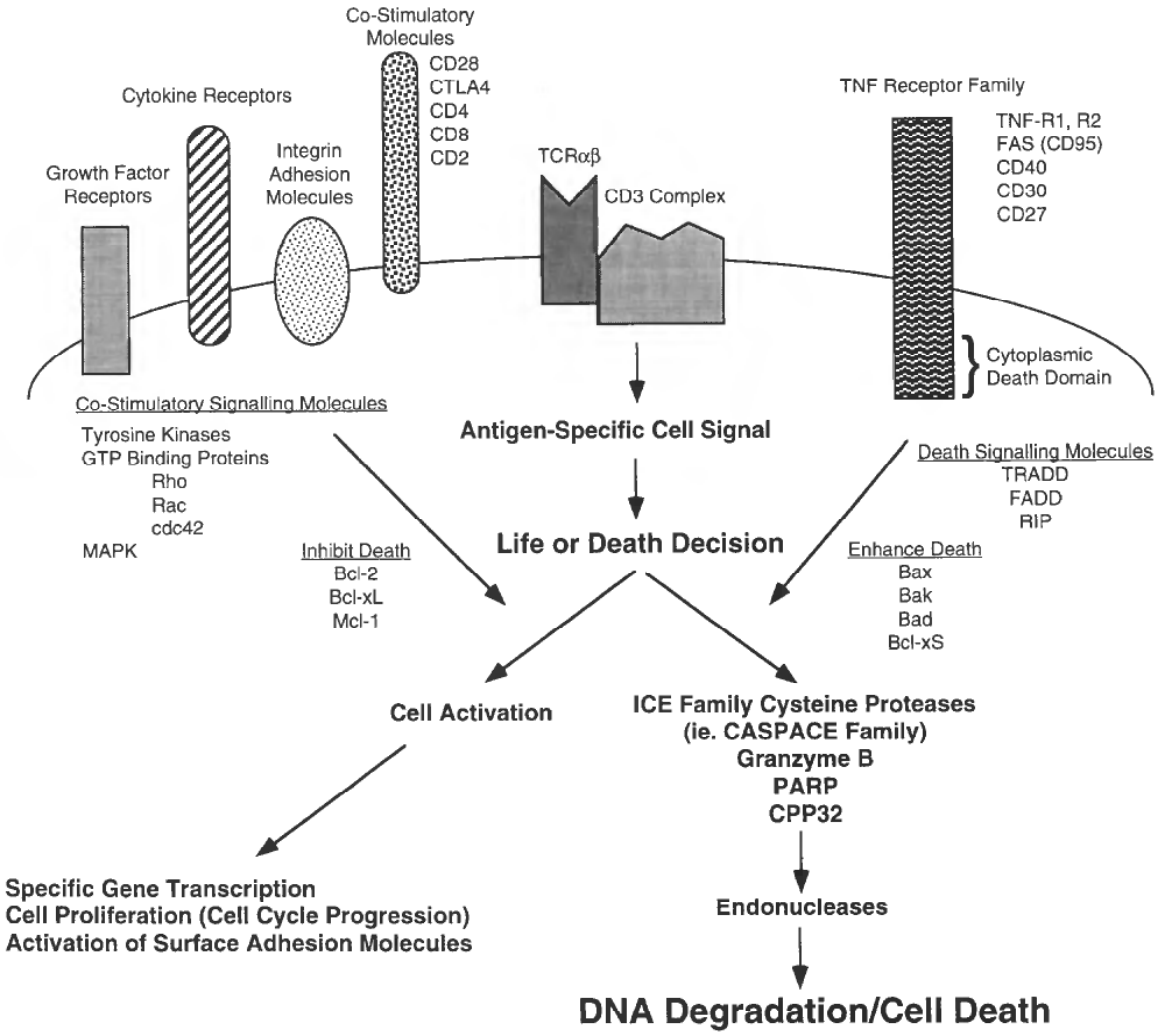

Figure 3 Apoptosis is a critical aspect of T-cell activation. Each cell is confronted in an inflammatory situation with a combination of activating signals (e.g., cytokines), growth factors (e.g., interleukins), and ligands to engage counterregulatory molecules such as FAS and the TNF receptor. These signals force a "life or death" decision on the T cell, which, in turn, determines the nature, timing, and extent of the immune response.

signals. The cytokine receptors, particularly interleukin (IL)-2, IL-4, and IL-7, are also critical; it is probably important that these three cytokine receptors share a common γ chain implicated in signal transduction. However, other growth factors, costimulatory molecules, and adhesion molecules have been implicated in this decision tree as well. In sum, the net result of T-cell signaling by the different combinations of these costimulatory receptors combined with antigen recognition is cell activation, proliferation, and cytokine release rather than cell death. Already described is another outcome of T-cell antigen confrontation, that is, anergy, when T cells recognize antigen without delivery of these costimulatory signals. However, anergic T cells do not die.

On the other side of this life-and-death decision are what may be called "death receptors," which include the tumor necrosis factor receptors and the CD95 or Fas receptor. These receptors have specific sequences called "death domains" on their cytoplasmic tails which mediate the binding of cytoplasmic signaling intermediaries such as Mort1, RIP, and TRADD (25,28,29). Physical stress including exposure to ultraviolet light, heat, and osmotic change may trigger cell membrane changes that also signal activation of the death domain pathways. These different signal pathways for apoptosis converge on the ICE/Ced-3 family of intracellular cysteine proteases (now called the CASPACEs). When activated, these enzymes can initiate the intracellular pathways, producing most of the biochemical and morphological changes of apoptosis (26). The CASPACE family of apoptosis-inducing enzymes is normally present in the cytoplasm as inactive precursors that must be cleaved for activation. The cell also has a complex regulatory network to control the activation of these precursors, which includes such molecules as Bcl2, Bcl-xL, and Mcl-1, which inhibit apoptosis, and Bax, Bak, Bad, and Bcl-xS, which promote apoptosis (25,30). In the final mechanism of the pathway, endonucleases are activated that mediate the degradation of chromosomal DNA in the cell nucleus and resultant cell death.

Apoptosis, like costimulation, cannot be understood in terms of simple positive and negative signals (e.g., growth factors versus FAS ligand). For example, the loss of a positive signal, such as the withdrawal of IL-2 or the breaking of the connection between an integrin adhesion molecule and extracellular matrix, may also initiate the apoptosis cascade. Thus, apoptosis should be considered as a complex and regulated process that is always in play within the cell. The cell is constantly checking its environment and the signals derived therefrom to decide whether to continue living or to initiate the process of programmed cell death.

Four applications of apoptosis theory may be directly relevant to transplantation and rejection. First, the adult T-cell repertoire is created in the thymus during T-cell development by the dual processes of positive and negative selection. Ninety-five percent of developing thymocytes are not successfully selected and die. Apoptosis is the mechanism for regulating this set of events. Therefore, the first application of apoptosis to transplantation is to establish the molecular basis of how T-cell clones are specifically eliminated in the thymus. The next step is to develop a strategy to eliminate the T-cell clones capable of recognizing donor alloantigen. One approach would build on the current use of antithymocyte antibody induction therapy, which destroys both peripheral and intrathymic T cells and, at least theoretically, forces the thymus to recapitulate maturation of the T-cell repertoire. However, such induction therapy does not produce the state of tolerance in human patients that was originally demonstrated in murine models of transplantation. Therefore, there must be another requirement for tolerance induction in humans necessary to eliminate the donor-reactive T-cell clones. One theory is that the placement of donor antigen into the thymus during the rematuration of the T cells in the thymus would manipulate the process to eliminate the donor antigen-reactive T-cell clones (31–37). If the mechanism of intrathymic

apoptosis were understood, it is also possible that tolerance could be created peripherally by selectively targeting circulating donor-reactive T cells before transplantation.

Second, it is clear that costimulatory signals for T cell activation can play a role in protecting cells from apoptosis. For example, cells activated by low levels of antigen may undergo an apoptosis that may be prevented by engagement of VLA5 ($\alpha 5\beta 1$) integrin with its ligand, fibronectin (38). There must be a logic to whether a cell becomes anergic or undergoes apoptosis when a second signal is not delivered with antigen recognition. Recent evidence suggests that only certain kinds of costimulatory or "second" signals can produce anergy. Perhaps there are also specific costimulatory signals that protect against apoptosis. For example, another pathway for inducing T-cell apoptosis is the Fas (CD95) receptor binding FAS ligand (FasL) (39,40). Fas is a member of the nerve growth factor/TNF receptor pathway and has been shown to mediate apoptosis by activation of the CASPACE family of intracellular cysteine proteases (41). The expression of both Fas and FasL is extensively upregulated during T-cell activation, and current thinking is that they are important in regulating whether activated cells continue the immune response, become dormant memory cells, or undergo apoptosis and die. If all activated T cells immediately underwent apoptosis when Fas-FasL was engaged, then the whole regulatory process would make no sense. Thus, it appears that the apoptosis signal delivered by the Fas-FasL pathway can be balanced by a costimulatory signal delivered via CD28 and possibly by other costimulatory signals such as the CD40-CD40 ligand (CD40L) pathway (26) implicated in the regulation of T-cell–dependent B-cell activation. I suggest that the immune response regulates its pace and outcome via interplay of a series of signal pathways that can mediate at least three conflicting outcomes: costimulation, anergy, and apoptosis.

Third, the Fas-FasL pathway has been implicated directly as a strategy for preventing rejection in transplantation. It has been known for some time that the testes is an immune-privileged site capable of supporting both allogeneic and xenogeneic transplants in several animal models. FasL is highly expressed by rodent Sertoli cells (42), although not by their human counterparts (43,44). Transplantation of Sertoli cells from strains of mice with a genetic loss of FasL resulted in rapid rejection as compared to transplantation of FasL-expressing Sertoli cells from control animals. The theory is that the FasL on the Sertoli cell surface triggers apoptosis of infiltrating T cells in the early stages of the immune response. This mechanism of targeting cells for death is supported by the fact that cytotoxic T cells use expression of FasL as one mechanism for killing their targets. Thus, it has been proposed that the purposeful expression of FasL on transplanted cells via gene therapy might form the basis for a novel strategy to accomplish successful cell transplantation (45).

Fourth, current understanding of apoptosis raises the question: How many ways can a cell die? For example, in transplantation, significant tissue ischemia is recognized as a consequence of arterial vascular rejection leading to cell necrosis. It is logical to propose that individual cell death in these pathological situations is caused by cell necrosis resulting from physical trauma and not apoptosis. It appears that various physical insults such as ischemia, heat, pressure, and exogenous toxins can also trigger the apoptosis pathways of the cell, completing the process of cell death. One theory is that there is a working relationship between pathological cell death and physiological cell death (apoptosis) (25). For example, a mild insult might injure some cells but the majority could still recover. In contrast, a more severe insult crossing some kind of cell threshold would either trigger apoptosis or, if severe enough, just physically destroy the cell. Therefore, strategies to protect cells from triggering apoptosis could be used to limit the final extent of rejection-induced cell death and tissue injury. Would this help protect patients with early rejection from chronic rejection?

Epithelial and endothelial cells are also subject to programmed cell death, since apoptosis is not a mechanism unique to lymphocytes. Epithelial cells use integrin adhesion molecules to stabilize their location and orientation to the underlying basement membrane (46–48). This integrin-mediated process is critical to creating and maintaining the three-dimensional organization of human tissues (see Sec. VII). In the context of apoptosis, it is understood that when epithelial cells lose their anchor to the basement membrane, they undergo apoptosis. One theory in the study of cancer is that malignant epithelial cells lose their sensitivity to this anchor-dependent apoptosis and, thus, can leave their normal location and metastasize. Kidney transplant rejection results in various mechanisms of tissue injury including ischemia and the release of proteolytic enzymes called metalloproteinases. These enzymes can profoundly alter the integrity of extracellular matrix structures such as the basement membranes of blood vessels and kidney tubules as well as the interstitial matrix frameworks that contribute to the shape and structure of the kidney. Therefore, if the destruction of these matrix frameworks is extensive, epithelial cells that otherwise may have escaped direct T-cell–mediated rejection and even ischemia may nonetheless undergo apoptosis.

IV. IMMUNE DEVIATION, CYTOKINES, AND THE Th1/Th2 HYPOTHESIS

Another area of transplantation immunology is the concept of immune deviation, or the Th1/Th2 hypothesis. It started with the discovery in mice that there are at least two major types of helper/inducer cells characterized by the production of different cytokines (49–51). The first observations were in a mouse model of *Leishmania* infection in which a cytokine response involving primarily IL-2 and interferon (INF)-γ protected one strain of animals while a response in a second strain characterized by the cytokines IL-4 and IL-10 favored disease progression and death. The investigators made the connection that helper T-cell progenitors could be channeled in at least two different directions, Th1 and Th2, during the development of an immune response. This was a major insight into the logic of a system in which at least 20 different cytokines had already been demonstrated to be produced by helper T cells in various systems. Currently, the evidence is that Th1 cells produce primarily IL-2, INF-γ, and TNF-β, and Th2 cells produce primarily IL-4, IL-5, IL-6, IL-10, and IL-13 (49,52). The Th1 pathway favors development of cell-mediated immunity, whereas the Th2 pathway favors humoral immune mechanisms and may also mediate a feedback suppression of the Th1 pathway (Figure 4).

Based on the dramatic immune effects of Th1 and Th2 responses in the murine infection model, it was suggested that transplant rejection represents a primarily Th1 response, whereas transplantation tolerance is favored by a Th2 response. It followed that any strategy to induce tolerance should be characterized by an immune deviation from a primary Th1 response, the normal destructive agenda of the immune system, to a Th2 response. A number of studies followed that documented relative increases in Th2 cytokine gene transcripts and Th2 cells in tolerant animals, favoring this immune deviation theory for tolerance (see Chap. 4).

Whereas Th1 and Th2 cells are defined by the cytokines they produce, cytokines can also regulate which type of T helper cells are expanded after antigen stimulation (see Fig. 4). Thus, naive helper cell progenitors responding to antigen are often referred to as Th0, with the assumption that they can produce any cytokine. Depending on the cytokine signals delivered in the microenvironment, the helper T cell response that develops is shaped to fit the Th1 or Th2 phenotype. The significance of this theory for transplantation is that, if the

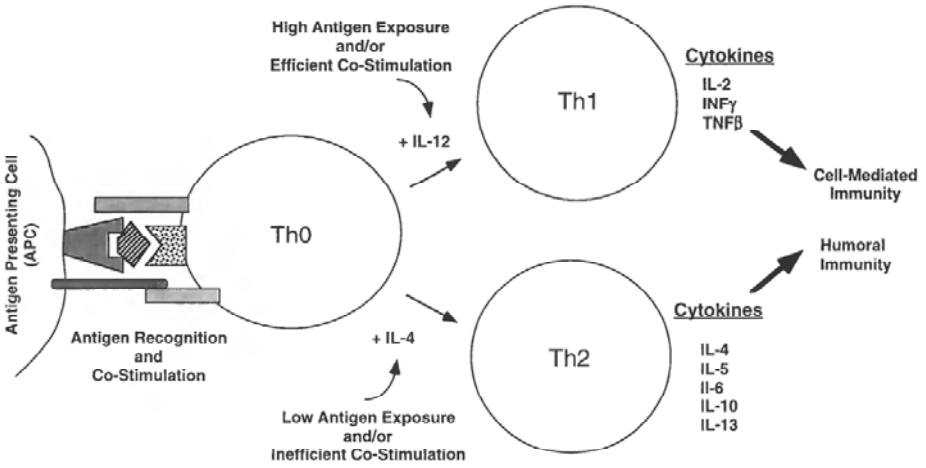

Figure 4 The theory of immune deviation is based on two concepts. First, a T-helper cell progenitor (Th0) can mature into either of two mature T-helper cells (Th1 or Th2) after antigen recognition and activation. These T-helper cells are distinguished by the pattern of cytokines they produce and, thus, the immune mechanisms they support. Second, the selection of Th1 or Th2 pathways is determined by the nature of antigen presentation and/or costimulation as well as by cytokine signals.

cytokines can be manipulated early in development of the T helper cell response, the immune response could be selectively deviated to induce tolerance. Thus, IFN-γ and IL-12 (from activated APCs) support a Th1 response and IL-4 favors a Th2 response. The potential clinical significance of this approach is emphasized by studies in which the administration of IL-12 or a single dose of anti–IL-4 early after *Leishmania* infection can completely alter the course of the disease in the mouse model, deviating the immune response to Th1 and favoring the animal's survival. Theoretically, it follows that administration of cytokine signals favoring deviation of the immune response to Th2 after transplantation would favor tolerance induction and protect patients from rejection.

A less obvious application of this theory is an explanation for the phenomenon of tolerance induced by extremely low or extremely high antigen concentrations, so-called low-zone/high-zone tolerance. Low-zone tolerance has been suggested as one mechanism for the protective effect of pretransplant random donor blood transfusions, and high-zone tolerance may be invoked for pretransplant donor-specific blood or bone marrow transfusions. In either case, the immune deviation theory predicts that either extreme of antigen exposure alters the release of cytokines from activated APCs or surrounding Th cells and favors a Th2-like immune response.

Despite the logical attractions of this simple binary Th1 versus Th2 paradigm for transplantation, there is increasing evidence that the immune response to transplantation in humans is more complex (24,52). For example, a simple application of the paradigm would predict the following result. In separating the peripheral Th cell populations of two groups of patients, one with acute rejection and another that never had a rejection, a distinct Th1 predominance in the rejecting patients and a Th2 predominance in the nonrejecting group should be found. However, this has not been reported. It could be countered that the Th cells in the peripheral blood may not be representative of the immune response to the transplant. Therefore, a number of investigators have studied Th1 versus Th2 cytokine message levels in biopsy specimens from rejecting patients but with no better results. On the other hand, studies are only beginning to use quantitative polymerase chain reaction (PCR) techniques, an approach that promises to provide a much better picture of relative cytokine profiles during rejection. It is important to wait for results of these quantitative experiments before making any final conclusions on the application of this paradigm to human transplantation.

Nonetheless, it is interesting that messages for both Th1 and Th2 cytokines are detectable by PCR in rejecting transplant biopsy specimens, and these immunosuppressed patients often demonstrate a preponderance of Th2 cytokines such as IL-4 and IL-10. This paradoxical result is not surprising based on the potent suppression of IL-2 and IFN-γ gene transcription by cyclosporine and of IL-1 and IFN-γ by steroids. Thus, the theory of immune deviation predicts that the use of immunosuppression effectively blocks the cytokines responsible for Th1 development, thus favoring immune deviation to the Th2 phenotype. This is supported by mouse and rat transplant model studies in which a short course of cyclosporine at the time of transplantation can produce a long-term tolerant state. Unfortunately, whatever immunosuppression is doing to Th profiles in the human patients, the fact that they are rejecting suggests that tolerance has not been produced. These contrasting observations in murine and human transplantation may eventually be explained by critical differences in the logic of the Th1/Th2 paradigm in higher mammals.

It is also important to acknowledge the possible connections between the theory of immune deviation and the potential roles of costimulatory signals described previously. Cytokine signals alter the expression of costimulatory ligands on the APC surface, and cytokine signals also determine the Th phenotype of the immune response. Thus, it is reasonable to suggest that certain costimulatory signals may favor Th1 or Th2 development

and, in turn, the nature of the cytokines present in a given environment may regulate the efficiency of antigen presentation.

In conclusion, there is still much to be learned about the nature of balance in the Th cell response. Simplistic views that increases in IL-4 or other Th2 cytokines will favor tolerance induction or mark a tolerant state after transplantation have not been supported. On the other hand, the concept that the immune response is regulated by a complex balance of cytokines produced by different Th cell clones is important. It remains clear that cytokines play a fundamental role in regulating the immune response at multiple levels. Even though murine models have shaped the current immune deviation theory, it appears likely that significant differences characterize the Th cell immune response in humans. Therefore, it is likely that the rapid advances in understanding of the Th1/Th2 paradigm in human systems will eventually allow its placement into a proper context in clinical transplantation.

V. ADHESION MOLECULES AND THE PHYSICS OF THE IMMUNE RESPONSE

Adhesion molecules have been defined in the context of stabilizing T-cell interactions with APCs during presentation of antigen and delivery of costimulatory signals. By this definition, CD4 and CD8, the TCR, and even HLA molecules are adhesion molecules. However, the term "adhesion" is too general to use without generating some confusion. "Adhesion" for the cell biologist has a different meaning and refers to the mechanisms by which all cells adhere to extracellular matrix proteins or adjacent cells. In this context, cell adhesion determines the fetal development of the organism, maintains the adult structure and function of the body's organs, and heals the body when injured. Therefore, to relate adhesion to transplantation, the roles of two different families of adhesion molecules must be considered: the integrins and selectins (53–57).

This expanded view of adhesion in transplantation immunology has two important components. The first explains how lymphocytes, leukocytes, and APCs can traffic to a site of acute inflammation, accumulate, and mediate an immune response (Figure 5). The second explains how local epithelial and stromal cells use adhesion molecules to repair tissue injury and restore organ function after acute inflammation subsides. This discussion deals with the first component. The second component is described in Sec. VII.

The selectin family consists of three molecules: E-selectin, P-selectin, and L-selectin (54,58,59). The designations refer to their expression on endothelial cells (E), platelets (P), and leukocytes (L). They are structurally related, all containing characteristic lectin and epidermal growth factor (EGF)-like binding domains, and all use a variety of carbohydrate moieties decorating cell surface proteins as their target ligands. A simple view is that the selectins are the first adhesion molecules used to initiate the cell adhesion of platelets, leukocytes, and lymphocytes moving across the endothelial cell surfaces in the circulation. Thus, it appears that lymphocytes or leukocytes literally roll across the endothelium by means of rapid, low-efficiency interactions mediating cell binding and quick release of selectins and their ligands expressed on endothelial cell surfaces. The discovery of this physical rolling phenomenon was a remarkable insight into how circulating blood cells monitor events taking place across vascular surfaces. Thus, if the local release of inflammatory mediators signals the upregulation of these molecules on adjacent endothelium, the rapid binding and release kinetics of the rolling cells will be critically slowed at this site and a more stable, high-avidity integrin-mediated adhesion is facilitated.

The integrin family of adhesion molecules is much more complex and consists of at least 17 different molecules (54,60,61). These are all heterodimers and are characterized by

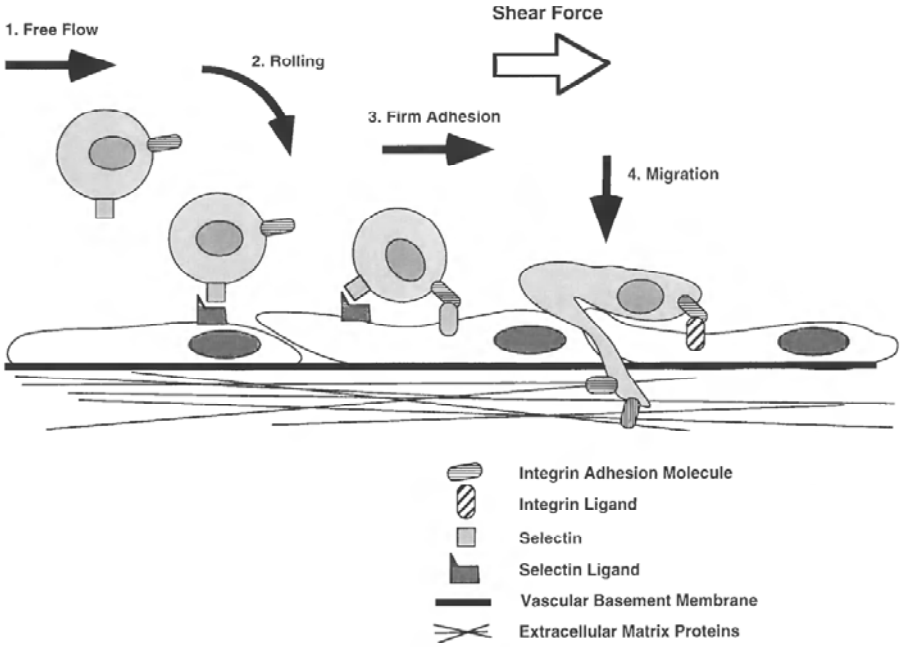

Figure 5 Four distinct stages in the process of taking a lymphocyte flowing freely in the bloodstream, to rolling along the endothelium (e.g., selectin-mediated), to firm adhesion at sites of inflammation or tissue injury (e.g., integrin-mediated), to migration across the endothelial wall and into the tissue (e.g., integrin-mediated).

a complex cation-binding domain important to receptor conformation and function. Integrins are receptors for a number of extracellular matrix proteins such as fibronectin, vitronectin, the laminins, and the collagens. Integrins can also bind several cellular ligands such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). Integrins can mediate cell attachment or firm adhesion with a high enough strength to fix the rolling lymphocytes and leukocytes on the endothelial surface. Integrins can also mediate cell migration, which requires a regulated mix of both adhesion and cell locomotion.

A key concept is that both integrins and selectins can be activated to enhance binding efficiency. For example, a number of inflammatory cytokines mediate integrin or selectin activation. These cytokines also stimulate the expression of the cellular ligands for these receptors; thus, IL-1 is a potent stimulator of endothelial VCAM-1 expression, which is the target ligand for two integrins, VLA4 ($\alpha 4\beta 1$) and $\alpha 4\beta 7$. Furthermore, certain integrins are also capable of delivering costimulatory signals required for T-cell activation (e.g., LFA-1 binding to ICAM-1). In contrast, there is no evidence that selectins are costimulatory.

The role of adhesion molecules must also be considered in the context of the physics of the immune response, that is, the role of blood flow in the mechanics of cell–cell interactions in the vascular lumen. It is a basic principle of physics that flowing fluid creates a shear force. Thus, shear stress is created within the vascular lumen by the dynamics of blood flow and it can be described and measured in precise physical terms. It is clear that an understanding of cell adhesion to the vascular surface must include consideration of the physics of shear stress applied to the cell (62). The physical complex of any adhesion molecule with its ligand has a tensile strength that must be measured under flow conditions (63). Thus, it is logical that adhesion in the high shear stress of the arteriole will involve receptor ligand interactions fundamentally different than those required for adhesion in the relatively low shear conditions of the postcapillary venule.

This hypothesis can be applied to transplantation as follows. The pathology of rejection demonstrates that early accumulations of inflammatory cells after transplantation and during initiation of classic acute transplant rejection begin in the postcapillary venules (low shear stress). The interstitial rejection infiltrate that results is characterized by the accumulation of activated lymphocytes, cytokine release, and cell-mediated cytotoxicity. Thus, this process would seem to involve efficient T cell adhesion and a prompt migration into the underlying tissue. That early stages in acute rejection are targeted to a low shear stress site makes sense because T cell activation and cytokine levels have not had a chance to upregulate adhesion molecules and their ligands to support function in higher shear stress sites. In contrast, the vasculitis associated with autoimmune disease or acute vascular rejection typically starts on the arteriolar side of the capillary bed (high shear stress) and is initially dominated by leukocytes followed only later by activated T cells. First, leukocytes may initiate local injury and ischemia, and release cytokines that increase the activity or binding efficiency of T-cell integrins as well as increase endothelial ligand expression levels. Increased adhesion receptor activity and higher ligand concentrations enhance the ability of the T cell to arrest its forward motion on an endothelial surface in the high sheer stress environment of the artery. These mechanisms may also explain why acute vascular rejection is more common in patients with primed or memory T-cell populations resulting from multiple antigen exposures from previous transplants, pregnancies, or after blood transfusions (e.g., sensitized or high PRA patients). A similar mechanism is likely to be important in the increased risk of acute rejection following cadaver organ donation, which clearly exposes the transplant including its endothelium to a series of insults that increases inflammatory cytokine release and upregulates adhesive ligands. Finally, any activation of the

clotting cascade or platelet aggregation creates disturbances in the flow of the vessel (e.g., decreases shear stress) and enhances the ability of inflammatory cells to stop and participate in the evolving immune response. Thus, the accumulation of T lymphocytes in classic acute cellular rejection may be favored by interactions between the integrin VLA4 and VCAM-1 presented early in the immune response by the postcapillary venules. In contrast, acute vasculitis appears to favor early accumulation of inflammatory leukocytes using LFA-1/ICAM-1 and L-selectin adhesion to stop their motion in the much higher shear stress of the arteriole. As rejection continues, however, a series of mechanisms cooperate to recruit T cells to the higher shear stress side of the vascular bed. This is one explanation for the fact that some patients with acute cellular rejection on the initial biopsy then demonstrate the development of acute vascular rejection on the next biopsy when the renal dysfunction does not respond to initial antirejection therapy.

These adhesion molecule pathways are promising potential targets for manipulating the immune response as therapy. If cells cannot get to the transplant, then antigen recognition will clearly be limited, particularly the direct route of antigen presentation. Similarly, if donor APCs cannot traffic from the transplant to sites of peripheral lymphoid antigen presentation (e.g., lymph nodes and spleen), then one mechanism of indirect pathway antigen presentation will be inhibited. Moreover, even if the first wave of T cells is activated, interfering with their traffic to the transplant or their migration into the interstitium could be used to suppress rejection. Blocking adhesion could even induce tolerance or T-cell anergy if any of the adhesion receptor–ligand combinations previously described are critical costimulatory signals for T-cell activation. A growing number of new reagents has been developed. These inhibitors of adhesion include humanized monoclonal antibodies against both adhesion receptors and ligands, novel soluble ligand fusion molecules that can compete with normal cell surface ligands for cell binding, and a new generation of peptide analogue inhibitors capable of selective integrin and selectin inhibition. A major challenge is to determine which adhesion pathways are most critical to transplantation immune responses and rejection. Otherwise, these strategies will create the same degree of global immunosuppression associated with current drug regimens. On the other hand, the fact that these same adhesion molecules, by definition, are also required for a wide variety of normal cellular mechanisms puts a special emphasis on carefully testing these new reagents.

VI. INTERCELLULAR AND INTRACELLULAR SIGNAL CASCADES

Once the T cell recognizes its target antigen, the next steps in T-cell activation occur in the cytoplasm. The current view of cytoplasmic signaling is as complex as the network of T-cell subsets, cytokines, and surface receptors determining the course of the immune response on the exterior of the cell (Figure 6). It is presently one of the greatest challenges in cell biology to elucidate the logic in the signal pathways triggered by engagement of various cell surface receptors. Although it is beyond the scope of this chapter to detail the signal pathways involved in the immune response, a few concepts are introduced herein.

There are at least four basic cellular roles for intracellular signaling relevant to immune-mediated rejection.

1. Cell activation. Cell activation has already been discussed in the context of antigen recognition and costimulatory molecules, the first and second signals.

2. Coordination and support of cell functions. For example, intracellular signals regulate the expression of costimulatory ligand (e.g., ICAM-1) and the production of various

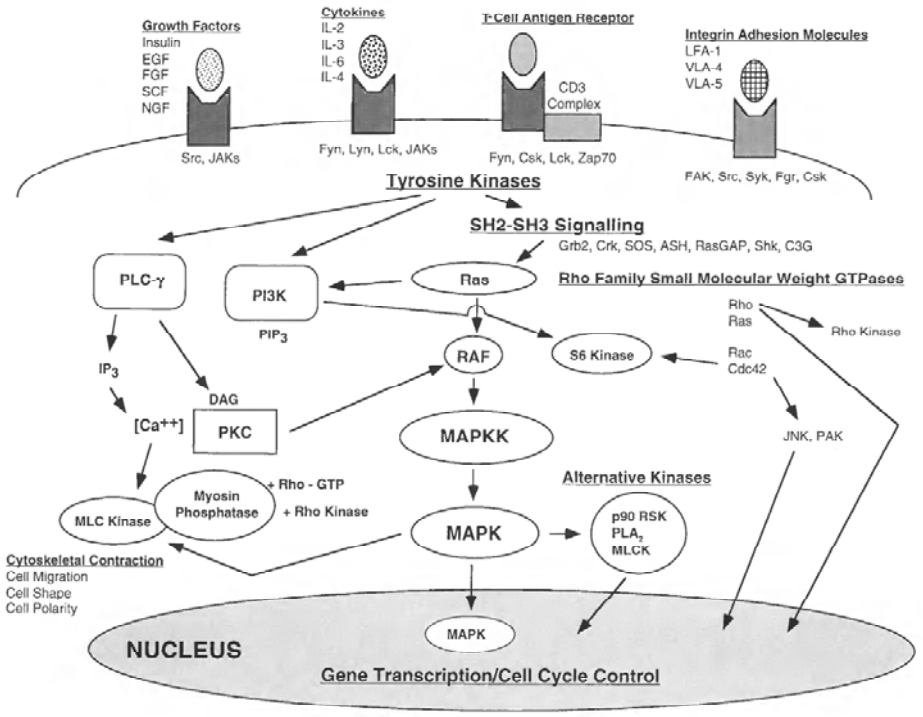

Figure 6 Intracellular signal pathways are responsible for linking events taking place at the cell's surface to various cellular responses such as cytokine release, migration, cell activation as well as to the nucleus, where specific gene transcription ultimately determines the cell's response and fate. Signaling can be viewed as a series of parallel pathways extending from surface to nucleus with a lot of crosstalk pathways allowing for integration of signals originating from multiple receptors.

cytokines, which, in turn, determine the consequences of cell activation and cell survival (e.g., apoptosis). Intracellular signals also regulate gene transcription for the synthesis of effector molecules such as granzyme and perforin required for killing by cytotoxic T cells.

3. Cell cycle control. The control of cell division is a complex and regulated process that proceeds from DNA synthesis to the physical separation of duplicated chromosomes into two new cells. Thus, a primary characteristic of the immune response is the proliferation of activated cells, which is amplified by costimulatory signals and cytokines (growth factors) such as IL-2, INF-γ, TNF, and IL-4.

4. Cell traffic—adhesion and migration. The immune process depends on the ability of cells to traffic to sites of inflammation, migrate into tissue, bind to the surface of target cells, and release specific enzymes involved in cell killing or provide costimulation or cytokine support for other cells migrating into the site.

Although the fundamental logic of intracellular signaling is unknown, there are several key mechanisms (see Fig. 6) (64,65). First, a majority of primary signals are delivered by intracellular enzymes called tyrosine kinases, which catalyze the phosphorylation of very specific tyrosine residues on target molecules. These target molecules are typically also enzymes. Tyrosine phosphorylation activates them, presumably by creating a conformational change in the molecule, which uncovers the catalytic site or, alternatively, dislodges an inhibitory molecule bound to the target enzyme. There are also serine-threonine kinases. The calcineurin complex is a serine-threonine kinase and is the target of both cyclosporine and FK506 when either drug is bound to its carrier protein. Second, there is a series of small molecular weight GTP binding proteins (e.g., Ras and Rho) that are activated by GTP and subsequently catalyze phosphorylation of various substrates such as mitogen-activated protein kinase (MAPK) and mitogen-activated protein kinase kinase (MAPKK), which have been directly linked to activation of nuclear-binding proteins and specific gene transcription or cell cycle activation. Third, a number of "connector" and "adaptor" molecules act as bridges between receptors and various GTP-binding proteins. These often bind to specific regions called src homology domains (e.g., SH2 and SH3) to mediate the construction of multiprotein enzyme complexes at sites of receptor activation. This is an important insight into how a cell "knows" where a signal is originating from in a three-dimensional space. In other words, if one surface of an activated lymphocyte binds to the endothelium during acute rejection, it clusters its adhesion receptors as well as its TCR–CD3 complex on the side of the cell in contact with the allogeneic target. The clustering of cell surface receptors mediates the clustering of intracellular signal enzymes, which, in turn, selectively activates the cytoskeletal elements of the cell in that portion of the cell. Thus, given a location and a direction for a particular signal, the cell migrates toward an inflammatory site in the transplant interstitium during acute rejection or into the intimal and muscularis layers of a vessel in acute vascular rejection.

Other aspects of intracellular signaling are understood in some detail. For example, the TCR–CD3 complex is directly involved in the generation of cytoplasmic signals for T cells. It is the CD3 molecule complex that is responsible for this signaling. A number of cytoplasmic tyrosine kinases such as src, zap70, and fyn can bind to the cytoplasmic tails of the CD3 complex proteins and are activated when the TCR engages its antigen on the exterior surface of the cell. Moreover, the close physical location of the costimulatory CD4 or CD8 molecule, which is binding to the same MHC molecule as the TCR, creates a bridge between the kinases bound to the cytoplasmic tails of the CD3 complex and kinases such as lck, which are bound to the cytoplasmic tails of CD4 or CD8 molecules. In close proximity are various adhesion molecules (e.g., integrins such as LFA-1), which stabilize T cell adhesion to the APC and interact with various tyrosine kinases or GTP-binding proteins

connecting cytoskeletal components into the mix. An excellent example of an adhesion-specific kinase is focal adhesion kinase (FAK), which rapidly localizes at the cytoplasmic side of clustered integrin molecules binding a cellular ligand (e.g., ICAM-1) or an extracellular matrix protein (e.g., fibronectin). Activation of nearby cytokine or growth factor receptors also link membrane-bound G-proteins and calcium channels with subsequent activation of phospholipase C and generation of diacylglycerol and the phosphoinositol intermediaries of cell activation.

Ultimately, most cellular functions described in the context of transplantation involve the transcription of specific genes. Gene transcription mediates the synthesis of cytokines, cell surface receptors, intracellular signaling molecules, and a variety of enzymes involved in processes as diverse as cell replication and cytotoxic T-cell killing. The connection between cell signaling and gene transcription is made via nuclear transcription factors. Transcription factors are proteins present in both the cytoplasm and nucleus. These proteins can bind directly to specific sequences of genes, called enhancer or promoter elements, which are basically required for transcription of the genes typically located immediately upstream of the factor binding sites. These factors in the cytoplasm are often complexed with inhibitor molecules that prevent their translocation to the nucleus. In some cases, phosphorylation, and, in other cases, dephosphorylation, result in the loss of the inhibitory complex, allowing the transcription factor to enter the nucleus, bind the promoter element, and initiate gene transcription. Thus, one objective of the intracellular signaling enzymes is to regulate the activation of these cytoplasmic transcription factors. More recently, it appears that specific transcription factors may be associated with specific signal pathways. The best support for this hypothesis is the association of the cytokine receptors for IL-2 and IL-4, with the Janus family kinases (JAK kinases) and members of the STAT family of transcription factors (66). Thus, it appears that both the IL-2 and IL-4 receptors share a common γ chain and thus can trigger the same set of JAK kinases after receptor engagement. If so, how can the cell know whether it has bound IL-2 or IL-4? The answer appears to be that it is the association of two different STAT transcription factors with each receptor that determines the specificity of the cell response that follows (67,68).

What remains a mystery is how all these individual signaling pathways are regulated to mediate specific cell functions. The insight from what is known about STAT may be one breakthrough. However, a good description of the current state of knowledge was recently offered by a senior scientist at the Scripps Research Institute when he referred to it as a "bar room brawl" that starts when two recognizable people at the bar get into an argument but which quickly escalates into such a melee that the details are impossible to describe coherently. Nonetheless, understanding of this complex system will reveal a whole new set of drug targets for more specific immunosuppression and tolerance induction. One feature of this endeavor is that cell signaling is a fundamental property of all cells, not just of lymphocytes. Thus, this entire field has moved transplant immunology into the mainstream of cell biology. Once the basic principles of these intracellular cell signal cascades are understood, then their specific applications in the immune response can be studied and the right targets for transplantation can be identified.

VII. A THREE-DIMENSIONAL VIEW OF IMMUNITY

Any site in the human body can be considered in terms of its physical or three-dimensional space. Thus, I propose that the mechanisms of cellular immunity must ultimately be understood in the context of real intercellular distances and events that are shaped by the com-

plex framework structures that determine and maintain the organization of normal tissues. The corollary of this three-dimensional view is that normal organ or tissue function requires a normal structure.

There is a tendency to view immune-mediated injury in two "dimensions." For example, activated T cells recognize antigen via the T-cell receptor and engage a cascade of inflammatory cytokines and cytotoxic cells resulting in generic tissue injury. However, to understand rejection, a third dimension of space and structure must be considered in which immune-mediated injury results in the disruption of normal cellular organization and function. This three-dimensional view also includes the epithelial, endothelial, and stromal cells, of the transplanted organ, which do not stand by passively waiting for the immune response by invading lymphocytes, APCs, and leukocytes. These epithelial and stromal cells actively create and maintain their environment. Moreover, they are prepared to protect their structure and heal the injury after a rejection episode is subdued.

Extracellular matrix (ECM) proteins are synthesized by stromal cells such as fibroblasts as well as by endothelial and epithelial cells. These ECM proteins create a three-dimensional structural framework that defines each organ system. Thus, during the development of a tissue, the cells participate actively in creating the normal structure within which they will function. However, the health of an adult tissue also depends on the constant remodeling of the ECM structure such as occurs in the bone with collagen and osteopontin (69) or in the skin with laminin (70,71). Stromal, endothelial, and epithelial cells also express an array of integrin adhesion molecules that recognize specific binding sequences on the ECM molecules. These integrins mediate the connection between the cells and the organ framework structures created by ECM. For example, by expression of integrins on specific surfaces, the cell can determine its polarity with respect to an underlying basement membrane structure. Thus, epithelial cells lining the kidney tubules express certain integrins on their basolateral surfaces which attach them to the laminin and collagen of the basement membrane in a polar fashion. These mechanisms of receptor-mediated polarity are similar to those described for lymphocytes in the context of directed migration across the endothelium during rejection.

In acute rejection, leukocytes and lymphocytes are initially attracted to a vascular site in the transplant. Cytokines mediate the upregulation of several adhesive ligands on the endothelial cell surfaces that mediate leukocyte and lymphocyte adhesion. One of these ligands, VCAM-1, can trigger cells attracted to this site via the integrin VLA4 (a4b1) to release various enzymes, including members of the metalloproteinase family. In turn, these enzymes can digest the ECM of the basement membrane and allow cells to access and migrate into the interstitial tissue (72). As injury evolves, the normal endothelial cell monolayer is increasingly disrupted and the underlying vascular basement membrane is exposed. In turn, this disrupts the vascular integrity of the tissue, increases downstream ischemia, and facilitates the extravasation of other cells and inflammatory mediators to fuel interstitial injury. In parallel, the lumen of the vessel is filled with a growing mass of activated platelets releasing a number of chemotactic cytokines and factors. The milieu of this vascular site is further complicated by the activation of complement components, which can act as chemokines or bind to integrin adhesion molecules to activate leukocytes. The clotting cascade, as well as local platelet aggregation and activation, are facilitated by the exposure of basement membrane tissue factors. Polymerization of fibrin in the vessel lumen and its extravasation into the interstitium exposes the cells to ECM proteins trapped from the circulation including fibrin, fibronectin, and vitronectin. The immobilization of these ECM proteins at the site of acute injury uncovers integrin binding sites that are normally hidden on these proteins in the circulation. The exposure of these ECM-binding sites for integrin attachment accelerates

cell recruitment and directs further migration of the activated cells into the underlying tissue spaces. Thus, the three spatially separated compartments (vascular lumen, vascular wall, and interstitium) each present a unique combination of ECM proteins and structural features that contribute to the immune mechanisms evolving during acute rejection.

Chronic rejection of a kidney transplant is typically associated with progressive interstitial fibrosis and vascular narrowing with ischemia. These changes produce a progressive loss of renal function. Some investigators have proposed that chronic rejection involves a series of unique immulogical injury mechanisms. Alternatively, chronic rejection may represent the final expression of a series of low-grade acute rejections that are further complicated by secondary mechanisms of progressive tissue injury. In either case, the start of chronic rejection may be viewed within the gross disruption of the three-dimensional tissue space created by acute rejection.

Chronic rejection can be integrated into the three-dimensional view as follows. A loss of ECM framework structure occurs in acute rejection as a consequence of cell and cytokine-mediated killing of epithelial and stromal cells, destructive enzymes released by activated inflammatory cells, and tissue ischemia. In the healing phase of this injury, the interstitial space is infiltrated with new cells arising from the stroma of the transplanted organ, mainly fibroblasts, which secrete new ECM proteins to heal the internal wounds. Other proliferating cells including monocytes may migrate from the vascular space and are largely patient derived. In parallel, the vascular basement membrane is reestablished by donor endothelial cells migrating into the damaged area, although clearly some vessel sites will be unreparable. In these irreversibly damaged sites, downstream ischemia is never resolved and whole glomerular units with their tubular structures are lost and replaced by interstitial fibrosis and scarring. The tubular epithelial cells of the kidney that were damaged in the original rejection and associated inflammation must find an intact tubular basement membrane upon which to attach their integrins, proliferate, and organize their polarity to the tubular lumen. Even though the epithelial cells can secrete ECM proteins to aid this process, they do require some underlying structure first. If they do not find this ECM structure, they undergo a form of programmed cell death or apoptosis signaled through their integrin adhesion molecules (46,48). These delicate epithelial ECM frameworks in the kidney were created originally during fetal development and cannot be simply repaired by the infiltrating fibroblasts and monocytes triggered in the healing wake of rejection-mediated injury. As a result, this critical organizational element of the tissue can be lost. Thereafter, the combination of tissue disorganization at the structural level and ischemia secondary to end-stage vascular injury results in increased organ dysfunction. Organ dysfunction creates a stress that activates secondary mechanisms of chronic injury such as hypertrophy and hyperfiltration in remaining nephrons. Even blood vessels that were only injured by the initial rejection may never heal to their original state, creating altered flow, ischemia, hypertension, and vessel wall stress. These physical elements may then initiate low-grade inflammatory cell and platelet activation, leading to progressive vascular injury in a process mechanistically similar to atherosclerosis.

The underlying logic of a three-dimensional view of transplantation and rejection is that all of the cellular and matrix structures of the transplant must be considered to place the mechanisms of immune-mediated injury in a proper context. It follows that the transplantation and long-term function of an organ is a complex story in which the epithelial, endothelial, and stromal cells of the donor organ are also important determinants of clinical success or failure. By considering the fundamental mechanisms and consequences of cell adhesion, apoptosis, and intracellular signalling, transplantation immunology has moved into the mainstream of cell biology.

VIII. CONCLUSION

This chapter introduced a series of paradigms that explain basic mechanisms in immunology in a context applicable to current experience with transplant rejection. It is a major challenge to integrate the increasing complexity of these paradigms with real clinical situations. The driving force for making the effort to do so is that a growing number of exciting new therapies for transplantation will be based on the understanding of these processes. Thus, the design and conduct of safe clinical trials will depend to some extent on the success of meeting this challenge. Simple explanations are often difficult to apply to complex clinical events, and several central realities currently confound this effort. First, the irony is that the immunological paradigms described here are not fully understood, so that current predictions are only starting points and will likely change as our scientific understanding continues to advance. Second, the ability to describe the immunological situation in a given patient at any specific time after transplantation is limited, and it is difficult to agree on the immunological significance of many pathological features identified in the biopsy in acute and chronic rejection. There are too many interrelated factors that influence the immune response in real life. Thus, while elegantly constructed animal models have been instrumental in developing these immunological paradigms, we must confront the frustration that transplantation in clinical practice is not an elegant model, at least not yet.

However, rather than being negative or overwhelmed, I believe that this is the most exciting period in the history of transplantation. The next phase will see the increasing refinement of these basic immunological paradigms balanced by a rapidly increasing understanding of their precise implications for clinical events in transplantation. A growing number of novel immunosuppressive agents and strategies will accelerate and shape this process. The unique feature of transplantation has always been a collaboration between clinicians and scientists in which the experiences at the bedside inform the decisions and experimental designs at the bench. Never before has this been more important or productive.

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Mechanisms of Cell-Mediated Rejection

DOUGLAS P. SLAKEY
Tulane University Medical Center, New Orleans, Louisiana

JUDITH M. THOMAS
University of Alabama at Birmingham, Birmingham, Alabama

I. INTRODUCTION

Since Medawar's early studies demonstrating the immunological basis of allograft rejection, experimental in vivo and in vitro models have been developed to examine the various immunological mechanisms responsible for allograft rejection. The alloimmune response has proven to be complex (1–3), and the precise nature of the various effector mechanisms continues to challenge immunologists. Allografts can be damaged by immunologically specific cellular and humoral mechanisms and by various nonspecific inflammatory mechanisms (4). Accurately defining cell-mediated rejection is complicated by the realization that, to varying degrees, cellular components of the immune system are involved in all types of primary vascularized allograft rejection (hyperacute, acute, and chronic). This chapter reviews the functions of immune cell populations which have been implicated at some level of the rejection process, including T lymphocytes, monocyte-macrophages, dendritic cells, B lymphocytes, and null lymphocytes of the natural killer (NK) or killer cell type. The current understanding of antigen presentation and recognition by immune competent cells is reviewed. Finally, the participation of the various cell populations as effectors (or suppressors) of the immune response in each type of rejection is discussed.

II. GENERAL CONSIDERATIONS FOR MODELS OF ALLOGRAFT REJECTION

A. Type of Tissue Transplanted

In an overview of effector mechanisms involved in allograft rejection, it is useful to bear in mind that the type of tissue transplanted influences the nature of the effector mechanisms generated to destroy it. There are apparent differences in the rejection of skin grafts, primary vascularized organ allografts (e.g., kidneys and hearts), and dissociated cell grafts, such as bone marrow transplants. Skin grafts, for example, are unusually susceptible to rapid rejection.

Factors that may explain differences in the sensitivity of various types of solid allografts to rejection include the type of circulatory connection of donor graft to host, for example, the primary large vessel anastomoses in organ transplants versus delayed vascular capillary connections like those seen with skin grafts. Another major factor is the extent to which recipient alloreactive cells come into contact with donor antigen presenting cell (APC) populations in the graft. APCs express major histocompatibility complex (MHC) class II antigens and include tissue macrophages, dendritic cells (Langerhans cells in skin), and some endothelial cells. These APCs, particularly the dendritic cells within the allograft, are highly effective stimulators of the rejection response (5–7).

Even basic surgical variables can influence putative immune factors. Examples include the volume of perioperative blood transfusions that has been postulated to contribute to heart and liver transplant success, possibly by diluting antibody titers (8). The degree of ischemia-reperfusion injury, related to cold and warm ischemic time, may damage graft endothelium, increasing immunogenicity and activating nonspecific inflammatory mediators.

B. Immunogenetic Variables

The nature of the alloantigen disparity between recipient and donor appears to be a dominant factor in the relative role of cytotoxic T cells, helper T cells, and antibody in rejection. Activation of CD4 and CD8 T cells is guided by the recognition of MHC class II and class I gene products, respectively (9). Different classes of histocompatibility antigen mismatch elicit variable immune effector mechanisms of rejection, a concept that has important implications for clinical transplant immunosuppression and specific tolerance induction strategies. Inbred rodent strains, particularly congenic strains, are invaluable for delineating these immunobiological variables. The relative in vivo roles of specific effector mechanisms of graft rejection in large outbred species, including humans, are more difficult to study, although certainly more relevant to clinical practice.

C. Recipient Immune Responsiveness

Immune response genes, which influence both quantitative and qualitative aspects of specific immune effector responses, are linked to the major histocompatibility complex (10). Antigen presenting cells regulate immune responsiveness, a property associated with MHC antigen expression, particularly MHC class II surface molecules. A unique aspect of alloimmunity is the ability of either host or donor allogeneic APC to present antigen (9). In the first situation, the host APC can present a complex of self-MHC and processed alloantigen peptides to the T-cell receptor of alloreactive clones. It has been assumed that the donor

APCs present native alloantigen, although other endogenous or exogenous peptides complexed to the allogeneic surface MHC molecules may also be presented to responding T cells. The two pathways of APC presentation of alloantigen impose different MHC restriction patterns on the reactive clones and likely influence the effector response.

There are suggestions from clinical transplant studies that some individuals are low or high responders to allografts. In humans, the putative immune responder genes have been difficult to define, although the DRw6 gene has been associated with high responder status in kidney transplantation (11). The issue of immune responder status in human transplantation is important and merits further study. For example, as seen in multiple transplants, there are patients who are seemingly unresponsive to transplants, irrespective of their degree of histoincompatibility, and others respond strongly despite only minor differences in histocompatibility.

D. Species Variability

The species of the recipient-donor combination must be considered in studies of rejection effector mechanisms. There are dissimilarities in the susceptibility of different species to certain alloimmune effector mechanisms. For example, complement-fixing antibodies against donor MHC alloantigens often induce hyperacute rejection in humans, whereas seemingly analogous antidonor antibodies in certain rat strains can enhance or prolong the survival of a rat allograft indefinitely. The biological basis for such discrepancies is not fully understood, but immunogenetic differences in serum complement activity and the expression of histocompatibility antigens on endothelial cells are possible explanations (12). A variety of procedures, including injection of limited amounts of specific antibodies, tissue antigens, or antigen–antibody complexes, may abrogate the rejection response, resulting in prolonged graft tolerance in rats, but apparently have limited effects in higher mammals. Despite some difficulties in extrapolation of rodent data to higher mammals, however, inbred rodent models have unique advantages for basic studies of allograft rejection. The availability of syngeneic, congeneic, and allogeneic strains allows the control of histocompatibility factors in experimental design.

III. IMMUNE CELLS INVOLVED IN REJECTION

A. Central Role of T Cells in Rejection

Thymus-derived (T) lymphocytes have an essential role in acute allograft rejection. Hosts naturally or experimentally deprived of T cells (e.g., nude mice, SCID mice, or thymectomized animals) are unable to reject allografts in a first-set fashion, a deficiency that is readily overcome in experimental transplant models by repopulating the host with viable syngeneic T cells (1,3,13,14). In human transplant recipients effectively depleted of circulating T cells by prophylactic treatment with rabbit antithymocyte globulin, acute rejection rarely occurs during the period of T-cell lymphopenia, but usually occurs during or after recovery of T-cell levels (15). Current evidence implicates T-cell involvement at many levels of the alloimmune response, including antigen recognition, immunoregulation, and cytolytic effector function. However, the actual cellular and molecular mechanisms by which T lymphocytes orchestrate the rejection scenario and the donor-specific versus nonspecific nature of the response are not yet completely understood.

B. T-Cell Maturation

A primary tenet in allograft rejection is that the recipient immune system has the capacity to distinguish between self and nonself. The process of T-cell maturation is largely responsible for this ability. During the ontogeny of T lymphocytes, stem cells committed to the T lymphocyte lineage travel to the thymus, and, under the influence of thymic hormones, they undergo a process of maturation and differentiation.

The realization that the thymus is required for T-cell production has come from the fact that T cells are rare in patients with thymic aplasia (Di George's syndrome) and in nude rodents. However, because nude mice are not totally devoid of T cells, some limited extrathymic differentiation of T cells is possible.

The intrathymic process of T-cell maturation involves a division of cells into distinct subsets based on CD4 and CD8 expression, gene rearrangement related to T-cell receptor (TCR) expression, and "selection" of clones of T cells to be released or deleted. Stages of thymocyte maturation can be correlated with phenotypic expression of CD4 and CD8 (16). T-cell stem cells lack expression of CD4 and CD8 (double negative, or DP). These cells also do not normally express TCR molecules and are normally the only subset that actively proliferates within the thymus. During maturation, DN cells are stimulated to express both CD4 and CD8, becoming double positive (DP) in the thymic cortex. $CD4^+$ $CD8^+$ cells account for 80% to 85% of thymic suspensions and express low levels of TCR molecules. Fully mature T cells are typically "single positive," being either $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ and express high levels of TCR.

The TCR is responsible for the ability of T cells to respond to diverse antigens. A single T-cell clone is recognizable by expression of identical receptor molecules. The ability to generate large numbers of unique T-cell clones (unique TCR populations) depends on DNA rearrangement of receptor genes. The TCR molecule is a heterodimer. On DN cells maturing into DP cells, a "prereceptor" heterodimer consisting of a β chain and another TCR chain termed gp33 is expressed (17). A small portion of DN cells express a $\gamma\delta$ TCR, but details regarding the destiny of these cells are uncertain (18). Most DN stem cells are committed to $\alpha\beta$ TCR expression by virtue of activation of a "γ-silencer" (19). During T-cell maturation, DNA rearrangement brings elements of discontinuous gene regions termed V (variable), J (joining), and C (constant) together to form different α and β chains (20).

T cells expressing an $\alpha\beta$ TCR must undergo a selection process involving positive and negative signals before release into the circulating lymphocyte pool. The T-cell selection process starts in the thymic cortex, where $CD4^+$ $CD8^+$ $\alpha\beta$ TCR^+ cells encounter MHC molecules. Both the TCR and the CD4/CD8 molecules participate as coreceptors. Class I MHC recognition results in a positive selection of CD4 (downregulation of CD8), whereas class II MHC recognition results in retention of the CD8 molecule (21,22). Positively selected DP T cells, therefore, mature to become SP (CD4 or CD8) T cells and migrate into the thymic medulla. Within the medulla, the T cells undergo a process of negative selection, during which cells with an unacceptably strong interaction with self-MHC become apoptotic (23). This is one mechanism of maintaining self-tolerance. Other mechanisms are discussed in Chap. 5.

C. Functionally Defined T-Cell Subsets

In terms of functional activities, T cells can be grouped into major populations that mediate either effector or regulatory functions. Effector T cells include those that mediate

delayed-type hypersensitivity (T_{DTH}) with associated mediator release, and cytotoxic T lymphocytes (CTL) that lyse target cells (24,25). Regulatory cells include helper T cells (Th), which upregulate immune responses, or suppressor T cells (Ts), which downregulate immune responses (26–28).

1. T_{DTH} Cells

The classic example of T_{DTH}-mediated function is Koch's observation in guinea pigs in which a severe skin reaction occurred 2 to 3 days after a second exposure to tuberculin (29). The cellular basis of this reaction was established by adoptive transfer experiments in which sensitivity was transferable to naive animals by lymphocytes (30). Later studies identified the infiltrating cells as $CD4^+$ helper T cells. T_{DTH} cells produce a variety of lymphokines, including interferon (INF)-γ, interleukin (IL)-2, IL-10, and tumor necrosis factor (TNF)-α, which elicit an influx of activated mononuclear cells and induce pathological changes characteristic of T_{DTH} reactions (31,32).

2. Th1 and Th2 Subsets

The CD4 T-cell subset mediates T helper and inducer activities and constitutes the major population responding in one-way mixed lymphocyte responses. Studies with cloned T cells showed that allogeneic Th cells are not homogeneous. In the mouse, at least two types of Th cells have been identified on the basis of functional parameters (25). One type contains the inflammatory Th cells, or TH_1 cells. These cells produce lymphokines involved in polyclonal activation (IL-2 and IL-3), DTH reactions (γ interferon), and cytotoxicity (lymphotoxin). TH_1 cells respond to IL-2 and express IL-2 receptors but lack receptors for IL-1. TH_2 cells are regulator cells that provide help for B-cell responses and some cytotoxic T lymphocyte (CTL) responses. TH_2 cells produce IL-3 (as do TH_1), IL-4, IL-5, and IL-6 and can respond to IL-1, IL-2, and IL-4 alone or in combination.

3. Suppressor T Cells

Suppressor T cells have been functionally identified in numerous in vivo and in vitro experimental models (33,34). Suppressor cells are most commonly associated with the CD8 phenotype, although exact phenotypic identification of suppressor T cells has been difficult to achieve. These regulatory cells inhibit the effector functions of other cells in the immune system. Suppression occurs either by cell–cell interactions or via the production of soluble suppressor factors that impair the T helper cell functions that are crucial for the function of effector cells (35,36). Suppressor and helper cells appear to regulate the activities of each other through immunoregulatory circuits that maintain homeostatic balance in the immune system.

4. Cytotoxic T Lymphocytes

Cytotoxic T cells (CTL) have a critical role in the elimination of virus-infected cells, syngeneic tumor cells, and allogeneic tissue or organ grafts (3,37). Allospecific CTL usually express the CD8 phenotype, although antigen-specific $CD8^-$ $CD4^+$ CTL populations have been described (24,38). The CD8 phenotype is also expressed by many nonspecific cytotoxic effectors (39).

Two distinct cytolytic mechanisms by which CTL destroy allogeneic target cells have been characterized. One, the perforin-mediated lytic process is biphasic and includes a

Mg^{2+}-dependent adhesion phase (inhibited by anti-LFA-1 antibodies) and a Ca^{2+}-dependent lethal hit phase (40,41). In the Ca^{2+}-dependent phase, perforin is inserted into the target cell membrane and cytolytic granules are released, resulting in osmotic lysis of the target cell. Structurally, perforin is similar to the membrane attack complex of complement (42). Studies indicate that perforin is not expressed in freshly isolated normal CD8 cells but is induced after CD8 cell activation (43). The second, the Fas-Fas ligand cytotoxic pathway, involves an interaction between the Fas antigen (CD95, APO-1) and the Fas ligand (44,45). Both cytotoxic T cells and NK cells can use either the perforin or Fas-L pathways to destroy target cells.

The expression of Fas-L mRNA and perforin mRNA directly correlate with the histological severity of acute rejection (46). There is evidence that, within the T-cell population, Fas-L–mediated cytotoxicity is principally a function of $CD4^+$ cells (47).

5. Division of CD4 and CD8 Populations

Distinction of two T-cell subsets (CD4 and CD8) followed the development of monoclonal antibodies that could recognize T-cell differentiation antigens. $CD4^+$ T cells recognize or are restricted by MHC class II molecules in contrast to $CD8^+$ T cells, which recognize or are restricted by class I (48,49). Assignment of certain T-cell functions based on cell surface phenotypes has generally proven to be inexact. In general, $CD4^+$ cells have been associated with helper functions and $CD8^+$ cells with suppressor or cytotoxic effector activity, but there are multiple functions attributed to each of the major T-cell subsets, and some functional overlap has been observed (50,51). $CD4^+$ $CD45R^+$ cells and $CD4^+$ $CD29^+$ phenotypes have been shown to identify naive and memory populations of helper cells (52). The expression of the CD29 antigen on memory Th cells may provide potential opportunities for immunomanipulation of this population, which is difficult to inhibit with conventional immunosuppressive agents.

There are phenotypic properties of $CD8^+$ T cells that appear to identify killer and suppressor effector cells. $CD8^+$ T cells that express the $CD11b^+$ phenotype, the $CD28^-$ phenotype, or the S6F1 phenotype have all been shown to have functional suppressor cell activity without cytotoxic activity (53,54). Flow cytometry analyses of fine-needle biopsies of infiltrating cells in human kidney allografts have yielded data that supports this concept. Low percentages of $CD8^+$ $CD11b^+$ cells have been observed in rejecting allografts; conversely, high levels of this subpopulation have been observed in nonrejecting grafts (55,56).

IV. DOES A SPECIFIC T-CELL SUBSET PREDOMINATE IN THE REJECTION PROCESS?

Allograft rejection involves antigen-specific and nonspecific functions of immunocompetent cells and their soluble products. The cells participating in rejection include phenotypically and functionally distinct effector cells, and the predominance of any one cell type or mechanism depends on a variety of factors. Influential factors include the genetic constitution of the recipient, such as the high-responder DRw6 genotype in humans (11), the degree of expression of MHC class II antigens on parenchymal cells of the graft (57), the type of immunosuppression used, the type of graft, and the nature of the mismatched alloantigens, that is, MHC class I only, class II only, both class I and II, or non-MHC antigen disparities (58,59).

A. Nonspecific Components of Rejection Response

The DTH response may be considered an example of the efficacy of the nonspecific immune response components, in that very few specifically sensitized T helper cells can be efficiently amplified by recruiting nonspecific effector cells to inflict tissue destruction. These nonspecific elements can be directly or indirectly related to the function of the TH_1 subset with its associated lymphokine products IL-2, IL-3, and INF-γ. Interleukin-2 amplifies the alloactivated T cell subsets and enhances the cytotoxic activity of CTL, natural killer, lymphokine-activated killer, and antibody-dependent killer cells in the graft (32,60). Interleukin-3 induces Thy-1 expression and promotes the expansion of macrophages and monocytes (61). Activated T cells secrete INF-γ, a potent upregulator of MHC class II gene expression and macrophage secretory functions (62). Activated macrophages and monocytes in turn produce tumor necrosis factor, which causes further tissue destruction (63).

B. Relative Importance of CD4 and CD8 Subsets in Rejection

Several lines of evidence suggest that the CD4 subset and its lymphokine products are the principal mediators of rejection (reviewed in [64]). In an adoptive transfer model in thymectomized, irradiated, bone marrow-repopulated (ATXBM) mice, it has been shown that only the CD4 (Lyt 1^+) subset could reconstitute the rejection response (65). In one study, CD8 cells failed to induce rejection even if administered with exogenous IL-2, although they collaborated with CD4 cells to increase the tempo of rejection (66). In other experiments, the restoration of brisk rejection occurred only when mixtures of CD4 and CD8 cells were infused (67), suggesting that, although CD8 cells are important effectors, they are not sufficient to independently orchestrate rejection in these models. Similar trends have been reported in rejection of multiple non-MHC mismatched grafts in SCID mice (68). The finding that rejection could be induced by infusion of cloned T helper cells supports the notion that CD4 cells are necessary and sufficient for rejection (69).

Studies of the cellular response to allografts expressing isolated MHC class I or class II disparities have furthered the understanding of the relative roles of CD4 and CD8 T cells. In general, adoptive transfer experiments have shown that $CD8^+$ cells initiate rejection of class I disparate grafts and $CD4^+$ cells initiate rejection of class II disparate grafts (16,70). However, this MHC class distinction is not absolute. $CD4^+$ cells alone have been shown to be sufficient for MHC class I mismatched allograft rejection, perhaps by providing help for the generation of alloantibody (71).

Although the evidence that CD4 cells mediate rejection is convincing, the extraordinary specificity of the rejection process in many experimental models is difficult to reconcile with a lymphokine mechanism (69,72). This suggests that a highly specific discriminating effector mechanism, such as T-cell cytotoxicity, is operative in such systems.

Studies of graft-infiltrating cells during rejection have demonstrated a predominance of $CD8^+$ cells (73). The experiments of LeFrancois and Bevan demonstrated that, although Lyt 1^+ (CD4) cells restored alloimmune competence and induced rejection of non-MHC–incompatible skin allografts in ATXBM-treated mice, the recipients generated graft-specific CTL, which proved to be of host origin (74). These observations are consistent with the hypothesis that alloreactive CTL have a role in the rejection process. Alternatively, the CD8 cells in the grafts of recipients reconstituted with CD4 cells may represent populations of broadly alloreactive NK cells or leukocyte-activated killer (LAK) cells, as others have suggested (75).

The controversy over which T-cell subset mediates rejection may be artificial. Most likely, the CD4 and CD8 subpopulations collaborate to a greater or lesser degree, depending on the nature of the tissue transplanted and the donor-recipient histocompatibility differences. Thus, in class I–incompatible grafts, CD8 cells are expected to have a predominant role, whereas in class II–incompatible grafts or combined class I– and II–incompatible grafts, CD4 cells and lymphokine-activated nonspecific effector cells have a dominant role. Several lines of evidence derived from adoptive transfer studies and monoclonal antibody treatment models support this hypothesis (58,59,76).

V. T-CELL SUBSETS IN HUMAN KIDNEY ALLOGRAFTS

Phenotypic analyses of infiltrating cells in biopsy sections of acutely rejecting human allografts reveal a predominance of the cytotoxic-suppressor T-cell subset. Furthermore, graft-infiltrating cells have been shown to exhibit specific donor-directed T-cell cytotoxic activity (3,77). Conversely, in quiescent or nonrejecting human allografts, the number of infiltrating cells expressing the phenotype of the T helper-inducer subset tends to be higher than the number of cells expressing the cytotoxic-suppressor phenotype (78). This general pattern prevails in both experimental animal and human allografts. However, there are notable exceptions, and some reports have described an opposite pattern, with a preponderance of the T helper subset in rejecting grafts, especially in irreversible rejection (79,80).

In general, the phenotypic ratios of helper or cytotoxic subsets in rejecting human allografts do not have clear prognostic implications for the reversibility of the rejection episode with immunosuppressive agents. Other factors, such as intensity and diffuse versus focal patterns of T-cell infiltration may be more clinically significant (81).

In early acute cellular rejection, there consistently appears to be an increase in the expression of the IL-2 receptor and of HLA DR, indicating an increase in the number of activated T cells (82). At this early stage, a predominance of $CD4^+$ cells in the graft has been described, although this was not consistently confirmed (80,83). Subsequently, $CD8^+$ cells (cytotoxic suppressor T cells) appear to become more numerous within the core biopsy specimens of rejected grafts (56,84). In a recent study, biopsy specimens from rejecting allografts contained significantly more $CD8^+$ and $CD45RO^+$ cells than biopsy specimens from nonrejecting grafts (85). Severe cellular rejection is associated with an influx of macrophages with strong DR expression (86).

Two-color monoclonal antibody labeling and analysis by flow cytometry has made it possible to further dissect mononuclear cell subsets. In rejection, various researchers have found elevated DR expression on CD3, CD8, CD16, and macrophage subsets, indicating an activated state in these cell populations (87). Furthermore, suppressor and suppressor inducer cells are less numerous in rejection (55,56). These morphological findings suggest that the cellular interstitial graft infiltrate consists of dynamically fluctuating cell populations. Decreased numbers of phenotypically defined suppressor-inducer and effector cells during rejection may indicate an active downregulatory mechanism in stable grafts. Reappearance of HLA DR on various subsets of infiltrating cells (and on the parenchymal cells of the donor organ) underscores the important role of this molecule in the process of immune activation.

VI. OTHER ASSOCIATIONS BETWEEN CTL ACTIVITY AND ALLOGRAFT REJECTION OR ACCEPTANCE

In most mammalian species, cytotoxic lymphocytes are frequently observed during allograft rejection (3,88,89). In contrast, allospecific antidonor CTL are rarely detected in

experimentally tolerant recipients or in nonrejecting human transplant recipients (52, 89,90). During allograft rejection, effector cells exhibiting specific antidonor cytotoxicity have been recovered from peripheral lymphoid tissue, from the circulation, and from the rejecting allograft (3,91).

The high number of sensitized effector cells required to kill relevant allogeneic target cells in vitro (commonly a 100:1 ratio) and the prolonged incubation period required to detect significant cytotoxicity in vitro (usually 4–8 hours) have cast doubt on the in vivo effectiveness of the CTL effector pathway in rejection. Furthermore, the observation of Dallman et al. that the kidneys of operationally tolerant rats contain donor-reactive CTL is difficult to reconcile with the CTL concept of rejection (92). Because lymphoblastoid targets were used in the assay system, it is possible that target cell–derived lymphokines may have overcome a state of maturational arrest of poised CTL present in the tolerant grafts. Ascher and colleagues demonstrated that precursor CTL undergo maturation directly within the allograft (88). Thus, there may well be local mechanisms in a rejecting graft to foster the differentiation of CTL. In contrast, in tolerant grafts, there may be in situ suppressive mechanisms to curb CTL maturation. Studies on tolerant primates support such a concept (89).

VII. NATURAL KILLER CELLS IN REJECTION

Studies of human kidney, human heart, and rat kidney allografts indicate large granular lymphocytes (LGL) are among the first cells to appear during rejection (93,94). The LGL population has been shown in most species to contain NK cells that destroy certain tumor and virus-infected target cells in the absence of known prior immunization (95). The NK cell population is derived from bone marrow and expresses unique phenotypic characteristics as well as phenotypic characteristics partially associated with T lymphocytes and monocytes. NK cells appear to have a role in control of malignancy and viral infections and in the rejection of bone marrow transplants (96).

Studies of LGL and NK activity in rat renal allografts have revealed differences in the timing and location of NK and CTL activity in the spleen and graft (94). Sequential phases of high NK activity were observed, first in the spleen, then in the graft, and again in the spleen, suggesting an NK traffic circuit between spleen and allograft. Findings in human renal allografts indicate that NK-like cells (phenotypically Leu 7^+) accumulate in allografts very early in the course of rejection episodes, before clinical manifestations of rejection are evident (97). Adoptive transfer studies in nude rats in which a $CD4^+$ cell inoculum restored rejection have implicated host NK and LAK cells as effectors of graft destruction (75).

It is possible that NK cells have a role in the maturation or regulation of CTL or regulation of other cells of the immune system. It is known, for example, that activated NK cells produce γ interferon (95). Alternatively, NK and CTL activity may be dual functions of a single subpopulation present in rejection grafts (98).

VIII. MACROPHAGES IN REJECTION

Studies of infiltrating cells from acutely rejecting allografts consistently show a high proportion of macrophages (20%–60%) in the infiltrate (99). Several lines of evidence indicate that increased vascular permeability, fibronectin, and T-cell derived lymphokines (MAF, INF-γ, and others) are responsible for the localization of macrophages in areas of inflammatory injury, including allograft sites. It is difficult to establish whether macrophages are

active effectors of allograft destruction, or whether they play an accessory role (100). It has been suggested that the presence of large numbers of macrophages in the rejecting allograft is associated with a poor prognosis (101). Attempts to study the role of macrophages in rejection are confounded by a number of factors, including the inability to establish in vivo models of macrophage depletion.

It has long been recognized that the macrophage has an important function in antigen processing for optimal elicitation of in vitro alloimmune responses. The role of the macrophage in inducing acute inflammation and in amplifying allograft reactivity is likely related to IL-1 production. Interleukin-1 has systemic effects, producing fever and negative nitrogen balance and can trigger a cascade of local inflammatory activities (102). Interleukin-1 stimulates T helper cells to produce IL-2, which, in turn, leads to expansion and amplification of activated cytotoxic T-cell clones, and augments suppressor T-cell activity as well as natural killer activity. In addition, IL-1 acts to stimulate B cell proliferation. Interleukin-1 is also known to stimulate fibroblast proliferation, which may contribute to the chronic inflammatory processes seen in chronic allograft rejection.

Recently, several studies have implicated macrophages as active participants in the rejection process. Monoclonal antibody staining techniques have demonstrated increased macrophage populations in acutely rejecting allografts (103–105). The identification of MRP8 and MRP14 (proteins expressed by differentiating monocyte/macrophages) in acutely rejecting renal allografts add further support to the importance of these cells in acute rejection (106). The presence of a large number of HLA-DR$^+$ macrophages has been correlated with a poor prognosis (107). Interestingly, activated intravascular macrophages may resemble thrombi filling the vessels (105).

Macrophages have been shown to be critical in the process of chronic rejection (108,109). Gamma lactone, an inhibitor of macrophages, has been shown to prevent the changes associated with chronic rejection (110).

Pharmacological agents that affect IL-1 production may offer important avenues for the clinical management of transplant recipients. Cyclosporine, a potent inhibitor of IL-2 production, has no apparent direct effect on IL-1 production. In contrast, both in vitro and in vivo pharmacological doses of steroids act to strongly inhibit or suppress completely the production of IL-1 (102). This provides a rationale to support the use of high doses of steroids in the early posttransplant period, when inflammatory reactions involving IL-1 may be occurring in the recently transplanted allograft, and as treatment of acute rejection.

Current understanding is still rudimentary regarding the biochemical mechanisms that regulate IL-1 production by macrophages as well as the in vivo relevance of IL-1 to the cellular immune effector mechanisms of graft rejection. Much work is necessary in this area before it is known what pharmacological agents may be useful in the modulation of production or activity of this potent stimulator of inflammation.

IX. LYMPHOCYTE MIGRATION PATTERNS DURING REJECTION

The migration of circulating lymphocytes into lymphoid and nonlymphoid tissues, such as an allograft, is guided by the expression and interaction of homing receptors on the lymphocytes and specific ligands, called vascular addressins, on the endothelial cells (111, 112). The continuous recirculation of lymphocytes into lymphoid tissues occurs at the site of the high endothelial venules (HEV) in the lymph nodes. This is presumed to be regulated by the surface expression of specific vascular addressins. Lymphocyte maturity, activation state, and perhaps initial activation site appear to be major factors involved in lymphocyte adhesion at HEV ligands.

The MEL-14 antigen on mouse T cells and the Hermes antigens on human T cells have been shown to be involved in lymphocyte-HEV adhesion. These 85–95 kD homing receptors are recognized by a variety of monoclonal antibodies and can be identified by an ex vivo adhesion assay (113). Activated T cells lose the MEL-14 receptor and are unable to home into lymphoid tissue (114). Immunohistological studies of mouse skin allografts revealed that MEL-14–positive cells are conspicuously excluded from the infiltrating populations (115,116). It is possible that particular T-cell subsets that are activated distally home into grafts after losing the MEL-14 receptor or, alternatively, are confined in the graft if activated in situ. Using lymphocyte-endothelium binding inhibition assays, Jalkanen and colleagues have demonstrated site-specific endothelial homing receptors (117). The endothelial addressins are differentially expressed at distinct locations, such as MECA 79 on peripheral lymph node HEV and MECA 367 on mucosal lymphoid tissue. The current concept is that the tissue-specific addressins control lymphocyte-endothelial cell interactions and traffic flow in and out of the blood. The activation state of the endothelial cells in sites of acute and chronic inflammation is also important because it alters the expression of vascular addressins, increasing lymphocyte-tissue traffic and sequestration patterns in rejecting allografts.

Hematopoietic cells have characteristic migration patterns. For example, most polymorphonuclear leukocytes appear to be marginated in the small capillaries of the body, and only a small portion circulate freely within the peripheral bloodstream. Lymphocytes migrate in massive numbers between the bloodstream and the lymphatic vessel space, finally converging via the lymphatics to the thoracic duct, where they are returned to the bloodstream. Modification of the lymphatic drainage of a skin allograft can change the patterns of rejection (118). On the other hand, the isolation of a primarily vascularized kidney allograft from its lymphatic connections was shown not to prevent or modify the rejection response (119). During the course of allograft rejection, immunocompetent cells appear to migrate among various lymphoid depots in the body, the peripheral circulation, and the allograft (120,121). The allograft is often distant from lymphoid compartments, and, presumably, the migration patterns of lymphoid cells are related to immunological mechanisms designed to recruit and concentrate immunocompetent cells in the allograft. Nemlander et al. studied the flow of white cell traffic to and from rat renal allografts in unmodified hosts and found that the traffic was bidirectional and increased exponentially until post-transplant day 4, after which there was little intercompartmental exchange (122).

Most of the infiltrating cells found in allografts do not exhibit donor-specific reactivity (121,123). The reasons that large numbers of nonspecific immunocompetent cells accumulate early after transplantation are not clear. Certainly, the ischemic injury and the trauma of reimplantation could elicit inflammatory stimuli associated with wound healing. In rat allograft models, even the early inflammatory cell influx during the first 4 to 5 days has been found to be largely nonspecific, containing high levels of macrophages and NK cells within syngeneic grafts and allografts in the immunoincompetent ATXBM rats (2,73,94).

In addition to the nonspecific inflammatory cells infiltrating allografts, there is an accumulation of specific donor-reactive cytotoxic and helper cells in the peripheral lymphoid compartments and in the allograft (120,121). In the rat renal allograft, donor-specific CTL are usually found in the spleen within 4 to 5 days after transplant and simultaneously, or shortly thereafter, within the allograft (122). In unmodified rejection, there seems to be a much higher level of CTL activity in the allograft than in any of the systemic lymphoid depots, but increases in blood CTL precursor frequencies have been reported during rejection of human kidney grafts (124). Whether this represents the local differentiation and

expansion of donor-reactive CTL or selective migration of these cells into the graft is not certain. However, the study of Ascher et al. supports the former notion (88). These investigators placed sponge matrix allografts on recipient mice and then irradiated the recipients on the 5th day after transplant, with one of the grafts selectively shielded from irradiation. Antidonor cytotoxic cells developed to a much higher level in the shielded sponge matrix allograft, leading to the conclusion that CTL precursors present in the allograft matured in situ, independent of the host lymphoid system after day 5 posttransplant. This result supports the findings of Nemlander's group indicating that there is little cellular traffic between rat lymphoid compartments and the allograft after day 4 (122). If this presumption is correct, then the migration of lymphocytes, inflammatory cells, and antidonor cells into the allograft is probably most important in the early stages of rejection, as opposed to the later stages when the immune response within the allograft may become independent of lymphoreticular traffic.

X. VASCULAR ENDOTHELIAL CELLS IN REJECTION

The endothelium of a primary vascularized allograft such as a kidney is a critical component of the rejection response. The importance of the endothelium is highlighted by the fact that vascular abnormalities are prominent in all forms of rejection. Even though it is evident that the interface between an allograft and the recipient's circulating lymphocyte pool is the graft endothelium, it has not always been considered an active participant in the immune response.

The antigens expressed on kidney endothelium include those of the ABO system, MHC class I and class II (the latter is inducible by exposure to INF-γ), and, perhaps, endothelial cell (EC) specific antigens (125). Host antibody directed against EC antigens may initiate hyperacute rejection despite ABO and HLA compatibility and has been implicated in later types of rejection as well (126).

In addition to passively expressing antigens on the cell membrane, EC have been implicated as functioning APCs capable of initiating a host antigraft immune response (127). Recent research has shown that T cells cocultured with EC proliferate and produce cytokines apparently in the absence of dendritic cells. The costimulatory molecules implicated in T-cell activation by EC include CD2-CD59 and CD 44, but not intercellular adhesion molecule-1 (ICAM-1), ICAM-2, vascular cell adhesion molecule-1 (VCAM-1), or CD28. Interestingly EC APC function may overcome the ability of cyclosporine and FK506 to suppress IL-2 synthesis (128,129).

With respect to immune cell response to the allograft, the expression of adhesion molecules is an essential aspect of recruitment of effectors of the immune response. Endothelial cells actively increase expression of adhesion molecules in response to both specific and nonspecific inflammatory mediators and tissue injury. For example, E-selectin (ELAM-1) expression is stimulated by oxygen-derived free radicals, histamine, and thrombin, and endothelial cell adhesion molecule–1 (ECAM-1) and VCAM-1 expression are stimulated by IL-1 and TNF (112,127). In addition to providing adhesion molecules, ECs stimulate macrophage entry into the graft by synthesizing MCP-1/JE, a monocyte chemoattractant, when exposed to IL-1β, TNF, and INFγ (130). Recent studies have suggested that the focal areas of high endothelial venules in organ grafts regulate the diapedesis of cells into the interstitium of the donor organ. An in vitro model of this phenomenon has been recently developed in which allogeneic CTL alter and traverse allogeneic endothelial cell monolayers (131).

Finally, the involvement of the endothelium in the effector arm of the immune response has become more apparent. Local release of nitric oxide and prostaglandin I_2

(PGI_2) increase vascular permeability, allowing immune cells to traffic to the graft interstitium. EC may be injured by, and even produce reactive oxygen species during inflammation, ischemia-reperfusion, or rejection. Endothelial cell injury may, in turn, increase immunogenicity of an allograft. Perhaps the ultimate consequence of immune-mediated allograft injury is thrombosis, a process in which the EC plays a key role. Thrombosis in hyperacute and aggressive acute rejection may be related to relative increases in EC-derived procoagulants, including tissue factor, factor V, and plasminogen inhibitor. The thrombotic process may be enhanced by inflammatory cytokines such as IL-1 and TNF (132,133). Antibody binding to EC is associated with loss of heparan sulfate (134). Additionally, the response of EC to inflammatory cytokines can result in activation of the coagulation cascade, platelet deposition, and increased vascular tone (resistance) (135).

XI. ANTIGEN PRESENTING CELLS

The initiation (afferent) phase of acute cellular rejection by recipient T cells requires that foreign antigen be processed and presented to T cells in association with MHC molecules, the "two-signal hypothesis" (136). This is the specialized function of several different cells collectively labeled as "antigen presenting cells" (APCs).

Antigen presenting cells include dendritic cells, macrophages, (activated) B cells, and endothelial cells. The efficacy of the APC–T-cell interaction depends on multiple factors, some of which are yet to be understood. The classical interaction is characterized by recognition of antigen in the context of MHC class II by the TCR. This stimulates T-cell activation, release of IL-2 and other cytokines, and expansion of the cytotoxic T lymphocyte pool.

It is known that, for a T cell to become activated and release IL-2, the APC must provide a number of costimulatory signals in the form of accessory molecules on its cell membrane. The most efficient APC such as dendritic cells and macrophages express large numbers of costimulatory molecules (e.g., B7, ICAM-1) (127). Less efficient APCs such as resting B lymphocytes express low concentrations of these molecules. The costimulator requirements for naive T cells are greater than those of memory T cells, so that only professional APCs, such as dendritic cells, have the capacity to stimulate resting naive T cells (137).

The APC–T-cell interaction does not always result in T-cell activation but may actually induce T-cell anergy. The site of the interaction within the host appears to play an important role in determining the lymphocyte response. In a study reported by Chung et al., intrahepatic, but not intravenous, injection of APC decreased T lymphocyte proliferation in response to restimulation by alloantigen (138).

An interesting feature of APCs is that they are supplied by both the donor and the recipient. This fact provides the distinction between "direct" and "indirect" antigen recognition. Direct recognition is allorestricted, that is, the foreign antigen (peptide) is presented to the host T cell associated with nonself-MHC molecules expressed by the cells of the graft. Indirect recognition is self-restricted as the host APCs present the graft (foreign) peptides associated with self-MHC molecules (see Chap. 1).

XII. REJECTION

Kidneys undergoing rejection by cell-mediated mechanisms usually show significant numbers of plasma cells (mature B cells) on histopathology. In addition, specific antibodies

(usually IgM and IgG) can be eluted from the rejecting kidney and are localized in most parenchymal areas of the kidney. Functioning immunoglobulin-secreting cells can be extracted from rejecting allografts and propagated in MLC cultures (139). Strong B cell immunoglobulin responses and mitogen-induced B-cell responses are associated with human kidney graft rejection (140). B-cell hyperresponsiveness has been reported in sensitized patients who rejected grafts, whereas B-cell hyporesponsiveness may be seen in long-term renal transplant recipients with a well-tolerated graft (141). These studies all suggest that the B lymphocyte plays a role in kidney graft rejection.

Other observations suggest that B-cell immunity is generally neither necessary nor sufficient to mediate allograft rejection. For example the athymic nude mouse and rat do not reject allografts in a normal fashion. Similarly, thymectomized, thoracic duct-drained, B-cell–reconstituted recipients do not reject allografts. However, the large numbers of B cells and plasma cells in some core biopsy specimens of rejecting allografts are unlikely to represent a family of "innocent bystanders." Certainly, the ability of antibody to mediate renal graft injury has been implicated in the pathogenesis of hyperacute, acute, and chronic rejection (see Chap. 6) (8,142,143). This knowledge underscores the numerous interfaces of humoral (B cell) and cell-mediated immunity (antibody-dependent cellular cytotoxicity, macrophage cytophilic antibody, endothelial cell–antibody reactions, eosinophil and mast cell–antibody reaction, and others) that challenge paradigms designed to artificially separate them for analysis. In short, there is reason to believe that B cell products actively participate in many types of rejection. Other cooperative interactions between T and B cells, such as those involving antiidiotype antibodies, regulate the T cell alloimmune response. Further studies should focus on the collaborative role of these two arms of the immune system in rejection.

XIII. IN REJECTION

The population of killer cells mediating antibody-dependent cellular cytotoxicity (ADCC) is phenotypically indistinguishable from the NK population (95). In addition to sharing common membrane antigen phenotypes, both NK and killer (K) cells have IgG Fc receptors capable of affixing cytophilic antibody. In normal individuals, there is usually a high correlation between ADCC and NK activity, although there are documented dissociations of NK and K activity in immunosuppressed and in tumor-bearing hosts (144,145). Unlike NK cells, which are directly cytotoxic to certain sensitive targets in vitro, K cells are indiscriminately cytotoxic to most nucleated target cells, provided that the latter are sensitized with IgG antibody. There are also instances in which K cells appear to exert direct cytotoxic activity when they are armed with cytophilic antibodies via Fc receptors (95).

The apparent phenotypic similarities between K and NK cells suggest that the same cell mediates both cytotoxic activities. Because NK cells have been found in rejecting allografts, the question is raised whether K cell activity (i.e., ADCC) could be involved in graft rejection. The available data are controversial but overall suggest that ADCC may be an ancillary allograft effector mechanism.

Tilney and colleagues demonstrated high levels of antidonor ADCC activity within the infiltrating cell population of rejecting rat heart allografts in unmodified recipients (3). Furthermore, at least half the cells recovered from rejecting grafts were found to exhibit Fc receptors. Nemlander et al. showed donor-directed ADCC in the spleens of unmodified rats rejecting renal allografts, although lower levels were found in the graft (94). Delayed rejec-

tion occurring in antithymocyte globulin (ATG)-treated primates is associated with both donor-directed CTL and ADCC activity (146).

In human transplant recipients, specific donor-directed ADCC has been associated with rejection in a number of studies (5,147). A recent report demonstrated endothelium-specific ADCC in HLA-identical graft rejection (126). However, other studies have failed to detect ADCC during rejection (148). Although several reports have demonstrated the presence of antidonor ADCC in recipients with functioning transplants, others have monitored antidonor ADCC in a series of long-term recipients (2–10 years posttransplant) and found a highly significant association with clinical and histopathological indications of chronic rejection (149). The ADCC-positive individuals all had functioning, but deteriorating, allografts. ADCC appears to represent a highly sensitive, ancillary effector mechanism that may have an important role in delayed or chronic rejection in immunosuppressed hosts and perhaps in genetic recipient–donor combinations that tend to preferentially elicit alloantibody responses.

XIV. TYPES OF REJECTION

A. Hyperacute Rejection

Hyperacute rejection (HR) occurs rapidly, often within minutes of establishing blood flow. The donor organ becomes mottled, cyanotic, and swollen, and transplant function ceases. Histologically, the reaction is characterized by antibody deposition, endothelial damage, fibrinoid necrosis, prominent polymorphonuclear leukocyte (PMN) infiltration, platelet thrombi, and relatively scant lymphocyte infiltration (12,150). This type of rejection is thought to be due to preformed humoral antibody directed against donor alloantigens expressed on endothelial cells. Theoretically, HR ought to be preventable by crossmatching, and improved techniques of crossmatching have markedly reduced the incidence of hyperacute rejection. Nevertheless, this reaction still occurs in perhaps 0.1% to 1% of kidney transplants. To date, the exact pathophysiology of hyperacute rejection has not been fully elucidated. Some investigators have suggested that heightened cell-mediated immunity may be involved (151). It is also possible that ADCC is involved. This is an attractive possibility because ADCC is a highly sensitive mechanism and uses multiple types of naturally occurring cytolytic effector cells that express Fc receptors for IgG. A recent hypothesis is that vascular endothelial cell–specific antibody induces hyperacute or accelerated acute rejection (see Chap. 5).

B. Acute Rejection

Acute rejection continues to be the most common cause of allograft loss in transplantation and can even occur in HLA-identical donor-recipient pairs; the majority of acute rejection episodes are initially reversed with steroids, antilymphocytic globulin, or OKT3. Woodruff, in 1952, noted that acute rejection occurred far more commonly in the initial 6 weeks after allografting and decreased in both incidence and severity after this time (152). The immunobiological basis for this phenomenon (termed immune adaptation) has not been established. Evidence to show decreased immunogenicity in the long-surviving grafts, particularly in larger animals, has not been convincing.

The histopathological features of acute rejection are described in detail in Chap. 11. There is considerable information on the pathology of allograft rejections from studies

using light microscopy and electron microscopy. The reaction is characterized by an acute mononuclear cell infiltrate, often associated with arteritis and perivascular cuffing of mononuclear cells. Immunohistochemical studies have shown that the infiltrating mononuclear cells are heterogeneous and consist of T cells, macrophages, B cells, and large granular lymphocytes, with occasional polymorphonuclear leukocytes, eosinophils, and basophils. The patterns of cellular infiltration may vary between organs and even within the same organ. The cellular mechanisms are complex, and the precise interaction of the various cell populations remains to be defined. Clearly, $CD4^+$ T cells are involved in the induction of acute rejection (153). Serial biopsy studies of rejecting renal allografts have shown infiltration by mononuclear cells, macrophages, and $CD8^+$ and $CD4^+$ T cells. Elaboration of cytokines results in activation and recruitment of specific (CTL, DTH-mediating T cells) and nonspecific (macrophage, NK cells) effector cells within the allograft. The involvement of CTL is supported by the detection of perforin gene expression in biopsy specimens (154). Recent studies have suggested that cytotoxic T-cell responses dominate acute rejection occurring early after transplantation, whereas DTH-type responses appear to predominate in acute rejection occurring late after transplantation (155). The importance of immune cells other than T cells (e.g., macrophages) in the acute rejection response is just beginning to be fully recognized. It can be difficult to interpret the results of histopathological studies of biopsy samples of rejecting allografts because of the uneven distribution of infiltrating cells in different areas (sampling error), difficulties in establishing accurate correlations between morphological and functional properties of mononuclear subpopulations, and the continual trafficking of immunocompetent cells in and out of allografts (120). Many of the lymphoid cells found in the allograft may be bystanders or nonspecific effectors activated by locally secreted lymphokines (75,123,156). For example, PMNs, basophils, and eosinophils present in rejecting allografts probably represent incidentally recruited effector cells.

Antibody is frequently associated with acute rejection episodes, and anti–class I antibody is associated with a worse prognosis (157). The target of these antibodies is the endothelium of arteries, microcirculation, and glomeruli (143). Once a threshold level of antibody deposition is exceeded, effector mechanisms are likely triggered that include recruitment of PMNs and mononuclear cells by virtue of Fc receptors and ADCC via recruitment of LGL.

As described, the graft endothelium is both the initial target for the rejection response and an active participant in the cell-mediated immune rejection process. The degree of vascular injury and the presence of antibody-producing cells in the graft have been associated with a poor prognosis for survival of the allograft (158,159). Permeability changes in the microvasculature may increase the traffic of immunocompetent cells into the allograft interstitium (160). Cellular infiltration, particularly focal infiltration within the interstitium of a graft, can often be rapidly and completely reversed by immunosuppressive agents, whereas vascular damage is more resistant to reversal. Endothelial damage is usually associated with endothelial repair, including infiltration by macrophages and foam cells and smooth muscle proliferation, in a process that resembles the pathological picture of atherosclerosis (133). The process of endothelial repair can result in irreversible damage to allograft vessels, including scarring that may culminate in the "chronic changes" seen in biopsies of allografts succumbing to the inexorable process of late allograft failure. It is important to remember, therefore, that acute rejection may be subclinical as individual episodes, but that the damage to the allograft from each episode may be cumulative, eventually resulting in failure of the graft.

C. Chronic Rejection

Studies of long-term transplant patients have revealed that most acute rejection episodes occur within the first 90 days after transplant, although in all vascularized organs some late acute rejections may be seen. In contrast, chronic rejection proceeds in a slow but inexorable course over months to years. Patterns of chronic rejection take many different forms in different organs. In the kidney, there is a triad of pathological findings that include arteriosclerosis and nephrosclerosis related to endothelial damage, tubular atrophy and interstitial fibrosis related to damage and repair, and glomerulopathy resulting from damage in the glomeruli. In human kidney transplants, the glomeruli appear to be a prime site of immunological attack in chronic rejection. The process of chronic rejection is poorly understood, and both antibody-dependent and -independent events have been implicated (161). Most likely, a cascade of events occurs, beginning with endothelial damage, leading to attraction and activation of circulating lymphocytes. Macrophage recruitment and activation appears to be essential—at least in the rat model—because treatment with gamma lactone (a specific macrophage inhibitor) prevents the functional, structural, and molecular changes of chronic rejection (110). The role of cell-mediated immunity in glomerular damage in allografts is not well understood, partly because glomerular epithelial and mesangial cells may be damaged by recurrent disease, immune complexes, and many other pathological processes unrelated to rejection.

The arterial endothelium is a prominent target organ for most types of chronic rejection and is evidenced in biopsy specimens demonstrating obliterative arteriopathy. Within the intimal hyperplasia of obliterative arteriopathy, there are varying degrees of smooth muscle proliferation and an inflammatory infiltrate of macrophages, T cells, plasma cells, and eosinophils (162). Endothelial cell activation may contribute directly through the synthesis and release of platelet-derived growth factor (133). This type of rejection is believed to be partially mediated by antibody (163,164). Some studies have suggested that chronic rejection involves antibody-dependent cell-mediated cytotoxicity, an effector mechanism that combines humoral and cellular components of the immune system (146,149). Because chronic rejection is the predominant cause of graft loss over time, it constitutes an important immunobiological process that needs to be better understood if the survival of incompatible allografts is to be prolonged for long periods of time. Because chronic changes in the allograft may also occur related to nonimmunological processes, the chronic structural changes in the allograft are usually best regarded as chronic transplant nephropathy in the absence of specific evidence of chronic rejection (see Chap. 7).

D. Xenograft Rejection

Recently there has been a surge of interest in the use of xenografts for transplantation, partially because of the limited availability of human donor organs and increasing demand. The major barrier to xenografting is organ rejection, and the success of xenografts will depend on better control of both humoral and cell-mediated effector mechanisms. This section focuses on cell-mediated immune mechanisms of xenograft rejection, recognizing that there are broad interfaces between cell-mediated immunity and humoral immunity that may be relevant to xenograft rejection. The basic distinction of discordant and concordant species in xenografting is primarily related to the degree of humoral immunity and cell-mediated immunity, respectively. Concordant xenografts appear to be rejected by cell-mediated immune (CMI) mechanisms, which are poorly understood. The earliest human histopathological studies of cell-mediated immunity in xenograft rejection in the human are

from the report of Reemtsma et al. in which 12 human kidney xenografts were implanted from rhesus and chimpanzee donors (165) and from Starzl's report of kidney and liver xenografts from baboons and chimpanzees (166). In the rhesus-human and chimpanzee-human grafts, clinical and histopathological manifestations of cell-mediated immunity were similar to those seen in human donor allografts, although they were generally more severe. Reemtsma et al. documented clinical reversal of xenograft rejection with steroids, with one of the chimpanzee-human xenografts surviving for 9 months. Histopathological analysis of the longest surviving xenograft showed preservation of kidney architecture with mild inflammatory changes resembling that of first-set allograft rejections. In contrast, Starzl's study of the baboon-human xenografts showed a clinically and histopathologically aggressive rejection reaction, with a marked infiltrate of mononuclear cells, evidence of hypersensitivity reactions, and early and severe destruction of the baboon xenografts (166). These important studies demonstrated variability in the rejection of concordant xenografts that depended on the particular species combinations.

The rejection pattern in xenograft combinations with high levels of preformed antidonor antibody or in discordant species is more severe than that seen in allografts (167). In the hamster-Lewis rat xenograft, one of the best examples of a xenograft with preformed antidonor antibody, histopathological findings in rejection showed endothelial destruction, edema, perivascular margination of leukocytes, capillary disruption, interstitial hemorrhage, and parenchymal cell death. The pattern of endothelial destruction shows some similarity to allogeneic hyperacute rejection, with PMN infiltration in the vessel wall and disruption of endothelial integrity. Interstitial hemorrhage and parenchymal cell death were at least partially related to infarcts and thrombosis of the blood vessels.

The possibility of endothelial cell–specific reactivity in xenograft rejection has been raised (168). In vitro studies in mixed human lymphocyte–mouse endothelial cultures showed that the proliferative and cytotoxic response to endothelial cells was usually 15 to 25 times greater than the response in mixed human lymphocyte–mouse lymphocyte cultures. In contrast, mouse–rat combinations (higher concordance than human–mouse) showed a weaker mixed lymphocyte endothelial cell response. Thus, both in vivo histopathological patterns as well as in vitro lymphocyte reactivity patterns suggest that xenograft non-MHC endothelial antigens or endothelial cell activation products may represent major targets in xenograft rejection. Previous studies have shown that levels of T-cell precursors, T helper cell reactivity, and T-cell cytotoxicity are low between xenogeneic combinations (169). However, the recent demonstration of self-restricted T cell recognition of xeno-MHC peptides in an in vivo mouse model implies a role for T cells (170). In addition, anti–human B7 antibody has prolonged human islet graft survival in mice (171).

Several observations implicate non–T-cell–dependent mechanisms in xenograft rejection. The role of natural killer effector cells (which mediate ADCC) in xenograft rejection has been suspected but has not yet been proven (172). A predominance of macrophages in the cellular infiltrate of rejecting porcine islets (in a rat recipient) was recently shown (173). The Chaussy group's finding that antimacrophage serum prolongs xenografts but not allografts is another unexpected finding that suggests macrophages are critical participants in xenograft rejection (174).

In summary, cell-mediated effector mechanisms play a role in some types of xenograft rejection, but they are probably different from those responsible for allograft rejection. It may be that the cellular rejection mechanisms activated in xenograft rejection are those developed by the host to respond to macroparasites (such as helminths), which are

not phagocytized, in contrast to the mechanisms activated in allograft rejection that are related to the immune response to virus-infected cells (173).

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3

Cytokines: Regulators and Effectors of the Immune Response

PHILIP F. HALLORAN
University of Alberta, Edmonton, Alberta, Canada

NELSON GOES
Allegheny University Hospitals, MCP, Philadelphia, Pennsylvania

SING LEUNG LUI
Queen Mary Hospital, Hong Kong, China

I. CYTOKINES IN TRANSPLANTATION: INTRODUCTION

Graft rejection involves interactions among many cells involved in the immune and inflammatory responses and other cells such as endothelial and parenchymal cells. These cells communicate through direct contact using recognition molecules located on their cell surfaces (e.g., major histocompatibility complex [MHC], T cell antigen receptor [TCR], CD4, CD8, CD40L, FasL) and through the production of soluble proteins called cytokines. Cytokines are produced by a cell to engage specific receptor proteins on other cells—remote (*endocrine*) or adjacent (*paracrine*)—or on the same cell (*autocrine*). Cytokines act typically in low concentrations over short distances, but some cytokines participate in systemic reactions induced by local inflammation. The cloning of the cytokines, their receptors, and their signal transduction mechanisms has provided many insights into these events and has opened new possibilities for intervention. This chapter focuses on concepts and general principles in the cytokine field. Further details are available in recent reviews of cytokines, cytokine receptors, and signal transduction mechanisms (1–5). (Note that chemokines are not covered in detail in this discussion but will be mentioned in some parts of the review, e.g., IL-8).

Many cytokines are detected in any inflammatory site, complicating the understanding of the role of the individual cytokines. The use of mice with disrupted cytokine or

cytokine-receptor genes is helping to unravel this complexity. The use of such "knockouts" gives a new view of the redundancy and the uniqueness of the processes in inflammation. However, cytokines are not usually loners: "be careful—they usually travel in groups" should be the operative consideration.

II. GENERAL FEATURES OF CYTOKINES AND CYTOKINE GENES

Cytokines are proteins of relatively low molecular weight (15–25 kD), which mediate interaction between cells through specific membrane receptors. Most cytokines consist of a single polypeptide chain of approximately 100 to 200 amino acids that functions as a single domain. Cytokine genes are usually small and typically consist of four to five exons, including a leader sequence. The 3′ untranslated region is often rich in AT sequences, which probably confers a very short half-life on the mRNA. This, together with the short half-life of the cytokines themselves, enables fine regulation of cytokine expression. The regulation of cytokine gene expression occurs mainly at the transcriptional level, although certain cytokines such as transforming growth factor-β (TGF-β) are also regulated posttranscriptionally.

A domain is a sequence or segment of a protein that is likely to form a discrete structural unit. Three criteria are used to identify a domain. First, domains are established by their tertiary structure, for example, being immunoglobulin-like. Second, superfamily segments that exist as the sole extracellular sequence, as well as sequence contiguous with hingelike regions, may be considered as domains. Third, superfamily segments coded by single exons, which can be readily spliced to form a new gene, may also be considered as domains.

The secondary and tertiary structures of cytokines are of several types, including a bundle of α helices, such as interferon (IFN), predominantly α helices with short β strands, such as interleukin (IL)-2, or extensive β pleated sheets, such as tumor necrosis factor (TNF)-α (6). Some examples of the three-dimensional structures of cytokine proteins are listed in Table 1. Cytokines are often glycosylated and they frequently undergo dimerization or multimerization, resulting in a higher molecular weight. For instance, IFN-γ is a dimer and TNF-α is a trimer.

III. CLASSIFICATION OF CYTOKINES

The term cytokine encompasses the interleukins, interferons, colony stimulating factors, tumor necrosis factors, and chemokines of the hematopoietic and host defense systems. Cytokines are best classified in relation to regions of amino acid homology and shared *secondary* and *tertiary structures*, for instance, the predominantly α-helical cytokines versus

Table 1 Three-Dimensional Structures of Cytokine Proteins

Cytokine	3-D Structure
IL-4	Left-handed 4-helical bundle with two overhead connections. The linker element between the helices are formed by long loops, small helical turns, or short strands.
IFN-γ	Homodimer with each subunit consisting of 6-α helices without β sheets.
TNF-α	Homotrimer with each subunit consisting of an antiparallel β sandwich. Individual subunits form the trimer by a novel edge-to-face packing of the β sheets.
IL-8	Monomer with triple-stranded antiparallel β sheets in a Greek key motif.

Table 2 Classification of Cytokines

1.	Haematopoietins, e.g., IL-2,
2.	Interferons, e.g., IFN-γ
3.	Tumor necrosis factors, e.g., TNF-α
4.	Chemokines, e.g., IL-8,
5.	Epidermal growth factors, e.g., EGF
6.	β-Trefoil, e.g., IL-1
7.	Cysteine knot, e.g., TGF-β

those with an extensive β pleated structure. Shared features of the cytokine receptors correlate with shared features of the cytokines themselves. Further homologies are derived from studying gene organization and, in some cases, chromosomal location of the cytokine and the receptor. Thus, most cytokines can be placed into one of at least seven different families (Table 2).

Another classification is based on the expression of distinct cytokine prolifes by different mouse CD4+ T-cell clones. Two patterns were originally recognized: the *Th1 pattern* (producing IL-2, IFN-γ, TNF-α) and the *Th2 pattern* (producing IL-4, IL-5, IL-6, IL-10). A simplified approach is shown in Figure 1. This classification is limited by the fact that cytokines are almost never made exclusively by one cell type and that intermediate patterns are much more frequent. Each cytokine is usually produced by cells without strict Th1 or Th2 characteristics. Thus, concepts such as "IFN-γ is a Th1 cytokine" can be confusing because IFN-γ is usually produced by a variety of T-cell phenotypes and (NK) cells, not predominantly by CD4+ T cells meeting strict Th1 definitions. Moreover, evidence that Th1 and Th2 are true subsets in vivo has been unconvincing (7). Although the term subsets is

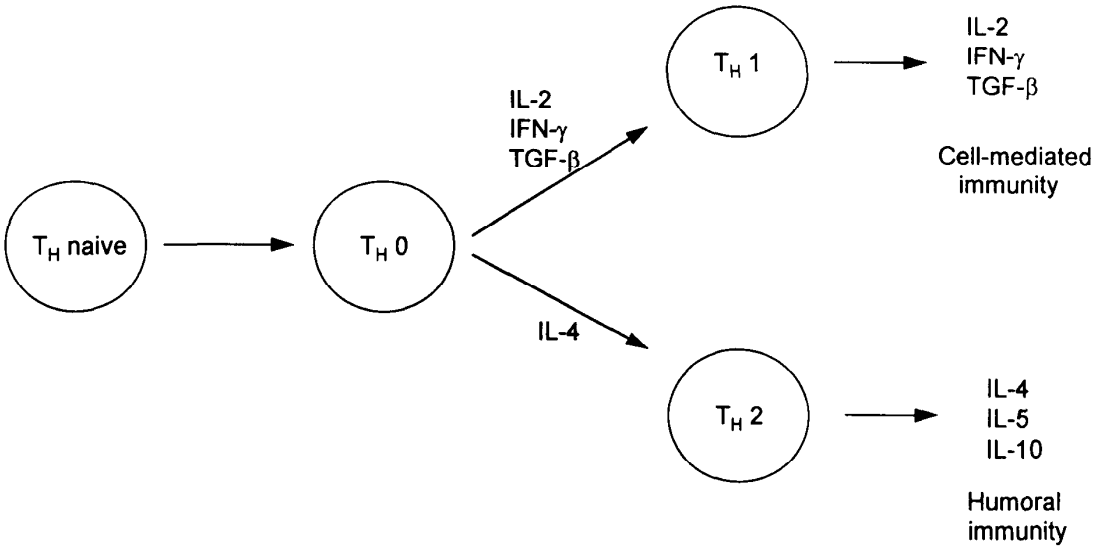

Figure 1 The differentiation of naive mouse CD4+ lymphocytes. Under the influence of cytokines, naive CD4+ lymphocytes differentiate into either Th1 or Th2 clones. The Th1 and Th2 clones are associated with cell-mediated and humoral immunity, respectively. This schema is known to be an oversimplification.

frequently used, it is more appropriate to think of overlapping population distributions, or at least to recognize that there are many phenotypes beyond Th1 and Th2 (8).

Cytokine–cytokine receptor systems can be restricted to few cells or can affect many cells. Thus, cytokines can be conceptualized as (1) produced by few cell types and received by few cell types, having potent effects on the target cell such as inducing mitosis (e.g., IL-2); (2) produced by few cell types but received by many or most cell types (e.g., IFN-γ), with effects on such a vast number of target cells not including induction of mitosis; and (3) produced by many cell types and received by many or most cell types (e.g., IFN-α, IL-1, IL-6, TNF-β). Effects of these cytokines depend on the context in which they are produced and received. Their effects are usually seen in complex interactions with other factors.

IV. APPROACH TO CYTOKINE SYSTEMS

A prototypic cytokine system is shown in Figure 2, and some possible components of a cytokine system are listed in Table 3. A complete understanding of a particular cytokine requires knowledge of (1) the producing cells; (2) the mode of regulation of cytokine production and release (extracellular or intracellular, positive or negative, transcriptional or posttranscriptional; (3) the structure, including the active form (monomeric versus polymeric) and the three-dimensional structure of that cytokine; (4) the regulation, structure and binding characteristics of its receptor and possible auxiliary receptors; (5) the signal transduction sequence initiated by the cytokine-receptor complex; (6) the ensuing biological consequences, including altered expression of target genes; and (7) the feedback loops that terminate the cytokine response.

A. Cytokine Systems Overlap with Membrane-Bound Ligand Systems

Although it is customary to consider cytokines in isolation from membrane-bound ligands, some cytokine systems overlap with membrane-bound ligand systems. The elucidation of the TNF family has complicated this distinction, because the TNF family includes both soluble and membrane-bound molecules. Soluble TNF has a membrane-bound form that interacts with the same receptors as the soluble form. The important examples are CD40 ligand (CD40L)-CD40 and Fas ligand (FasL)-Fas; other TNF-like molecules include CD27L and CD30L. Many other members of the TNF family function only as membrane-bound ligands, interacting with membrane-bound receptors. These ligands remain inducible by some of the same mechanisms that induce soluble TNF: for example, CD40L and FasL are inducible in T cells by antigen via a cyclosporine-sensitive pathway. The receptors for these ligands (i.e., CD40 and Fas) are closely related to the receptors for TNF. The signal transduction systems may also be similar, with a tendency to control the mechanisms of apoptosis and cell death. Thus, discussion of these membrane-bound members of the TNF family are included, despite the fact that these membrane-bound ligands are not strictly cytokines.

B. Cytokine Production Is Transcriptionally Controlled

The regulation of each cytokine response is usually focused on cytokine production, although the cytokine receptors (e.g., IL-2R) may also be regulated in some cases. Transcription is a key control point for production of most cytokines. Moreover, many cytokine receptors exert their effects by changing the expression of specific genes in the target cell. This chapter briefly describes the importance of phosphorylation in cell signaling events, and then discusses some general features of the control of transcription.

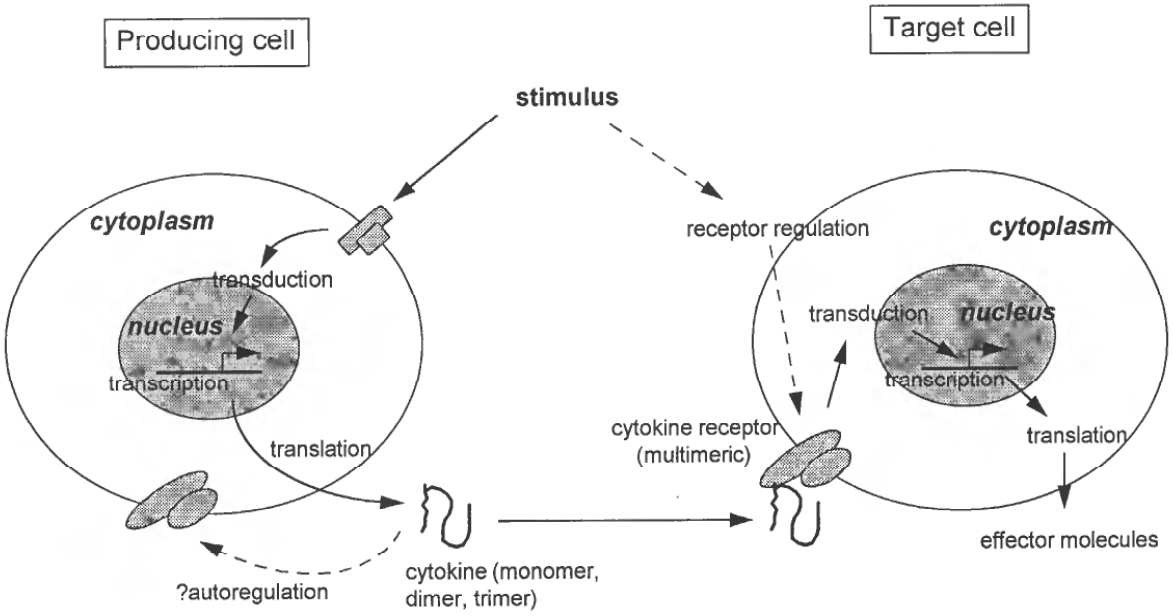

Figure 2 A prototypic cytokine system: The stimuli for inducing both cytokines and cytokine receptors include antigens, adhesion molecules, and other cytokines. Receptor regulation may involve gene transcription, release of stored subunits, aggregation of currently expressed subunits, or a combination of these. Signaling from the cytokine receptor often involves gene transcription, but may also involve nonnuclear pathways (not shown).

Table 3 Possible Components of a Cytokine System[a]

1. Control of steady-state mRNA levels
2. Stimuli for transcription
3. Posttranscriptional control
4. Secretion and activation of product
5. Cytokine binding to receptors and nonreceptor structures
6. Receptor: Structure and regulation
7. Signal transduction systems
8. Effects in the target cell
9. Synergy, antagonism, autologous effects
10. Role in circuitry

[a]Some items do not apply to some cytokines. For example, activation of the protein is not needed by IL-2 but is needed for TGF-β.

C. Phosphorylation in Signal Transduction from Membrane Receptors

Tyrosine and *serine threonine kinases* play many roles in signal transduction. The engagement of antigen-specific receptors in T cells (TCRs), with their antigens, and the engagement of cytokines to their receptors frequently activate protein tyrosine kinases (PYKs), which phosphorylate key tyrosines in the receptor or an associated protein. One important family of PYKs is the src group, including $p56^{lck}$ and $p59^{fyn}$. In the target protein, the three amino acids immediately adjacent to the phosphotyrosine group ("pY+3") are recognized by receptor sites in other signaling proteins (9,10). The targets for specific pyruvate kinases (PKs) are called src homology, 2 or SH2, domains, and permit the signal of receptor engagement to be transmitted to intracellular enzymes (such as phospholipase Cγ, permitting changes in intracellular inositol triphosphate (IP_3) and calcium. In general, tyrosine kinases associate with the receptor complex and initiate pathways that often lead to activation of serine-threonine kinases such as mitogen-activated protein kinases (MAP kinases) and the extracellular signal-regulated kinases ERK-1 and ERK-2. Another key group of tyrosine kinases is the Janus kinases (JAKs), which are associated with many cytokine receptors.

Phosphatases have many roles in intracellular signal transduction, although little is known of their role in signaling through cytokine receptors. The serine/threonine phosphatase calcineurin (the target of the cyclosporine–cyclophilin complex and the FK506–FK506 binding protein complex) is required to elicit increased cytokine mRNA through stimulation of the T-cell receptor. On the other hand, some T-cell responses can be elicited via the CD28 pathway, which is independent of calcineurin and resistant to cyclosporine, and may itself involve a tyrosine kinase. The CD28 pathway may be responsible for the cyclosporine-resistant activation of some lymphocytes. Responses to cytokines are generally independent of calcineurin and resistant to cyclosporine.

The receptor for transforming growth factor β is unique in using a serine-threonine kinase mechanism. The receptor consists of two chains with serine-threonine kinase activity (11). The TGF-β molecule binds one chain of the receptor together to form a "heterotrimer." In addition, other molecules such as endoglyn can bind TGF-β and facilitate its

attachment to the TGF-βR. Thus, endoglyn is an example of a facilitating receptor that concentrates a cytokine to present it to the main receptor.

D. General Features of Regulation of Gene Transcription

Gene transcription is controlled by proteins known as *transcription factors (TFs).* Transcription factors can be classified as the *general TF,* which act on all genes, and *specific TF* or *transcriptional activators,* which act on specific genes and give each gene its characteristic pattern of expression. The fundamental problem of gene expression is how to program controlled expression in response to signals from the environment such as hormones, growth factors, or cytokines, yet prevent accidental expression in many cells. For detailed discussions of the regulation of gene transcription, see current reviews (12–16).

Under normal circumstances, much of the DNA in each cell is bound up with histone proteins and packed into *nucleosomes,* which are inactive and inaccessible for transcription. The histone proteins contribute to the organization of chromatin and to the function of nucleosomes in inducible gene expression. The general TF may disrupt or relocate the nucleosomes to render the key DNA sequences accessible for transcription. Signal transduction pathways can also affect the chromatin structure by acetylation and phosphorylation of histones, which weaken their interaction with DNA, facilitating access of transcription factors to DNA. In general, cytokines such as IL-2 are highly restricted in their expression, and the IL-2 gene would be inaccessible in most cells.

Transcription of genes is achieved by a large multimeric protein complex designated *RNA polymerase II* (pol II). The RNA pol II is recruited to the *transcriptional initiator site* (Inr) by an assembly of general TF known as the *preinitiation complex* (PIC) (17–20). The PIC is composed of an array of more than 20 proteins, which include (1) TATA binding protein (TBP) which binds to the TATA box of the target gene located immediately 5′ to the Inr, (2) TBP associated factors (TAFs), which together with TBP, is also called TFIID, and (3) TFIIA, B, D, E, F, and H (Figure 3).

Transcription by pol II is regulated by the specific transcription factors or *transcriptional activators.* These activators give the gene its distinctive regulatory features. Transactivators are *modular* in the sense that they contain different domains to mediate DNA binding, dimerization, and transcriptional activation. The DNA binding and the dimerization domains contain motifs such as the helix-turn-helix, zinc finger, and leucine zipper. The activation domains can be classified as acidic, proline-rich, and glutamine-rich. The acidic domains probably interact directly with the PIC, whereas the glutamine-rich and proline-rich domains may have to interact with factors containing acidic domains (18).

The transcription activators bind to specific regulatory sites, principally 5′ to the TATA box, in the promoter region but also in remote sites 3′ or 5′ to, or within, the gene (enhancer sites). The regulatory elements of the gene are termed *cis*-elements, and the specific transcription activators are termed *trans-activators.* The transcription activators regulate gene expression either by disrupting repressive chromatin structures, possibly to expose key sites such as the TATA box, or by influencing the efficiency by which the transcriptional machinery utilizes the TATA box.

Transactivators often bind to DNA in dimers, either heterodimers or homodimers. They create a three-dimensional array that leads to transcription, as has recently been discussed for the IFN-β promoter (21). To assemble face-to-face homodimers, palindromic sequences (or dyad symmetry) are frequent. (For example, a dyad sequence is found

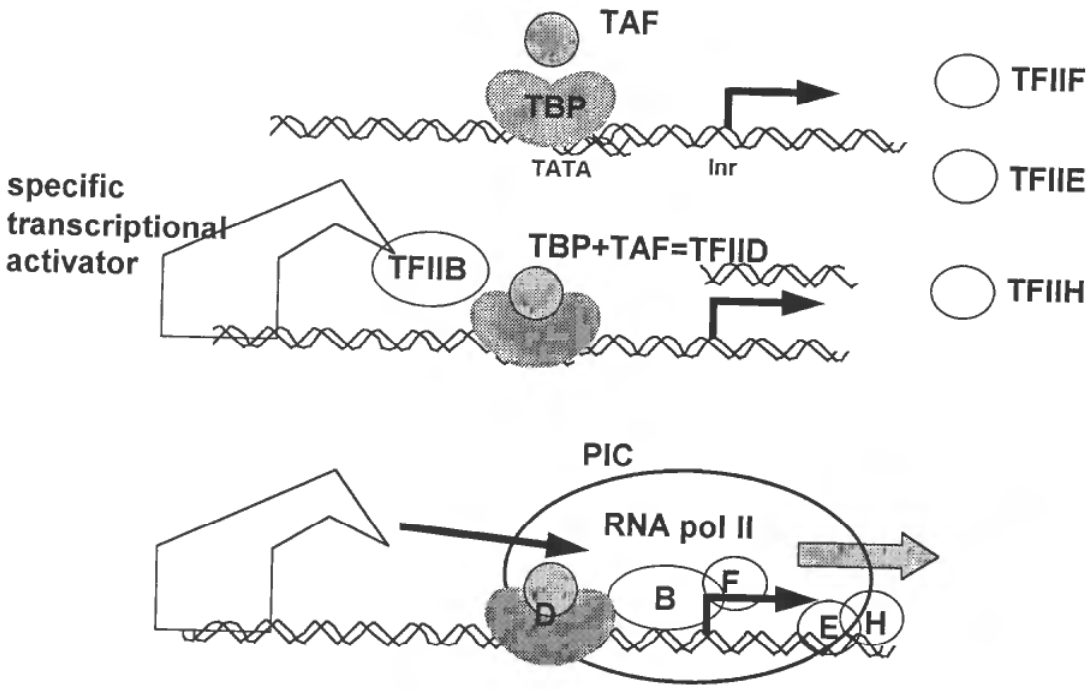

Figure 3 Regulation of gene transcription: Transcription of genes is achieved by RNA polymerase II (pol II). The RNA pol II is recruited to the transcriptional initiator site (Inr) by the preinitiation complex (PIC). The PIC is composed of an array of more than 20 proteins, which include (1) TATA binding protein (TBP), which binds to the TATA box of the target gene located immediately 5′ to the Inr, (2) TBP associated factors (TAFs), which together with TBP, is also called TFIID, and (3) TFIIA, B, D, E, F, and H. Transcription by pol II is, in turn, regulated by specific transcription factors or transcriptional activators.

upstream of all class I genes, termed the class I regulatory element: GGGGATTCCCC. The sequence TCCCC, as seen in the complementary bases of the opposite chain in the DNA, would be AGGGG. Considering both DNA strands together, this symmetry permits the site to bind dimeric proteins, such as the members of the transcription activator family NF-κB.)

Some cytokines (e.g., IL-2, IFN-γ) are highly cell type and tissue specific and are only expressed in certain cell types, such as T cells or NK cells, in response to stimuli. Others, such as IL-1, are made by many cell types. Some cytokine receptor genes (e.g., IFN-γ receptor α chain) are housekeeping genes and are expressed in most cell types. For each cytokine, the explanation for cell-specific expression and the detailed regulation by stimuli will probably be in the regulatory sites in the gene itself and the proteins expressed by the particular cell type.

E. Transcription Factors Involved in Cytokine Expression

Transcription factors implicated in the induction of cytokine gene expression during an immune response can be classified as preexisting and as induced. The preexisting factors are summarized as follows:

1. *Signal transducers and activators of transcription* (STATs) form a unique family of TF, which have been named to reflect their dual role as signal transducers as well as

activators of transcription. They are activated by tyrosine phosphorylation by members of the JAK family, which are cytoplasmic protein tyrosine kinases found in association with many receptor types. STATs are responsible for the activation of a large number of genes and are involved in the signal transduction pathways of a variety of cytokines. For instance, the transcription of many genes controlled by IFN requires STAT1 and/or STAT2, whereas the expression of the acute phase response genes induced by IL-6 requires STAT3. In general, STATs appear only to be involved in functional, as opposed to mitogenic, responses to cytokines.

2. The *ETS transcription factors* (e.g., Elk) are preexisting transcription factors activated via serine/threonine phosphorylation by MAP kinases ERK-1 and ERK-2. They regulate sites in some cytokine promoters.

3. *Corticosteroid receptors* are intracytoplasmic receptors that bind to corticosteroids to form active corticosteroid–receptor complexes. The corticosteroid–receptor complex then migrates to the nucleus and interacts with the corticosteroid response elements in the target gene, stabilized by interactions with other proteins. This receptor system is responsible for the well-known effects of corticosteroids.

4. *Activation protein-1 complex (AP-1)* consists of the Jun and Fos proteins. AP-1 is regulated by a combination of serine/threonine phosphorylation and induced transcription. AP-1 recognizes the "TPA response element" (TRE). Jun members can be activated by Jun N terminal kinases (JNK-1 and JNK-2) and they can form homodimers.

5. *Cyclic-AMP response element binding protein (CREB)* binds to the cyclic-AMP response element (CRE). CREB is often activated through the ligand binding of receptors of the G-protein–coupled seven-transmembrane spanning receptor superfamily, such as the chemokines. The effects of prostaglandins on cytokine expression may involve this system. The importance of CREB in cytokine production and immunocompetence has recently been shown in transgenic mice expressing a dominant negative form of CREB (22).

6. *NF-κB:* The components of the NF-κB/rel family include p50, RelA (p65), and c-Rel. The members of this family form various dimer combinations (p50–c-Rel, p50–RelA, and RelA–c-Rel), but their functional differences in relation to transactivating activity or cell type specific distribution are not well understood. They all share an Rel homology site domain that is responsible for DNA-binding site recognition, dimerization, nuclear localization, and interaction with a group of nuclear translocation inhibitory molecules, the Iκ-Bs (Iκ-Bα and Iκ-Bβ). Phosphorylation of Iκ-B causes it to dissociate, thereby leading to the activation and translocation of NF-κB. This family of transcription factors mediates many effects of T-cell receptor and mitogen activation, and has many influences on inflammation, immunity, viral transcription, and normal growth and development. The full activation of NF-κB in T cells requires one feature characteristic of T-cell–activating stimuli: the combined action of a calcium-dependent and a protein kinase C-activating signal. Thus, the immunosuppressives cyclosporine and FK506 can only partially inhibit NF-κB. NF-κB participates in the transcriptional activation of IL-1, IL-2, TNF-α, and the α chain of the IL-2R and many other cytokines.

7. The *nuclear factor of activated T cells* (NFAT) was originally described as acting on sites in the IL-2 promoter. The NFAT was described as a multimer of two components: NFATn, the nuclear component, which in some cases is a form of AP1, and NFATp, the preexisting cytosolic factor regulated through the calcium-dependent, cyclosporine-sensitive pathway. The NFATn and NFATp are members of a family of transcription factors regulated by serine dephosphorylation. In T cells, TCR triggering causes high intracellular

calcium, which activates the calcium-sensitive enzyme calcineurin, which is a serine phosphatase and dephosphorylates NFATp. This pathway is crucial to the activation of transcription of many T-cell cytokines such as IL-2, IL-4, TNF-α, IFN-γ, and others. Cyclosporine and tacrolimus act by inhibiting calcineurin and thus prevent the activation of calcineurin-regulated transcription factors.

F. Induced Transcription Factors

1. Interferon Regulatory Factors 1 and 2

The DNA binding domain of interferon regulatory factors 1 and 2 (IRF-1 and IRF-2) are structurally conserved and recognize the same DNA sequence within the promoter of the IFN-α and IFN-β genes. However, they are functionally distinct; IRF-1 functions as an activator for IFN-α and IFN-β, whereas IRF-2 represses the effect of IRF-1. Additional IRF family members have been identified: IRF-3, -4, -5, and -6. The IRF binding sites are found in some cytokine promoters.

2. Activation protein-1 complex

The AP-1 is partly regulated transcriptionally by the induction of Jun and Fos family members. The synthesis of Fos is regulated by ETS transcription factor Elk, and Jun is partially autoregulated by Jun activation. AP-1 is induced by the Ras-MAP kinase pathway and by costimulators such as CD28; the induction of sufficient AP-1 may be a key requirement for successful T-cell activation and cytokine transcription. In vitro, AP-1 is often activated via activators of protein kinase C.

G. Transcriptional Control of Cytokines in T Cells (e.g., IL-2, IFN-γ)

Engagement of the T-cell receptor and CD4 or CD8 by antigenic peptides in the context of MHC activates protein tyrosine kinases associated with the intracytoplasmic portions of the receptor, which, in turn, activates several signal transduction pathways. The key pathways are the calcineurin pathway and the ras-MAP kinase pathway.

1. *The TCR-calcineurin pathway.* Src-type tyrosine kinase Lck plus ZAP 70 activate PLC-γ1. PLC-γ1 hydrolyzes a membrane lipid, phosphatidyl inositol bisphosphate (PIP_2) to yield inositol triphosphate (IP_3) and diacylglycerol (DAG). The IP_3 binds to receptors on the endoplasmic reticulum to release stored calcium. The high cytosolic calcium is then sustained by changes in membrane transport. The high cytosolic calcium activates the calcium-dependent phosphatase calcineurin. Calcineurin, in turn, activates a cytosolic transcription factor NFATp, which then translocates to the nucleus where it binds to other transcription factors (such as NFATn) to form the full NFAT complex. The NFAT complex binds to the promoter site in the IL-2 genes and initiates the transcription of IL-2, IFN-γ, IL-4, and TNF-α mRNA (Figure 4). Calcineurin also may activate other preexisting transcription factors, namely Jun (23) (via JNK) and NF-κB (24).

2. *The TCR-ras-MAP kinase pathway.* Src-type tyrosine kinase Lck plus ZAP 70 activate connector proteins, which eventually activate ras to the ras-guanosine triphosphate (GTP) or activated form. Ras then attracts Raf to the membrane, which permits it to activate MEK, which is a kinase that activates the MAP kinases ERK-1 and ERK-2. These, in turn, have many effects, one being to activate the ETS transcription factor Elk. Elk then participates in the transcriptional activation of the Fos gene, and probably many cytokine promoters. MEK1, ERK-1, and ERK-2 are required for IL-2 transcription (25).

3. *The costimulator pathways.* Costimulation is received via receptors such as

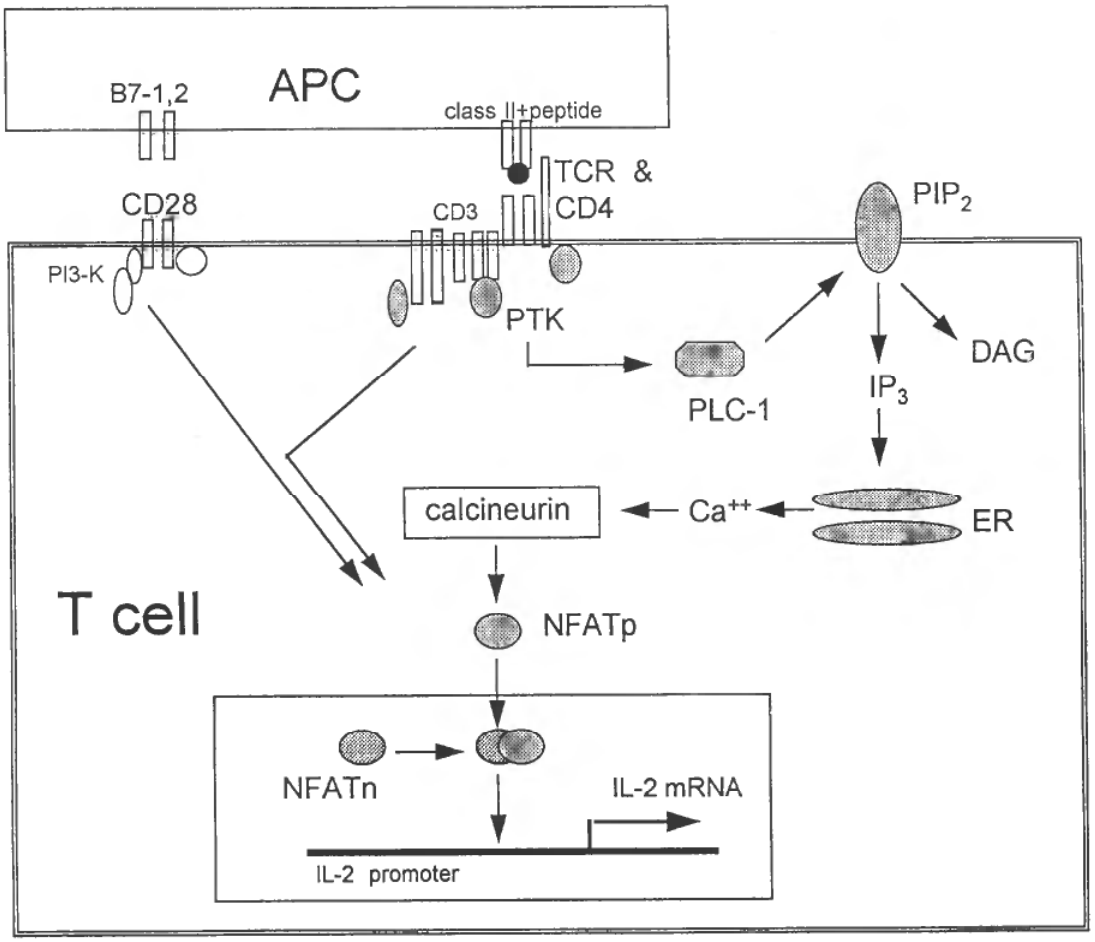

Figure 4 The transcriptional control of IL-2. Engagement of the T-cell receptor (TCR) and CD4 by antigenic peptides in the context of MHC activates TCR-associated protein tyrosine kinases (PTK), which, in turn, activates PLC-γ1. PLC-γ1 converts phosphatidyl inositol bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). The IP_3 induces the release of stored calcium from the endoplasmic reticulum (ER). The high cytosolic calcium is sustained by changes in membrane transport. The high cytosolic calcium activates calcium calcineurin (CN), which then activates a cytosolic transcription factor NFATp, which translocates to the nucleus, where it binds to NFATn to form the full NFAT complex. The NFAT complex then binds to the promoter site in the IL-2 genes and initiates the transcription of IL-2 mRNA.

CD28 engaging B7-1 and B7-2, and CD2 engaging CD58. In the case of CD28, the pathway involves both a tyrosine kinase and a lipid kinase phosphatidyl inositol 3 OH kinase (PI-3K) which is "a pivotal pathway in T cell activation" (26). The effect of CD28 triggering on cytokine transcription may be through synergy with JNK to activate AP-1. The CD28 also increases the $T_{½}$ of certain T-cell cytokines. Other pathways exist for costimulation from CD28 and from other costimulators such as CD2 and CD43 (27). In CD28 knockout mice (28), there are some immunologic defects, but overall the costimulation mechanisms may have a degree of overlap and redundancy.

H. Cytokine Receptors

Cytokine receptors can be classified into different families according to their structural similarities. Conserved amino acids in superfamilies are often clustered in domains or repeats, but even then the homology may be low, with the conserved amino acids clustered in small

sequences throughout the 40 to 110 residues that make up superfamily domains and repeats. Classes of receptors also tend to share functional characteristics. The main superfamilies recognized are the following:

1. Hematopoietic receptor superfamily
2. Interferon receptor superfamily
3. TNF receptor superfamily
4. Immunoglobulin receptor superfamily
5. G-protein-coupled seven-transmembrane spanning receptor superfamily

It is useful to consider receptors for growth factors TGF-β in the context of cytokines (Figure 5).

The principal receptor chains often have an extracellular ligand-binding domain and an intracytoplasmic signal transduction domain. Each intracytoplasmic domain is further divided into the membrane-proximal region and the membrane-distal region. The membrane-proximal region is responsible for the overall function of the receptor and is impor-

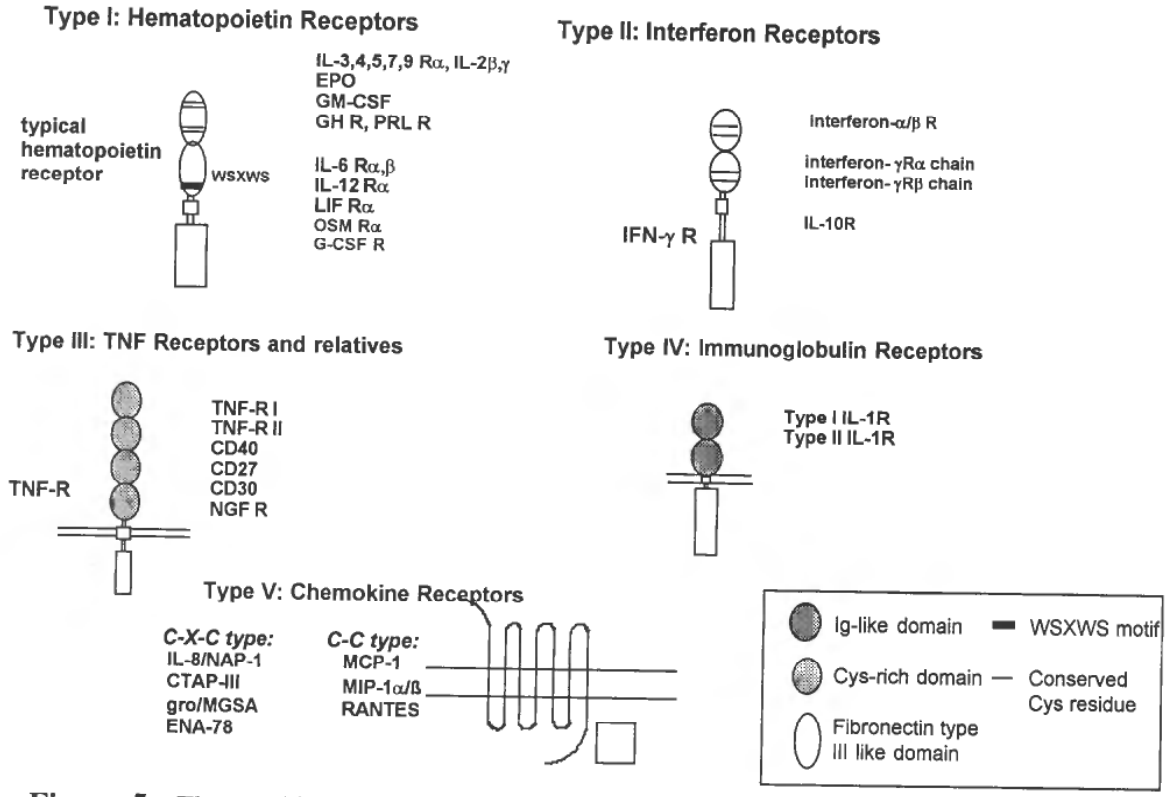

Figure 5 The cytokine receptor families. Representative members from each cytokine receptor family are also shown. Abbreviations: R, receptor; EPO, erythropoietin; GM-CSF, granulocyte and macrophage stimulating factor; GH, growth hormone; PRL, prolactin; LIF, leukemia inhibitory factor; OSM, oncostatin M; G-CSF, granulocyte colony stimulating factor; TNF, tumor necrosis factor; NGF, nerve growth factor; NAP-1, neutrophil-activating peptide-1; CTAP-III, connective tissue-activating protein III; gro/MGSA, growth-related oncogen/melanoma growth—stimulating activity; ENA-78, epithelial-derived neutrophil attractant-78; MCP-1, monocyte chemoattractant protein-1; MIP-1 α/β, macrophage inflammatory protein-1 α/β; RANTES, regulated on activation normal T cell expressed and secreted; Ig, immunoglobulin; Cys, cysteine; WSXWS, W = tryptophan, S = serine, X = nonconserved amino acid. (Adapted from Paul WE, Cell 1994; 76:241, and Taniguchi T, Science 1995; 268: 251.)

tant for mitogenesis. The membrane-distal region is not required for mitogenesis but is required for signal transduction and recruitment of signal transduction factors such as STATs.

Cytokine receptors usually consist of multimers of different transmembrane proteins. This is exemplified by the IL-2R, which is a complex of three chains: the α-chain, which binds IL-2 with low affinity but does not signal; the β-chain, which binds IL-2 with intermediate affinity; and the γ-chain, which is important for cell proliferation signals. Mutations of the γ-chain have been shown to cause X-linked severe combined immunodeficiency, a lethal disease characterized by absent or greatly reduced T cells and severely depressed humoral immunity. The γ-chain is also a functional component of the IL-4R, IL-7R, and IL-15R. The γ-chain is nonredundant: deficiency of the γ-chain in humans or mice produces a severe combined immunodeficiency.

Individual ligand-binding subunits are usually of low affinity ($K_d \sim 10^{-8}$–10^{-7}). However, the formation of a heterodimeric receptor complex results in high affinity ($K_d \sim 10^{-11}$) binding. The second subunit, which is commonly shared by different cytokines, is frequently responsible for signal transduction. For example, the receptors for IL-3, IL-5, and granulocyte and macrophage colony-stimulating factor (GM-CSF) are αβ heterodimers; each α chain is unique, but the β chain is common (in humans, KH97) (29). As a result, cross competition between these cytokines can occur (30). In addition, the use of common receptor components among different cytokines may explain why different cytokines may have overlapping biological effects.

V. THE PROTOTYPIC PATTERNS OF THE MAIN CYTOKINE LIGAND-RECEPTOR SYSTEMS

In general, the main patterns of cytokine systems are as follows:

1. *The hematopoietin pattern,* e.g., IL-2. A bundle of α helices engages all three chains of the IL-2R and triggers the effector activities of the IL-2Rβ and γ chains, activating tyrosine kinases of the JAK and src families, and ultimately activating JAK-STAT, ras-MAP kinase, and PI-3K-TOR-Kip pathways. There is extensive sharing of certain chains between receptors, for example, the γ_c, or common chain, is found in the IL-2, IL-4, IL-7, and IL-15 receptors, and the IL-2β chain is found in the IL-2 and IL-15 receptors.

2. *The interferon pattern,* e.g., IFN-γ. Interferon-γ dimer cross-links the α chains of the IFN-γ receptor, and thereby forms a binding site for the β chain, thereby activating JAK1 and JAK2. These tyrosine kinases phosphorylate and activate STAT1, which dimerizes to activate interferon-inducible genes (including the IRF-1 family of inducible transcriptional activators), which then induce other genes such as MHC class I and class II.

3. *The TNF family pattern.* Tumor necrosis factors are homotrimers that assemble three copies of their receptor and then induce the ceramide-sphingomyelin pathway. The TNF trimer "jelly roll" (31) has grooves between the three TNF molecules. The external portions of the TNF receptors are strings of cysteine-rich domains, which bind to the grooves between the TNF monomers in the trimer. Thus, the TNF trimer trimerizes the TNFRs (32). There are two TNFRs: the p55 chain (R1) and the p75 chain (R2), differing principally in their intracytoplasmic cellular signal transduction domain. The receptors are not redundant, serving some separate functions. For example, the p55 chain has a crucial role in inducing adhesion molecule expression (33) and leukocyte infiltration of organs (34). Both TNFRs are required for cytotoxicity in some cell lines (35). TNFR1 knockout mice show decreased sensitivity to TNF and a defect in resistance to *Listeria* infections

(36). TNFR2 knockout mice have decreased sensitivity to TNF (37). It is likely that the general pattern of TNFR triggering and signaling applies to other family members, including the important FasL- Fas and CD40L-CD40 pairs.

4. *The chemokine pattern.* Chemokine receptors are seven-pass receptors, that is, the protein chain has its N terminal outside the membrane and then passes through the membrane seven times before its final C terminal portion is left intracytoplasmic. Thus, the N terminal and three loops are outside the cell, and the C terminal and three loops are inside the cell. Chemokine receptors have some promiscuity not found in other cytokine receptors; thus, IL-8 can bind to more than one receptor, one of which it shares with other cytokines. Chemokines (usually monomeric) engage their receptors in a pocket formed by the N terminal and the external loops, and induce a conformational change, perhaps in the intracytoplasmic portions, perhaps in the C terminal, which interacts with the signal transduction apparatus, the heterotrimeric G-protein α, β, and γ chains.

A. Cytokine Receptor Signaling

There are two mechanisms by which cytokines trigger their receptors (4,38): aggregation and conformational change. The hematopoietin, interferon, and TNF families of receptors may be activated primarily because of aggregation by multivalency of the ligand. Receptor aggregation brings together and activates the JAKs and other tyrosine kinases. The chemokine receptor is activated by a conformational change.

The signal transduction mechanisms associated with cytokine receptors are being elucidated and include tyrosine kinases (e.g., $p56^{lck}$, JAKs), serine-threonine kinases (e.g., TGF-βR), ceramide-sphingomyelin mechanisms (TNF family receptors), and G-proteins (the chemokines).

The signaling pathways that are activated by hematopoietin and interferon family cytokines are the JAK-STAT pathway, the ras signaling pathways, PI-3K, and the TOR pathway.

1. JAK-STAT Pathway

The JAK family consists of Jak1, Jak2, Jak3, and Tyk2. With the exception of Jak3, which is primarily expressed in hematopoietic cells, JAKs are constitutively associated with the membrane-proximal portion of the intracytoplasmic cytokine receptor chain. Ligand-induced dimerization of the receptor brings the receptor-associated JAKs into close proximity to allow transphosphorylation and activation of catalytic activity. The activated JAKs then phosphorylate other JAKs and the receptor chain at multiple sites including key Y sites. The STATs are then recruited to the activated receptor through SH2 domains and become phosphorylated by the activated JAKs. The STATs then undergo dimerization through the intermolecular association of the SH2 domains. The dimerization triggers the dissociation of STATs from the receptor complex and translocation to the nucleus where they bind to specific response elements and, in general, activate gene transcription (Figure 6). Some JAK and STAT members are being shown to have unique and nonredundant roles: disruptions produce deficiencies in knockout mice, and mutations have produced immunodeficiencies in humans.

2. Ras Signaling Pathway

Ras and related members of the family of small GTPases act as "on-off" switches for many activities associated with transformation and activation of cells, including increased membrane activity, increased motility, and cell division. A number of cytokines activate the ras

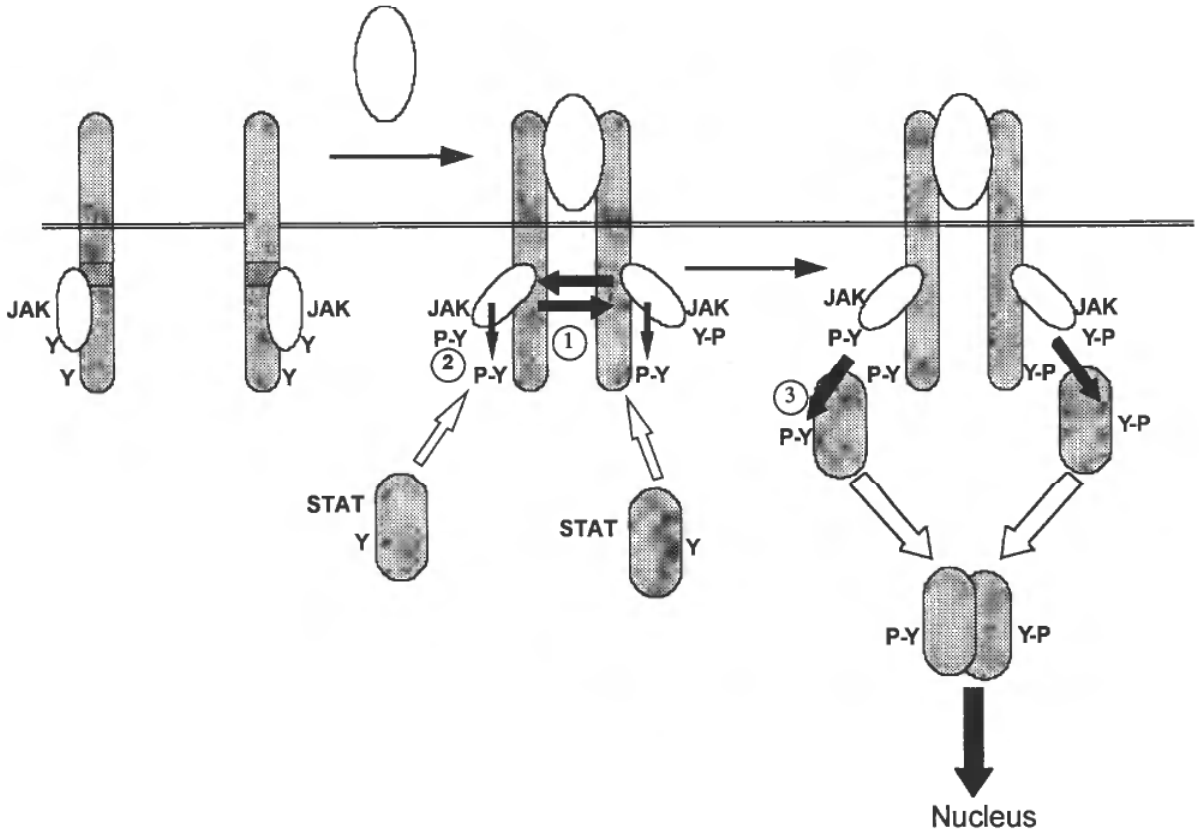

Figure 6 The JAK-STAT pathway. JAKs are constitutively associated with the intracytoplasmic portion of the cytokine receptor chain. Ligand-induced receptor aggregation brings the receptor-associated JAKs in close proximity to allow transphosphorylation and activation of the catalytic activity. The activated JAKs then phosphorylate other JAKs and the receptor at multiple sites. STATs are recruited to the receptor complex and become phosphorylated by the activated JAKs. The activated STATs then undergo dimerization, dissociate from the receptor complex, and translocate to the nucleus to activate gene transcription Note three phosphorylations: 1, JAKs; 2, receptor chains; 3, STATs.

signaling pathway, usually those which transform and activate the target cell. (Cytokines that act on many or all cells like IFNs will not usually activate Ras.) Ras is a guanosine triphosphatase (GTPase), a molecular switch for signals that control growth and differentiation. Like all GTP-binding proteins, ras cycles between an inactive form, that is, guanosine diphosphate (GDP)-bound, and an active, GTP-bound, conformation. Ligand binding of the receptor results in the tyrosine phosphorylation of SHC, its association with an adaptor protein complex Grb2-Sos. Interaction between the guanine nucleotide-releasing activity of Sos and the membrane-bound ras leads to the conversion of GDP-bound ras to GTP-bound ras. This, in turn, activates Raf, which then triggers the MAP kinase cascade. Stimulation of ras leads to an increase in the activity of two serine-threonine MAP kinases, Erk-1 and Erk-2, which subsequently translocate to the nucleus where they phosphorylate key transcription factors such as Elk, ribosomal S6 kinase, and MAPK-activated protein kinase (39) (Figure 7).

3. PI-3K and the TOR Pathway

In addition to SHC, tyrosine phosphorylation of many cytokine receptors also provide binding sites for the 85 kDa regulatory subunit of PI-3K. The function of PI-3K is unknown but it probably initiates the target of rapamycin (TOR) pathway, which regulates entry into cell division. It may also contribute to preventing apoptosis and/or activate the ras pathway by

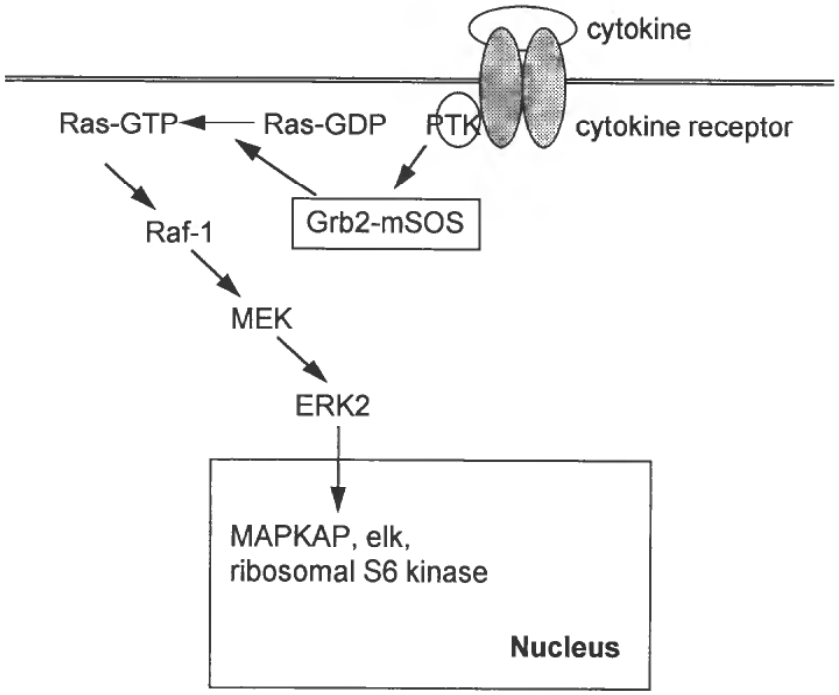

Figure 7 The Ras pathway. Ligand binding of the receptor results in the activation of receptor-associated protein tyrosine kinase (PTK) and its association with an adaptor protein complex Grb2-mSos. Interaction between the guanine nucleotide-releasing activity of Sos and the membrane-bound ras leads to the conversion of GDP-bound ras to GTP-bound ras. This, in turn, activates Raf, which then triggers the MAP kinase cascade. Stimulation of Ras leads to an increase in the activity of two serine-threonine MAP kinases, ERK-1 and Erk-2, which subsequently translocate to the nucleus where they phosphorylate key transcription factors such as Elk, ribosomal S6 kinase, and MAPK-activated protein kinase. Abbreviations: PTK, protein tyrosine kinase; SOS, Drosophila 'son of sevenless'-like protein; GTP, guanosine triphosphate; GDP, guanosine diphosphate; ERK extracellular signal-regulated kinase.

an SHC-independent mechanism (4). PI-3K also plays a role in the signaling pathway by CD28 (40).

The TOR pathway has been mapped by virtue of its sensitivity to the experimental immunosuppressive agent rapamycin (sirolimus) (Figure 8). Probably the pathway is initiated by PI-3K, which is activated by ligand binding to its receptor. PI-3K releases PIP_3, which may activate protein kinase B. This then directly or indirectly activates TOR, which autophosphorylates by virtue of its kinase domain. The TOR, in turn, directly or indirectly exerts downstream effects to activate several events. It activates S6 kinase, which may regulate protein synthesis, and inactivates an inhibitor of cell cycling, p27 KIP. KIP normally functions to inhibit cyclin-cyclin–dependent kinases from advancing the cell through the G_1 phase to initiate the S phase. Cytokines such as IL-2 or IL-4 cause lymphocytes to cycle at least in part by causing KIP to be degraded, releasing cyclin-cyclin–dependent kinases to mediate G_1 progression to S.

Rapamycin is a macrolide antibiotic that prevents growth-promoting cytokines from activating mitosis. Rapamycin is similar to tacrolimus in that it binds to the immunophilin FKBP to create the active complex. However, the complex does not engage calcineurin; instead, it binds to the protein TOR, which has a kinase domain. Rapamycin prevents the autophosphorylation of TOR. The TOR appears to be a nonredundant step on the pathway

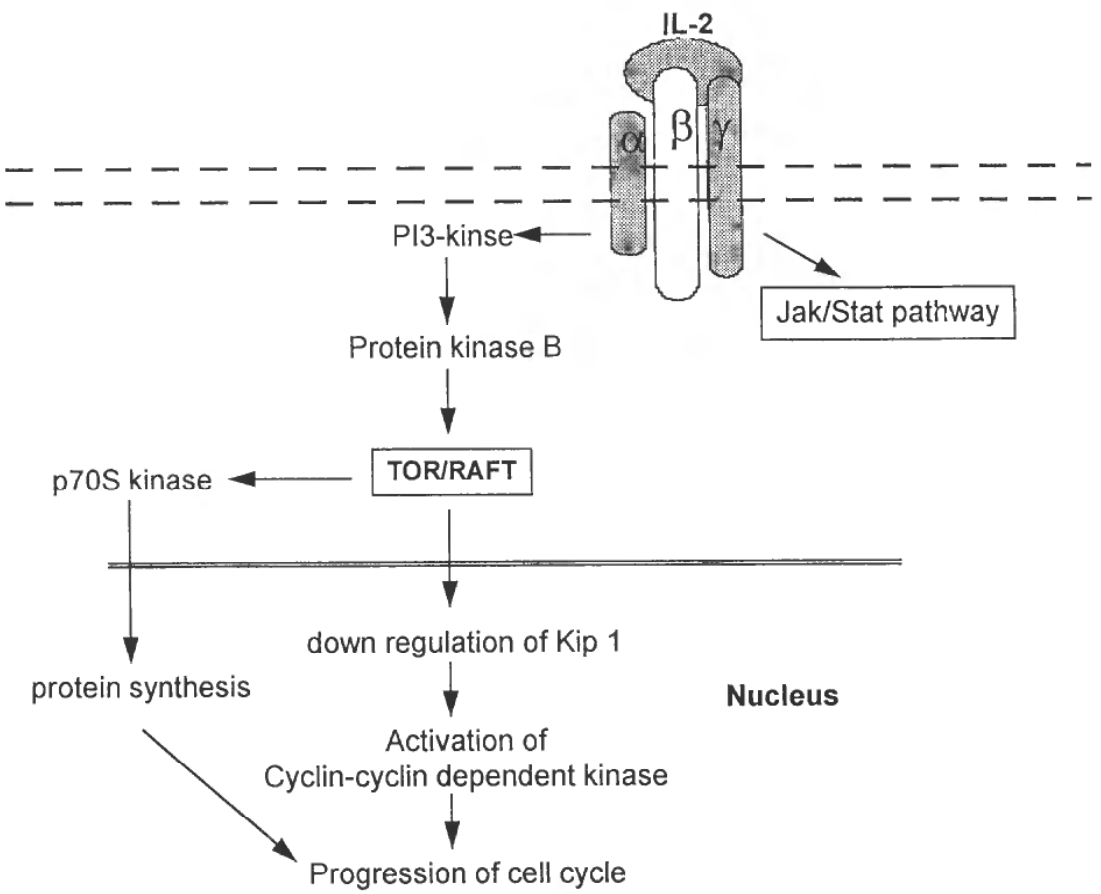

Figure 8 The TOR pathway: Binding of IL-2 to its receptor activates PI3 kinase. PI3 kinase releases PIP_3, which activates protein kinase B. This then directly or indirectly activates target of rapamycin (TOR), which autophosphorylates by virtue of its kinase domain. The TOR, in turn, directly or indirectly exerts several downstream effects, including activation of p70S6 kinase and inactivation of KIP1, an inhibitor of cell cycling.

from many cytokine receptors to the cell cycle. As a result, rapamycin is a powerful immunosuppressant because it prevents clonal expansion.

4. Sphingomyelin-Ceramide Pathway

The pathway activated by TNF family members is the sphingomyelin-ceramide pathway. This activates NF-κB, possibly indirectly, via phosphorylation, inactivation, and degradation of IκB. The TNFs have a strong tendency to affect apoptosis of lymphocytes, via the death pathways, through such mediators as CED.

The components of the FasL or TNF signal transduction apparatus (41) include the following: the specific domains of the intracytoplasmic portions of the receptors such as the "reaper" and "cell death" domains (42); a protease called interleukin-1β–converting enzyme and related proteases (43); sphingomyelinase, which breaks down sphingomyelin to ceramide, an important intracellular second messenger (44,45); the protein kinase receptor interacting protein (46); the TNF receptor-1–associated death domain protein, which is associated with the TNFR-1 and plays a role in signaling cell death and activated NF-κB (47); the protein TNF receptor–associated factor 2, which mediates the induction of NF-κB

by TNFR-2; and the protein Fas-associated phosphatase-1, an Fas-associated protein tyrosine phosphatase (48);

The signaling pathway activated by chemokine and other seven pass receptors is through the heterotrimeric G-proteins: the α chain, β chain, and γ chain releasing cyclic-AMP. Activating the α chain causes it to bind GTP. It will be slowly inactivated by the GTPase activity of the βγ chains. This activates adenyl cyclase to create cAMP, which is the second messenger for these receptors. Cyclic AMP then activates CREB, which binds to cAMP-regulated elements (CREs) in the promoters of certain genes, often in association with CBP. Chemokines can also activate other pathways.

B. Redundancy and Antagonism in Signaling Pathways Among Cytokine Receptors

Many links and interactions between cytokines, and their tendency to be acting in groups, complicate the assignment of unique signal transduction mechanisms and pathways to individual cytokines in "real-time" immune and inflammatory responses. Thus, in an inflamed tissue, TNF expression is often accompanied by expression of many other cytokines, and many cells are subjected to many cytokines simultaneously. It is likely that this is normal for the signaling systems to operate in this way. Perhaps there is a set of relatively few key points in key cells in which a signaling system must reach a critical level to achieve an effect, and that this key point (e.g., activation of MAP kinases in T cells) reflects and integrates positive and negative signals from many sources.

C. Regulation of Cytokine Responses

In general, the amount of cytokine produced is rate-limiting step in determining how much cytokine effect is available. Thus, cytokine responses are triggered via initiation of the production of the cytokine by transcription of the cytokine gene. Cytokine responses may be regulated at other levels, such as posttranscriptional changes in cytokine production, cytokine receptor expression, and postreceptor signal transduction. Certain cytokines regulate their own expression by positive or negative feedback mechanisms. For example, IFN-γ induces IFN-γ mRNA expression (49,50). In other cases, the response of one cytokine may be regulated by another cytokine. Finally, some cytokines compete at the receptor level because both receptors use and require the same component (e.g., the common, or γ_c, chain of the cytokine receptor), which is present in limiting quantities (51). However, the data on cytokine regulation tends to be derived from in vitro systems. More information is needed about in vivo controls.

Some cytokines regulate other cytokines, for example, there is cross-regulation of IFN-γ responses by IL-4 and of IL-4 responses by IFN-γ. The Th1 and Th2 paradigm indicates that, under some circumstances, cytokines classified as Th2 cytokines will antagonize Th1 responses, and vice versa. Many of these effects are indirect, operating on intermediate cells such as antigen presenting cells. However, the many exceptions make it difficult to rely on this generalization.

Many T-cell cytokines are controlled by the calcium-calcineurin pathway and are completely inhibited by calcineurin inhibitors (in vitro). The production of IL-2, IL-4, IFN-γ, GM-CSF, and TNF in T cells is controlled in this fashion. On the other hand, some cytokines are not controlled by the calcineurin pathway in T cells; IL-10 is resistant to calcineurin inhibitors.

VI. ESTABLISHING ROLES FOR INDIVIDUAL CYTOKINES IN TRANSPLANTATION

During allograft rejection, a large number of cytokines are present in the allograft. It is difficult to assign specific roles to each cytokine. In cell biology in vitro, it is common to use transfection, mutagenesis, expression of dominant negative mutations, and overexpression to validate the role of a particular protein in vitro. Some useful approaches to assigning roles to individual cytokines in vivo are as follows:

1. *Establishing the presence of the cytokine or its mRNA.* Cytokines can be detected in body fluids or in tissue sections by specific monoclonal antibodies. Similarly, the levels of steady-state cytokine mRNA can be demonstrated by Northern blotting and reverse transcriptase polymerase chain reaction (PCR). No cytokine is made exclusively by one cell type, however, a factor that limits their value as cell markers.
2. *Demonstrating characteristic or unique effects of the cytokine.* Certain cytokines exhibit unique effects that are nonredundant. A typical example is the induction of MHC class II products in nonlymphoid tissues that have been shown to be IFN-γ dependent. Induction of MHC class II may therefore be associated with the expression of IFN-γ.
3. *Blocking the production or the effects of the cytokine.* Cyclosporine and FK506 are two immunosuppressive agents that act by interfering with the calcineurin pathways in cytokine production by T cells and NK cells. They can therefore provide indirect evidence for the involvement of specific cytokine systems. Monoclonal antibodies against cytokine receptors are also instructive. However, they may sometimes alter or kill the cells or trigger the receptor, rather than simply block the receptor, rendering the observed effects more difficult to interpret. New strategies include antisense mRNA experiments and hybrid "ligand-Ig" molecules in which the receptor-binding portion of the cytokine is combined with the Fc portion of immunoglobulin.
4. *Producing transgenic mice* by introducing exogenous DNA into fertilized mouse eggs for the subsequent expression of a specific protein product. For instance, the generation of the IFN-γ transgenic with the insulin promoter has provided an excellent model of autoimmune diabetes and strongly suggests a major role for IFN-γ in the pathogenesis of this disease (52).
5. *Generating knockout and mutant mice* by introducing mutation to both copies of the target gene in the mouse ova, thereby preventing their expression. If the cytokine is involved in development, the host may emerge developmentally abnormal as well as deficient in the cytokine response. However, the importance of the "knocked out" cytokine may be underestimated by the knockout mice because they are under pressure to maximize the redundancy in the remaining cytokines. Knockouts exist for a wide variety of cytokines and TFs important in cytokine expression. These include IL-2, IL-4, IL-6, IL-10, TGF-β, IFN-γ, IFN-γR, GM-CSF, IRF-1, and STAT-1α. They are summarized in Table 4 (36,53–66).

VII. CYTOKINE AND CYTOKINE RECEPTOR KNOCKOUT MICE IN IMMUNOLOGY AND TRANSPLANTATION RESEARCH

Cytokine and cytokine receptor knockout mice have already provided some lessons on redundant and nonredundant effects, which are relevant to transplantation. Examples are as follows:

1. *Confirmation that TGF-β has some antiinflammatory activities.* The TGF-β

Table 4 Important Cytokine and Signaling/Transcription-Related Gene Knockouts

Knockout	General Phenotype
GM-CSF	Normal development with surprisingly normal basal hematopoiesis. Pulmonary alveolar proteinosis progressively develops in these mice.
IFN-γ and INF-γR	Normal development, defective natural resistance, especially killing of intracellular pathogens. Lower basal MHC class I, especially in arterial endothelium; normal basal class II, defective induction of class I and class II.
IL-2	Normal T and B cell development; development of ulcerative colitis.
IL-4	Normal development. Serum levels of IgG_1 and IgE strongly reduced.
IL-10	Retarded growth, anemic, and development of chronic enterocolitis.
IRF-1	Profound reduction of $TCRab^+CD8^+$ T cells, defective IFN-γ–dependent responses. Lower basal MHC class I, especially in arterial endothelium; normal basal class II.
STAT1a	Complete lack of response to IFN-α or IFN-γ. Highly sensitive to infection by microbial pathogens and viruses. Normal basal MHC class I and II; defective induction of class I and class II.
TGFβ-1	No gross developmental abnormalities; 2–3 weeks after birth they die with a wasting syndrome accompanied by multifocal inflammation and tissue necrosis. Elevated MHC class I and II prior to any evidence of inflammatory infiltrates.
TNFRp55	Normal development. TNF signaling is largely impaired, as judged by the failure of TNF to induce NF-κB. Severely impaired defense against microorganisms and their pathogenic factors.

knockout mouse has a profound inflammatory disease affecting almost all organs other than kidney and brain. The mice die of their inflammation, particularly in their gastrointestinal tract, at about 4 weeks of age (67). TGF-β knockout has high MHC expression (66). The apparent reason is a loss of the antiinflammatory effects of TGF-β, underscoring the potential importance of this agent in controlling immunological injury. TGF-β is also increased under some circumstances by cyclosporine, although its role in the mechanism of immunosuppression by cyclosporine is controversial. However, TGF-β is a widely expressed cytokine and probably has many roles in inflammation and tissue remodeling. It would be misleading to consider it an immunosuppressive or antiinflammatory cytokine when it has so many other roles in response to tissue injury.

2. *The redundancy of most individual cytokines.* One question that arises in most knockout mice is why they are so "normal" compared with the theoretical importance of the cytokine. For example, the IL-2 knockout mouse might be expected to be profoundly immunosuppressed, but it is immunocompetent (57). The IL-2 knockout mice also reject allografts vigorously. This rapidly became the rule for all cytokine knockouts. This apparent paradox should not be interpreted as evidence that the IL-2 cytokine is completely redundant, but the theory of IL-2 being sine qua non for immune responses was obviously incorrect.

3. *Nonredundancy of some early events in T-cell activation.* Human primary immunodeficiencies and knockout mice have established the nonredundant role of several early events: TCRαβ engagement of MHC class I and II and activation of protein tyrosine kinases of the src family and ZAP 70; calcineurin phosphatase activity (68,69); and cos-

timulation, possibly because ras, MAP kinase, and AP-1 generation is rate limiting for activation of cytokine transcription by some cytokine promoters (70,71).

4. *Nonredundancy of some cytokine receptor chains and signal transduction pathways.* Cytokine receptors are always heterodimers or trimers. Thus, disruption of each receptor chain must be discussed separately. Some cytokine receptor chains are used by several cytokines, some by only one. The IL-2 receptor α chain is used by only the IL-2R. Thus IL-2Rα knockouts simulate the redundancy of the IL-2R knockout. In comparison, the IL-2R γ_c chain is shared by IL-4, IL-7, and IL-15 (72–74) and is critical for immunocompetence. The γ_c chain gene maps to a locus implicated in X-linked severe combined immunodeficiency (XSCID) (75,76). Furthermore, three unrelated XSCID patients have been shown to carry mutations within their γ_c chain genes. Among the five distinct types of X-linked immunodeficiencies, XSCID is characterized by a markedly reduced level or a complete absence of both mature and immature T cells. These findings demonstrate that γ_c chain plays a critical role in thymic maturation of T cells and in cytokine responses. The γ_c chain is probably nonredundant because of its unique ability to bind JAK3 (?) and activate STAT (?) (77–79). The evidence that JAK3 is nonredundant is also based on human immunodeficiencies (79,80) and knockout mice (78). The answer to this question is that some cytokine R chains are used by several cytokines and may be nonredundant because they activate unique signal transduction pathways (JAK-STAT mechanisms or other tyrosine-kinase or lipid kinase signal transduction systems). Similarly the effectiveness of the immunosuppressive rapamycin (sirolimus) implies that the TOR is nonredundant. Many cytokine and growth factor receptors (e.g., PDGF) can signal cell division through the TOR pathway. Thus, there is redundancy in receptors but nonredundancy in pathways.

5. *The nonredundant role of IFN-γ and IFN-γR.* Both IFN-γ and IFN-γR knockouts have been described, and they establish the unique role of IFN-γ and IFN-γR in MHC regulation. The phenotype of the IFN-γ and IFN-γR knockouts is low MHC class I expression in the basal state, particularly on arterial endothelium. Thus, even in the unstimulated or "basal" state, the normal host relies on the IFN-γ and IFN-γR system to induce class I expression. Moreover, mice, unlike humans, lack class II on their endothelial cells, whereas many arterial endothelial cells in the normal human express class II. We believe that some class II expression by normal endothelial cells may be induced by IFN-γ.

VIII. THE CRITICAL ROLE OF CD40L-CD40 IN GRAFT REJECTION

Recently, it has been shown that simultaneous blockade of the CD28 and CD40 pathways using monoclonal antibodies against the CD28 and CD40 ligands promotes long-term survival of fully allogeneic skin grafts and inhibits the development of chronic vascular rejection of primarily vascularized cardiac allografts (81). However, blockade of either of these pathways alone is not sufficient to suppress allograft rejection. These findings suggest that the CD28 and CD40 pathways are both critical but independent regulators of the T-cell response during allograft rejection.

IX. POTENTIAL ROLES OF CYTOKINES AND CYTOKINE-LIKE MEMBRANE MOLECULES IN TRANSPLANTATION

Cytokines and TNF-related membrane molecules such as CD40L are important immunological mediators in transplantation. In general, no single cytokine is completely nonredundant, that is, no cytokine knockout has created a primary immunodeficiency in human

or a severe immunodeficiency in mice. Many phenomena observed in the immune response to a transplant are mediated by several cytokines, acting redundantly or synergistically. On the other hand calcineurin induces many cytokines, including CD40L, calcineurin, cytokine γ_c chain, and TOR, which all serve nonredundant functions.

The response of the host to an allograft can be conceptualized as a series of molecular events (82) and can be represented by the tale of three signals (Figure 9): Signal 1 is the engagement of T-cell receptors with antigenic peptides in the context of MHC antigen. This leads to the activation of tyrosine kinases associated with the T-cell receptors. The tyrosine kinases, in turn, trigger secondary messengers and initiate several signaling pathways (e.g., the calcineurin pathway), which activate TFs, that regulate the transcription of genes encoding for several key cytokines (e.g., IL-2, IFN-γ) and cytokine receptors. Cytokines regulate MHC expression and the peptide generation and processing pathways. MHC expression is regulated by IFN-γ with additional or synergistic effects from other cytokines: IFN-α and β, TNF-α and β, and possibly with inhibitory effects of TGF-β.

Signal 2 is the costimulatory signal delivered by the antigen presenting cells to the T cells, via receptors on the T cells. The best known stimuli are B7-1 and B7-2, which are regulated by cytokines. Other aspects of the T cell–APC interaction are also affects by cytokines, such as CD2-CD58 and LFA1–ICAM-1. Finally, some aspects of costimulation may be delivered by monokines, such as IL-1 and IL-6, or by chemokines. Thus, some costimulators are regulated by cytokines, and some costimulation may be mediated by cytokines themselves.

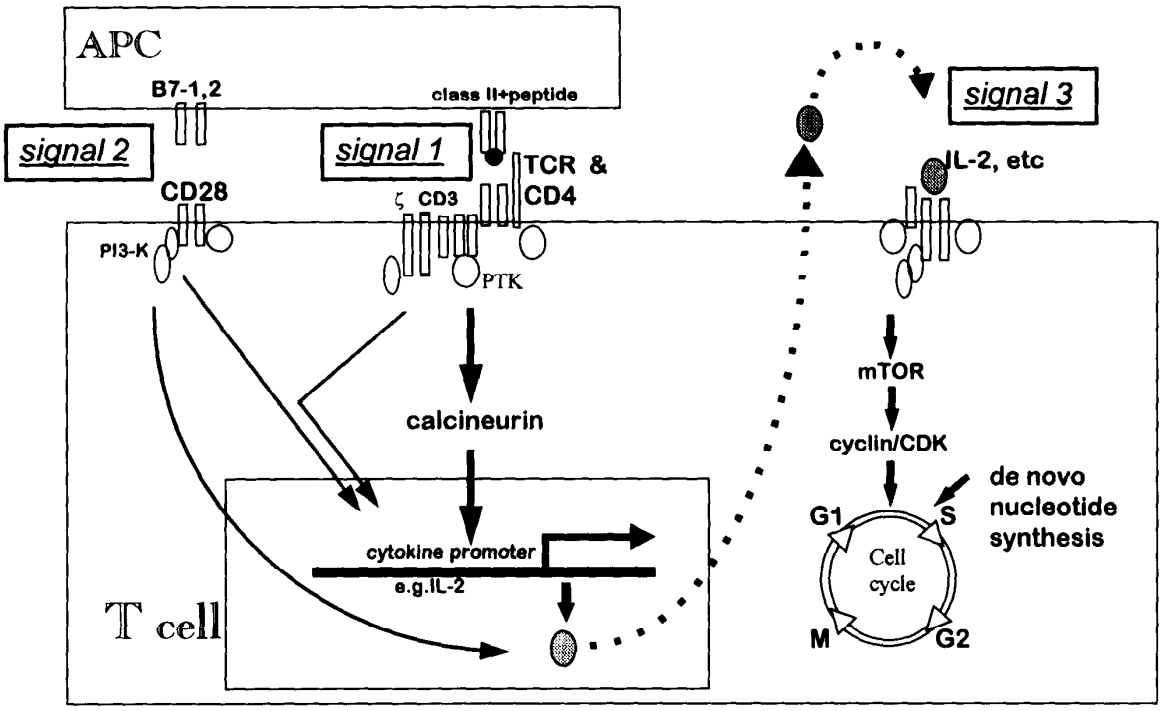

Figure 9 The tale of three signals. Engagement of the T cell receptor (TCR) with the antigenic peptide in the context of self MHC class II molecule leads to the activation of the calcineurin pathway and results in the induction of cytokine genes (e.g., IL-2). Signal two involves the costimulation of CD28 with members of the B7 family. Interaction between cytokine and its corresponding receptor leads to induction of cell division, probably through the target of rapamycin (TOR) pathway. This constitutes signal 3.

Signal 3 refers to the growth signal delivered by growth factor cytokines such as IL-2. This results in the triggering, commitment, and clonal expansion of T and B lymphocytes and emergence of effector functions. Donor cells in the graft are specifically lysed by these activated lymphocytes. If irreversible rejection does not occur, host/graft adaptation may ensue. Cytokines may mediate or control these events in a number of ways.

A. Graft Antigenicity and Adhesion Molecule Expression

The antigenicity of an allograft is determined to a large extent by the expression of MHC antigens, adhesion molecules, and costimulatory molecules in the allograft. Under basal conditions, the expression of these molecules on parenchymal cells is too low to permit T-cell recognition. Certain cytokines such as IFN-γ, TNF-α, and IL-10 may increase the antigenicity of an allograft by inducing the expression of MHC class I and II, adhesion and signaling molecules, and cytokine receptors. Moreover, IFN-γ increases the transcription of the large multifunctional protease (LMP) genes and the genes for transporters associated with antigen processing (TAP), which are encoded in the class II region of the MHC, and could influence the peptides available for binding to the class I grooves. Under some circumstances, peptide availability could be rate-limiting for MHC expression and for allorecognition. Costimulatory molecules such as B7 can also be induced by IFN-γ.

B. Triggerring, Growth, and Differentiation of Specific T- and B-Cell Clones

The role of cytokines in the differentiation and clonal expansion of T and B cells is well established. T-cell receptor triggering is dependent on the presence of costimulating factors (such as CD28 engaging B7-1/B7-2) and may be promoted by the binding of certain cytokines produced by the APC to their cognate receptors (e.g., IL-1, IL-6, IL-12) on the T cell. The subsequent lymphocyte differentiation and clonal expansion also require that certain cytokines produced by T cells (e.g., IL-2, IL-4, IL-7, IL-13) engage their receptors on T cells, producing paracrine or autocrine effects. The aggregate strength of those signals may be a key rate-limiting step in the immune response, but the role of soluble TNF-like ligands such as CD40L must also be emphasized. Some of these signals must be present to rescue the T-cell and B-cell clones from programmed cell death when they encounter antigen. The receptor components and the cytokines may themselves be regulated by cytokines. The roles of IL-15 (reviewed in [83]) may be as a partner to IL-2 in support of lymphocyte clonal expansion.

C. Organization of Inflammation, Including Chemotaxis, Recruitment, and Activation of Nonspecific Cells

Cytokines also play a crucial role in the organization of inflammation in a rejecting allograft. Cytokines can activate endothelial cells and affect their interactions with leukocytes and platelets, as well as their regulation of vasomotor tone and fluid movement. Endothelial and inflammatory cells activated by cytokines express adhesion molecules, such as VCAM-1 and increased amounts of ICAM-1, and are capable of producing many inflammatory mediators, including other cytokines (IL-1, IL-6, TNF-α, IL-10, GM-CSF), toxic oxygen intermediates, nitric oxide, eicosanoids, and enzymes. Chemokines are responsible for recruiting inflammatory cells such as neutrophils to the site of inflammation by chemotaxis. Chemotaxis of T cells in vitro is influenced by many other cytokines: IL-2, IL-4, IL-10, IL-13, and IFN-γ (84).

D. Specific and Nonspecific Injury to Donor Cells

The effector mechanism of a rejection response involves cytotoxic T cells that have developed under the influence of cytokines. There are two major mechanisms of cytotoxicity: degranulation of perforins and associated granzymes, and interaction of a TNF-like molecule (Fas ligand) on a cytotoxic T cell and a TNF receptor–like molecule (Fas) on the target cell. Membrane-bound and soluble TNF-α may also function in killing by cytotoxic T cells. The p55 receptor of TNF may mediate some cytotoxic effects and the induction of nitric oxide synthesis, although this is controversial (85,86). The p75 receptor may have proliferative effects and may bind the TNF to transfer it to the p55. However, the TNFR2 may also induce cytotoxicity. Recently there has been increasing dissatisfaction with existing models for cell death in allografts because knockouts of perforins and disruption of the Fas and TNF mechanisms have been unable to arrest graft rejection. Thus, the main mechanism of cell injury in organ transplants is currently unknown (87).

Cytokine-activated macrophages may be a nonspecific mechanism of graft cell injury through production of TNF-α, nitric oxide, eicosanoids, and many other activities.

E. Host/Graft Adaptation

Host/graft adaptation is a state of incomplete antigen-specific unresponsiveness induced by exposure to the antigens. Tolerance occurs spontaneously if irreversible rejection is prevented. One consistent feature of anergy models is the limitation of the production of proinflammatory and growth-promoting cytokines. Thus, the principal basis of anergy in vitro seems to be a rate-limiting reduction in cytokine production, perhaps reflecting the importance of steps such as AP-1 generation.

Tolerance may involve clonal deletion or abortion in some models, and the FasL-Fas pathway is crucial in some of these events. The molecules B7-1 or B7-2 engaging CTLA4 is also a powerful signal for apoptosis.

Efforts to explain anergy by Th1-Th2 theory have been controversial, with support being given in some models but not in others. The interaction between the Th1 and the Th2 cytokines could mediate some of host adaptation. In general, Th1 activation has been seen by some investigators to be associated with rejection and Th2 activation with induction of tolerance (88–90). Other investigators have pointed out that Th2 cytokines could lead to chronic rejection, although there is no agreement on this point. The ability of IL-10 to inhibit IFN-γ production suggests that administration of IL-10 could terminate a rejection response. However, experimental use of IL-10 to promote graft survival has been disappointing. Indeed, IL-10 expression in transplants correlates with rejection, not stability (91). But, rejection involves many mechanisms, and there is no emerging consensus that Th2 cytokines cause tolerance or anergy or stability. Efforts to deviate the T-cell response toward Th2 cytokines could predispose the subject to antibody production and to chronic rejection, and such efforts have not yet been shown to be desirable.

The TGF-β has been shown to possess antiinflammatory activities and may be important in the development of graft adaptation (92), but it has the ability to promote scarring and fibrosis.

F. Responses to Injury and Healing

Cytokines are produced in response to many forms of injury, including ischemic injury. For instance, in renal ischemic injury (acute tubular necrosis), the production of IL-2, IL-4, IL-10, TNF-α, IFN-γ, and TGF-β is increased, as is the expression of MHC class I and II prod-

ucts and adhesion molecules. Thus, cytokines are not only involved in inflammation but are also found in association with repair and scarring. These cytokines in injured tissue may promote rejection and be responsible for the association of tissue injury with impaired outcomes (93,94). Thus, cytokine expression is often a consequence, rather than a cause, of tissue injury. The exact mechanism of the cytokine responses to various forms of injuries is incompletely understood. Nevertheless, with the shortage of organ donors and the acceptance of more suboptimal donors, the role of cytokines in the response to injury becomes increasingly important. Cytokine response may be manipulated to promote tissue healing and remodeling. By administration of growth factors, we may learn to promote healing within the allograft (e.g., by the use of growth factor therapy) (95).

G. Infectious Agents Frequently Enlist Cytokine Mechanisms to Serve Their Purposes

Infectious agents have adapted mechanisms from cytokine systems to manipulate the host defense responses. If imitation is the sincerest form of flattery, these examples emphasize the importance of the cytokine system. For example, Epstein-Barr virus (EBV) has the "BCRF gene," which is homologous with the IL-10 gene, suggesting that EBV uses the antiinflammatory and immunosuppressive capabilities of IL-10 to suppress host defense, possibly contributing to the immunosuppression that occurs during EBV infections. A rabbit pox virus has been shown to have a truncated IFN-γR-like molecule (96). Such a molecule could antagonize the IFN-γ–mediated genes in host defense in a manner favorable to the virus. Human immunodeficiency virus (HIV) uses cytokines in several ways to promote the virus, including the NF-κB site (common in cytokine genes) in the "promoter," the long terminal repeat, of the virus. A chemokine receptor acts as a receptor for HIV entry into lymphocytes (97,98). *Trypanosoma brucei* can apparently trick $CD8^+$ T cells into releasing IFN-γ, which promotes the growth of the organism. Thus, this organism has developed mechanisms for both triggering production of a cytokine and using the cytokine for its growth. The theme of microbes manipulating cytokine responses may be common in the pathogenesis of infectious diseases and may play a role in the infectious complications of transplantation.

Table 5 summarizes the principal source and the speculative roles for the best known transplantation cytokines. The main events involved in the immune response to a transplant are listed in Table 6.

The production of effects of cytokines are important targets for immunosuppressive drugs. For example, the production of many individual cytokines is inhibited by the immunosuppressive agents that inhibit calcineurin (e.g. cyclosporine, FK506). Thus, even though individual cytokines are redundant, a group of cytokines and cytokine-like TNF molecules is essential and nonredundant. Similarly, rapamycin inhibits the growth signal delivered by several cytokines, which are individually redundant. But the growth signal is nonredundant and many experimental agents (e.g., IL-2 toxin, anti–IL-2 receptor monoclonals) are probably responsible for the side effects of other immunosuppressive agents, particularly ATGAM and OKT3.

X. CLINICAL APPLICATIONS IN TRANSPLANTATION

The field of cytokines offers considerable potential for application to clinical transplantation, in terms of understanding, diagnosis, therapy, and monitoring.

Table 5 Examples of Cytokines in Transplantation

Cytokine	Chr[a]	MW (kD)[b]	Principal Source(s)	Significant Features
IL-1	2	17.5	Reticuloendothelial cells; a monokine	-Widely produced and widely received -Proinflammatory -Synergies with many other cytokines
IL-2	4	15	T cells, NK cells The first cytokine produced by newly triggered unsensitized T cells	-Important growth factor for the initiation of T-lymphocyte activation and proliferation -Generally proinflammatory with effects on B cells and macrophages
IL-3	5	14	T cells	-Pluripotent growth factor for marrow-derived stem cells
IL-4	5	20	T cells, mast cells	-Important effects on B cells. Enhances proliferation of activated B cells -Ig synthesis and Ig class-switching; some antagonism of INF-γ
IL-5	5	18	T cells, mast cells	-Important stimulus for B cells and eosinophils
IL-6	7	26	Reticuloendothelial cells, T cells, endothelial cells	-Important in production of acute-phase reactants -Induces growth and differentiation of a wide variety of cells
IL-8	17	8.3	Reticuloendothelial cells, endothelial cells, T cells, fibroblasts, platelets	-Induces chemotaxis, exocytosis, and respiratory burst in neutrophils -Probably chemotactic for lymphocytes
IL-10	1	19	Reticuloendothelial cells, B cells, T cells	-Main targets are APCs -Inhibition of Th1 cytokines (especially INF-γ) -Suggests role in tolerance
IL-12	35		Macrophages, B cells	-Stimulates the production of IFN-γ from T cells and NK cells -In vitro, causes naive T cells to differentiate into Th1 cells
IL-13	10		T cells	-Induces morphological changes, enhances class II, and induces CD23 expression in monocytes -Can inhibit production of some monokines
IFN-γ	12	17	T cells, NK cells	-Major proinflammatory agent with effects on many cells -Primary inducer of MHC in inflammation -Primary stimulus for adhesion molecules
TNF-α	6	17	T cells, reticuloendothelial cells, NK cells	-Proinflammatory and widely produced by activated immune cells -Important factor in "cytokine release syndrome," septic shock, and cachexia
TNF-β	6	20	T cells	-Similar to, and shares receptor with, TNF-α
GM-CSF	5	22	Reticuloendothelial cells, endothelial cells, fibroblasts	-Stimulates growth and differentiation of granulocytes and macrophages -May be involved in cytokine feedback loops
TGF-β	2	12.5	T cells, reticuloendothelial cells, other	-Widely produced, although secreted in an inactive form -Present throughout the inflammatory process -Likely an important factor at multiple steps -Both proinflammatory and immunosuppressive effects reported

[a]Human chromosome number

[b]Molecular weight of the unglycosylated monomeric peptide

Table 6 Main Events in the Immune Response to a Transplant

Reperfusion	Role of chemokines, e.g., IL-8
Injury response	Many cytokines expressed
T-Cell response activation	
G_0 to G_1	
Signal 1	MHC expression regulated by IFN-γ
Signal 2	B7 and other costimulators induced by cytokines and CD40L
G_1 to S (clonal expansion)	
Signal 3	Growth-promoting cytokines (e.g., IL-2, IL-4, IL-7, IL-13, IL-15) trigger growth signal
	De novo purine synthesis
T-cell help for B cells, macrophages	Cytokine-like CD40L, IL-4, IL-2, others
T-cell cytotoxicity	Expression of molecules associated with cytotoxic activity: TNFs, perforins, granzymes
Graft infiltration	Chemokines
Effector activity	
Disruption of extracellular matrix	Elastase, for example
Cytotoxic T cells	Role of TNF, cytokine-like mTNF, FasL-Fas, perforin/granzyme
Delayed-type hypersensitivity	Cytokines that activate macrophages: IFN-γ, TNF-α, IL-4, many others.
Antibody and complement	
Anergy/tolerance	Potential role for Th2 cytokines unproven
	Possible antiinflammatory effects of many cytokines, e.g., TGF-β
Immune suppression	
Decreased cytokine production	Rate-limited cytokine production limits effects of cytokines on grafts and limits effects of cytokines on clonal expansion of T and B cells, e.g., calcineurin inhibition; decreased clonal expansion.
	Rate-limited clonal expansion limits all effector mechanisms, e.g., TOR inhibitors.
	Cyclosporine induces TGF-β expression, but role in immune suppression remains unclear.
Side effects of anti-CD3 and ALG	Cytokines are important mediators of "first dose" effects of OKT3.

A. Understanding

Transplant rejection and induction of tolerance are complex processes involving the interaction of a large number of cytokines and cell types. The elucidation of the role of cytokines and the TNF-like membrane molecules in transplantation will enable a better understanding of the mechanism of rejection.

B. Diagnosis

The association of the expression of cytokines and their receptors in the allograft or body fluid with clinical events may enable the development of clinical laboratory tests that can be used for diagnosing rejection and monitoring the effects of immunosuppressive drugs. One caution is that, in diagnosis, one cytokine can never be used as a marker for a cell type.

The idea that IL-4, for example, can be used to identify a CD4-positive Th2 cell is erroneous. An IL-4–positive cell can be a variety of T and non-T cell types.

C. Therapy

Cytokines or cytokine receptors involved in allograft rejection may serve as target for pharmacological intervention. Purified proteins, engineered proteins, or domains of proteins can be used to manipulate cytokine responses. Certain cytokines possessing intrinsic immunosuppressive activity (e.g., IL-10) have been used to treat rejection. TGFβ1 and TGFβ2 also have antiinflammatory properties. Soluble cytokine receptors (e.g., soluble IFN-γR) can modulate cytokine effects and may be useful as a therapeutic agent. Humanized monoclonal antibodies directed against the IL-2 receptor have been developed and are currently undergoing clinical trials. Soluble TNF receptor is also showing some promise as a therapeutic agent (99,100).

In theory, a humanized monoclonal can be developed against any cytokine receptor or cytokine. The long half-life of the humanized Mabs makes it possible to neutralize these systems specifically for weeks on end, with a single dose of intravenous Mab.

D. Monitoring

Some of immunosuppressive agents in current use (e.g., cyclosporine, FK506) exert their effects by influencing cytokine expression. These drugs have a narrow therapeutic index, and the current practice of measuring the drug levels is of limited value because it does not predict the level of immunosuppression on an individual basis. The direct measurement of the effect of the drug on the target system (i.e., the expression of cytokines) may provide a better and more reliable means of adjusting the dosage of the drug. The practice of immunological monitoring in which the immunosuppressive prescription is titrated against a real-time measurement of its effect may be the next horizon in immunosuppression.

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4

Regulation of Allograft Rejection by Anti-Idiotypic Responses

DOUGLAS M. SMITH

Aastrom Biosciences, Inc., Ann Arbor, Michigan

CHARLES G. WINANS

The University of Chicago, Chicago, Illinois

I. INTRODUCTION

The immune response to an allograft involves a complex series of cellular and molecular events leading to activation of B and T lymphocytes, which express specific cell surface receptors for antigen. Individual B- and T-cell receptors also express unique serologically detectable determinants, or idiotopes, which collectively determine the idiotype. The role of idiotypic interactions in regulation of humoral immunity was first proposed in the network theory by Neils Jerne (1–4). The immune response to an allograft also may be regulated by anti-idiotypic antibodies and anti-idiotypic T cells directed against the receptors of alloreactive lymphocytes.

Recent evidence suggests that anti-idiotypic responses may be involved in the establishment, selection, and homeostasis of receptor repertoires against self and nonself antigens in normal individuals (5–7). Anti-idiotypic responses to autologous lymphocyte receptors are a component of a preexisting self-protective mechanism associated with resistance to autoimmune (8–10) and graft versus host diseases (GVHD) (11–14). Elucidation of the cellular mechanisms responsible for regulation of responses against self antigens has led to the development of receptor-based preclinical strategies such as T-cell vaccination and immunization with T-cell antigen receptor (TCR) peptides for therapy of autoimmune disease. In contrast to autoimmune disease, a high level of receptor diversity is observed

during responses to alloantigens. However, TCR families have been identified in the immune response to particular alloantigens, and public idiotypes may be effective targets for selective immunosuppression of T-cell and B-cell responses in organ transplantation.

This chapter examines the role of B- and T-cell receptor interactions in the generation and regulation of the immune response to alloantigens. First, this chapter discusses the role of immunoglobulin idiotypes and anti-idiotypic responses in regulation of humoral immunity using Jerne's network theory as a conceptual framework. This discussion includes the genetic and structural basis for T- and B-cell receptor idiotypes and the relationship between anti–major histocompatibility complex (MHC) receptors on T and B cells involved in allorecognition. Further discussion includes the role of anti-idiotypic responses in regulation of alloreactivity in experimental model systems and in the clinical situation as a means to overcome the transplantation barrier in man.

II. THE IDIOTYPIC NETWORK THEORY

The concept of antibody idiotypy originated in 1963 from the studies of Henry Kunkel (15) and Jacques Oudin (16). They described serologically detectable antigenic determinants localized in the variable regions of rabbit and human antibodies. These antigenic determinants were unique to antibodies from a particular individual within a species; such unique determinants have been termed idiotopes. Collectively, the set of immunogenic idiotopes on an antibody molecule determines the idiotype. Thus, antibody diversity can be defined based on idiotypy as well as antigen-binding specificity. Each unique antibody possesses a set of serological determinants comprising the idiotype and an antigen-binding site or paratope; these structures are encoded by V-region genes.

The observation that antibody molecules have unique immunogenic epitopes that can be recognized by other anti-idiotypic antibodies provided the basis for the network theory as proposed by Neils Jerne in 1974 (2–4). Jerne suggested that lymphocytes interact in ontogeny and during an immune response through recognition of idiotopes on their own antigen receptors. The following postulates are central to the network concept.

1. The variable regions of antibody molecules are immunogenic and can elicit anti-idiotypic responses in autologous as well as allogeneic or xenogeneic systems.
2. Lymphocyte receptors are connected in an immunological network by a web of idiotypic and anti-idiotypic interactions.
3. Antibody molecules can recognize any foreign or self-epitope.
4. Antibody idiotopes can mimic the three-dimensional molecular structure of any foreign or self-molecule.

According to the network theory, self-reactive anti-idiotypic antibodies are not excluded from the mature antibody repertoire, and anti-idiotypic autoreactivity is the basis for immune regulation. Jerne postulated that any set of idiotopes can be recognized by a corresponding set of paratopes, and conversely that any paratope can recognize a set of idiotopes (2,3). Jerne defined the set of antibody molecules produced in response to a particular antigenic epitope as Ab-1; the set of anti-idiotypic antibodies elicited by Ab-1 has been termed Ab-2; further anti-idiotypic responses to Ab-2 have been termed Ab-3. Thus, connections are formed in the immunological network by interactions between self-idiotypes and complementary autologous anti-idiotypic antibodies.

Novotny et al. have used x-ray diffraction studies to locate antigenic epitopes on exposed or accessible regions of antibody molecules (17). These studies predict the exis-

tence of about 40 distinct idiotopes located in the framework and hypervariable regions of an antibody molecule. Therefore, it is conceivable that a particular idiotype could be recognized by as many as 40 different anti-idiotypic antibodies, resulting in a highly connected network. Regulation of the immune response may depend on the frequency and affinity of connecting anti-idiotypic interactions (18,19) and on the functional phenotype of the interacting cell types.

Jerne postulated that some network connections are stimulatory and others inhibitory to the target lymphocyte. Binding of anti-idiotypic antibody to a cellular receptor may deliver either a positive or negative signal to the target cell, leading to activation, proliferation, and differentiation or to tolerance and unresponsiveness (2). T and B cells with unrelated fine specificities and functional phenotypes can be connected in an immune response by complex antigens with multiple epitopes. Antigen processing and presentation by an appropriate accessory cell is critical for this type of antigen-mediated connectivity. Similarly, idiotypic interactions may connect functionally distinct B and T lymphocytes (20). The helper or suppressor effector function of an interacting idiotope-specific T cell may determine the responsiveness of the complementary B-cell clone.

Jerne proposed that multiple positive and negative interactions lead to a dynamic steady state as elements of the immune system interact and as some elements decay and new elements emerge (2). Antigenic challenge causes an increase in the concentration of idiotypic receptors specific for antigen. As Ab-1 stimulates production of Ab-2 and Ab-3, an entire network of interconnected cells and molecules is perturbed. Eventually, through a series of stimulatory and inhibitory interactions, the system reaches a new equilibrium.

During ontogeny, multiple germ line V-, D-, and J-immunoglobulin gene segments rearrange randomly to generate a diverse receptor repertoire. There may be as many as 100 million different antibody specificities in the mouse (2). The repertoire of specificities initially is not biased toward recognition of self- or nonself-antigens. Therefore, the receptor repertoire should include antiself-reactive antibodies specific for self-idiotypes and an array of other nonimmunoglobulin self-molecules, in addition to receptors reactive with foreign antigens. This random rearrangement of V-region genes suggests that a unique immunological network develops in each individual. Therefore, tolerance to self must be learned during development and cannot be encoded in the germ line (2,3).

The idea that every individual is capable of generating regulatory responses to self-idiotypes is an intrinsic property of the network theory. According to Jerne's view, V-region genes have been selected by evolutionary pressure to encode receptors reactive with self-antigens of the species. Self-reactive T- and B-cell clones are not purged from the repertoire, but are regulated by mechanisms involving complementary antagonistic anti-idiotypic antibodies or suppressor cells (4). Escape from idiotypic regulation by a small fraction of lymphocytes may cause autoimmune disease. Presumably, differences in responsiveness to self-idiotopes as compared to all other conventional self-antigens are related to differences in network stability. Apparently, the network can be perturbed by foreign antigen, leading to Ab-1 (self-idiotypes) and Ab-2 (anti-idiotype), but is stable and resistant to perturbation by conventional nonimmunoglobulin self-antigens.

As a result of the random recombination of V-gene segments, an antibody variable region can mimic the molecular conformation of any molecule within an organism or in its external environment. V-gene rearrangement leads to a vast array of molecular conformations. In experiments in which mice are immunized with an antigen leading to Ab-1, the usual response is the production of a complementary set of anti-idiotypic antibodies designated Ab-2. Ab-2 can bind idiotopes outside of the antigen-combining region of Ab-1 or

within the antigen-combining site. Thus, a subset of paratope-specific anti-idiotypic Ab-2 antibodies can mimic antigen binding to Ab-1; these Ab-2 molecules have been termed internal images by Jerne (2,4).

It has been shown that xenogeneic, allogeneic, or syngeneic Ab-2 can induce specific immune responses in the absence of antigen (21–24). The range of molecular structures that can be mimicked by antibody is striking; Ab-2 can substitute functionally for nucleic acids, monosaccharides, or polysaccharides including tumor antigens (25–27), drugs, and other polypeptides in a variety of biological systems (reviewed in 28). Anti-idiotypic antibodies directed against the paratopes of hormone-specific antibodies have been shown to mediate hormone-specific effects in several systems (29, reviewed in 30). It has been reported that anti-idiotypic antibodies against Ab-1 specific for trypanosomes (31), reovirus hemagglutinin (32), or hepatitis B surface antigen (33) can induce immunity in vivo to the respective antigen (reviewed in 34,35). Antibody mimicry can be explained by amino acid sequence homology between Ab-2 and the normal ligand. Alternatively, entirely different amino acid sequences may assume nearly identical three-dimensional molecular conformations. Therefore, it is not essential for the Ab-2 internal image of an antigen to share primary sequence homology with the antigenic epitope or ligand recognized by Ab-1 (28). These findings are consistent with the idea that mimicries within the immunological network may function to expand lymphocyte receptors specific for external pathogens prior to antigenic challenge.

III. GENETIC BASIS OF THE IDIOTYPIC REPERTOIRE ON B- AND T-LYMPHOCYTE RECEPTORS

Specific recognition of antigen during the immune response involves two diverse receptor systems: (1) surface immunoglobulin functions as the antigen receptor for B lymphocytes, and (2) T cells express an MHC -restricted surface receptor that recognizes antigen in association with self-MHC structures. These two sets of receptor molecules share structural homology at the protein level. In addition, the immunoglobulin genes and the genes encoding the T-cell receptor share extensive sequence and organizational homology, suggesting a common origin in evolution (36–38).

A. Genetic Basis for the B-Lymphocyte Idiotypic Repertoire

Functional immunoglobulin genes for the heavy chain variable regions are assembled from separate variable (V_H), diversity (D_H), and joining (J_H) gene segments; the light chain variable region genes are constructed only from V_L- and J_L-gene segments during early stages of B-cell development. The germ line encodes approximately 100 V_H, 20 D, and 4 J_H H-chain gene segments, together with 200 V_K and 4 J_K L-chain V-gene elements (39). A diverse repertoire of antibody specificities is generated from the large number of different germ line V-region gene segments, the combinatorial assortment of multiple V-, D-, and J-elements, and by variation of the site at which gene segments are joined (junctional diversity). Additional antibody diversity is introduced by the combinatorial association of different heavy and light chains and by somatic hypermutation of V genes in the existing repertoire after the heavy chain isotype switch from IgM to IgG (40–42). Thus, the complexity of the idiotypic repertoire reflects the rearrangement, somatic modification, and expression of the heavy and light chain V-gene segments.

The variable regions of immunoglobulin heavy and light chains each consist of relatively conserved or framework amino acid sequences interrupted by three hypervariable regions. The hypervariable sequences loop away from the V-region backbone to form anti-

gen-contact areas, which are referred to as complementarity-determining regions (CDRs) (41,43). There are six CDRs on an antibody molecule: the H- and L-chain V-region genes each encode three CDRs. CDR1 and CDR2 of the H-chain V-region are encoded by the V_H gene segment; CDR3 is generated from the D element and by junctional diversity at adjacent V-D and D-J joining sites. Similarly, CDR1 and CDR2 of the L-chain V-region are encoded by the V_L gene segment, and CDR3 arises from the V-J joining region. Therefore, diversity is generated in the first and second CDRs from the polymorphism of multiple germ line V_H- or V_L-gene segments; the third CDR is generated largely from junctional diversity (41,44).

Idiotopes have been identified in both the framework and hypervariable regions of Ab-1 using monoclonal anti-idiotope antibodies (45). Anti-idiotypic antibodies that recognize idiotopes in the CDRs may inhibit binding of Ab-1 to antigen. Alternatively, anti-idiotypic antibodies that recognize idiotopes in the framework regions may not block binding of Ab-1 to antigen or may induce conformational changes leading to loss of antigen-binding activity (41,45). Therefore, the operational definition of idiotopes as binding-site–related or non–binding-site–related based on blocking studies may not correlate directly with antibody structure.

Rajewski and coworkers have used monoclonal anti-idiotope antibodies to identify idiotopes on a monoclonal anti-NP antibody (44–46). A public idiotope designated Ac38 was localized to a region outside the hapten-binding cleft; a second idiotope designated Ac146 was localized to a region close to the hapten-binding site based on competition between hapten and anti-idiotypic antibody for binding to Ab-1. Anti-NP monoclonal antibody variants, which had lost the Ac146 idiotope, were derived; these variants simultaneously lost hapten-binding specificity. Another series of anti-NP variants was selected for loss of the Ac38 idiotope. Mutation of a single amino acid encoded by the D-gene segment led to the modification of one idiotope and the loss of six additional idiotopes on the parent anti-NP antibody molecule (44,46). Interestingly, the variant Ab-1 retained hapten-binding activity. Thus, minor substitutions in primary amino acid sequence can cause dramatic changes in expression of idiotypic determinants without affecting antibody specificity. Idiotopes that are associated closely with antibodies of a particular specificity may not be directly involved in formation of the antigen-binding site.

Idiotopes have been identified on isolated H or L chains; however, chain reassociation experiments suggest that both heavy and light polypeptide chains usually are necessary for full expression of idiotypic determinants (21,44,47). Interaction between idiotope and anti-idiotope may involve distant areas of the protein surface and cannot be attributed to a single continuous sequence of amino acids (44,48). Several studies indicate that the D region is critical for determination of idiotopes localized to CDR3. However, amino acids in V_H and V_L also contribute to idiotypic interactions and anti-idiotypic antibodies can recognize conformational determinants specified by residues located in separate hypervariable regions (46,48). Clevinger et al. have analyzed the hypervariable region structure and idiotype expression of anti–dextran-reactive monoclonal antibodies (47). In this system, cross-reactive or public idiotopes were localized to CDR2, which is encoded by the V_H-gene segment; private idiotopes were localized to the D-gene segment, which determines CDR3.

Molecular genetic analysis of antibody responses dominated by public idiotypes, including the responses to phosphorylcholine, dextran, arsonate, and nitrophenyl, suggest that each response is based on expression of a single or very few germ line V_H genes and a single or very few germ line V_L genes (reviewed in 41,44). Rearrangement of these V genes in combination with several D- and J-gene segments forms the basis for expression of the few antibody families observed in these responses. Additional families of idiotypically

related antibodies are created by H/L combinatorial diversity and somatic mutation. Thus, in these model systems, public idiotypes are controlled by the expression of a few antibody structural genes; private idiotypes may arise randomly by somatic mutation of these germ line genes. According to this interpretation, sharing of idiotopes may reflect sharing of V-gene segments. Therefore, antibodies with differing specificity but related idiotype may share the same V genes in combination with differing D or J segments.

B. Genetic Basis for the T-Lymphocyte Idiotypic Repertoire

The surface receptor for antigen on most T cells is composed of a single alpha polypeptide chain and a single beta chain, which form a disulfide-linked heterodimer. There are approximately 50 different α-chain V-region genes and 50 different J-region genes in mouse and man (49). Combinatorial rearrangement of these gene segments would allow production of at least 2500 different mature α-chain genes. In addition, germ line–encoded nucleotides can be deleted at the junction of the V- and J-gene segments leading to N-region diversity. Similarly, a functional β-chain gene is constructed from one of approximately 50 V-region genes, one of two D-region genes, and one of 13 J-region gene segments; N-region diversity is introduced at the V-D and D-J joining sites. Therefore, at least 4000 different β-chain sequences are possible in man. This number may be less in mice because there are only about 21 β-chain V-genes and 12 J-region genes. Thus, the potential diversity of αβ T-cell receptor sequences before thymic selection may be comparable to the number of different antibody molecules (49).

Infante et al. produced anti-idiotypic antisera against alloreactive T-lymphocyte clones using either an F_1 anti-(P anti-F1) protocol or by immunization of syngeneic mice (50,51). Subsequently, several groups of researchers derived clonotypic monoclonal antibodies reactive with the T-cell receptor for antigen on T-cell clones and T-cell hybridomas (52–56). Monoclonal anti-idiotypic antibodies were shown either to block or stimulate proliferation, lymphokine release, and cytolysis in vitro by the appropriate immunizing clone but not by other closely related T-cell clones. These findings extended early studies using polyclonal anti-idiotypic antibodies and heterogeneous T-cell populations (57,58) and demonstrated directly that the T-cell receptor for antigen expresses unique idiotypic determinants that can be recognized by clonotypic antibodies.

Recent studies have attempted to elucidate the structure of the TCR-binding site by correlating particular specificities for antigen or MHC with expression of particular V_α- or V_β-gene segments. The $CD4^+$ T-cell population is reactive with or restricted by class II molecules, whereas the $CD8^+$ population is reactive with or restricted by class I MHC molecules. However, there is no direct correlation between the expression of a particular α- or β-chain and associative recognition of class I versus class II self-MHC structures; the repertoire of α- and β-chains used by the $CD4^+$ and $CD8^+$ T-cell subsets is overlapping (59–63).

The TCR repertoire in response to an individual antigen or antigenic epitope can be highly degenerate with expression of multiple different V, D, and J segments (61,64). In many systems, a simple relationship does not exist between receptor specificity and gene segment usage (64–66). Bill et al. report that the TCR repertoire in the response of C57BL/10 mice ($I\text{-}A^b$) to the mutant class II $I\text{-}A^{bm12}$ alloantigen is extremely heterogeneous (67). This response involves a disparity of only three amino acids between the mutant and wild type I-A molecule. V_α-gene expression in 178 bm12 reactive T-cell hybridomas was not significantly different from a panel of random BL/10 T-cell blasts. However, the pattern of V_β-gene usage was not completely random. A slight bias for expression of the $V_\beta 14$, $V_\beta 15$, and $V_\beta 16$ genes was observed without dominant expression of any single β-chain. A general bias for

$V_\beta 8$-gene usage also has been reported for the response to mutant class I H-$2K^{bm1}$, H-$2D^{bm14}$ MHC molecules (68) or H-$2L^d$ (69,70), and skewed V_α- and V_β-gene expression was observed by Garman et al. in populations of alloreactive T cells derived by mixed leukocyte culture (MLC) across a full MHC difference (61).

In other systems, the receptor repertoire appears to be less diverse, and predominant specificities are apparent (71–74). Hochgeschwender et al. have shown that 40% of T-cell clones isolated independently for H-$2K^b$/TNP reactivity express identical germ line α- and β-chain gene segments (71). At least half of these clones were derived from independent precursors and are not replicate clones derived from the same precursor during the immune response. Sorger et al. derived Cyt C–reactive T-cell clones with related but distinct reactivity patterns based on the fine specificity for particular Cyt C peptide fragments, recognition of self-MHC restricting elements, and antigen cross-reactivity patterns (73). The expression of particular α-chain elements correlated well with antigen reactivity, whereas the use of particular V_β region segments appeared to determine MHC recognition (72).

Responses to particular alloantigens also may correlate with gene usage. Kappler et al. have derived a monoclonal antibody that is reactive against an epitope encoded by the $V_\beta 17a$ gene segment; T cells from a variety of strains that express this V gene have an unexpectedly high frequency of reactivity to allogeneic I-E molecules (49,75). Greater than 90% of T cells that express the $V_\beta 17a$ gene from SWR mice react with an allogeneic I-E molecule regardless of the expression of other TCR gene segments. These findings are consistent with the idea that germ line TCR genes are biased toward recognition of MHC antigens as suggested by Neils Jerne (1). Similarly, MacDonald et al. have demonstrated a direct correlation between $V_\beta 6$ expression and T-cell reactivity to minor lymphocyte stimulating (Mls) antigens (62). Shirwan et al. have demonstrated that T lymphocytes infiltrating rat cardiac and renal allografts express a limited TCR-Vβ repertoire (76,77). However, the α-chain repertoire appears to be diverse (78). Restricted use of TCR V genes has been reported in the responses to HLA-B7 (79), HLA-B27 (80,81), HLA-DP (82), and HLA-DR (83,84). Dominant TCR rearrangements in human allograft–infiltrating T cells have been reported by several investigators (85–87).

Collectively, these findings suggest that the relationship between V_α- and V_β-gene usage and T-cell specificity for MHC and antigen is complex. It is likely that the antigen-combining site is composed of contact residues contributed by the complex interaction of both receptor chains together, as described for the B-cell receptor, rather than by a single α- or β-chain alone. However, the use of particular V-gene segments may significantly bias T-cell recognition. Thus, elimination of T cells expressing these TCR V genes may allow selective inhibition of allograft rejection (69,70).

IV. IDIOTYPE EXPRESSION ON ANTIBODIES TO TRANSPLANTATION ANTIGENS

A. Idiotypes on Murine Anti-H-2–Specific Monoclonal Antibodies

The serological response to a complex foreign antigen may involve the production of several hundred distinct antibody molecules (88). These antibodies may be heterogeneous in fine specificity, affinity for antigen, and idiotype. The set of antibodies that express a particular idiotype usually is very small. Such idiotypes have been termed private idiotypes. However, some idiotypes occur relatively frequently during the immune response by individuals within a species to a particular antigen and are referred to as public or cross-reactive idiotypes. As a result of extensive sharing of public idiotypes in the Ab-1 response, the

anti-idiotypic Ab-2 response of one individual would be expected to cross-react widely with Ab-1 from other individuals.

Bluestone et al. have used mouse monoclonal antibodies directed against individual allogeneic MHC molecules as a source of Ab-1 for production of xenogeneic anti-idiotypic antisera (21). Swine were immunized with a panel of 14 different alloreactive mouse monoclonal anti-H-2 antibodies that recognize a variety of class I and class II antigens. Only 3 of the 14 monoclonal antibodies expressed idiotypes found widely in conventional polyclonal antibody responses. A proportion of the swine anti-idiotypic antibodies specific for 28-13-3, an anti-H-2K^b mAb, was shown to recognize combining-site–related idiotopes (21). However, anti-K^b antibodies with fine specificities different from 28-13-3 shared idiotypic determinants.

In two additional systems, public idiotypes were detected on anti-L^d (89) or anti-I-E^k monoclonal antibodies (90). A strong correlation was observed between idiotype and fine specificity in these systems. Antibodies that shared reactivity to a particular epitope cluster also shared idiotypes; antibodies with fine specificity for other epitope clusters on the same alloantigen did not express the idiotype detected by the swine anti-idiotypic antiserum (21). Thus, a general correlation exists between the antigen-binding specificity and idiotype of an antibody. However, as described, this correlation is not absolute and antibodies with completely unrelated specificities can share idiotypic determinants as shown by Oudin and Cazenave in 1971 (91).

The majority (11 of 14) of the mouse monoclonal anti-MHC antibodies studied by Bluestone et al. expressed relatively private idiotypes that were not widely detectable in conventional polyclonal alloantisera. These findings may reflect the heterogeneity of the immune response to alloantigen. The predominant expression of an idiotype in a complex serological response may be determined in part by the immunodominance of a particular antigenic epitope. Alternatively, differences in the predominance of expression of a particular idiotype might be explained by somatic diversification of germ line V-gene sequences after the isotype switch from IgM to IgG. Two of the three monoclonal antibodies that express a public or dominant idiotype belong to the IgM heavy chain class. Antibodies of the IgM class exhibit fewer V-region somatic mutations than IgG antibodies (21,40,42). Therefore, IgM antibody might be less diverse than IgG and more closely resemble germ line genes. Somatic mutation after the isotype switch would lead to more highly diverse antibody idiotypes. Thus, public or recurrent idiotypes may be markers for germ line V genes, whereas somatic variation may give rise to private idiotypes.

The effect of anti-idiotypic antibody on the immune response to MHC antigens was studied in vivo by administration of xenogeneic anti-idiotypic antiserum to adult mice. Administration of swine anti-idiotypic antiserum (Ab-2) directed against public or private idiotypes on various monoclonal anti-MHC antibodies (Ab-1) consistently induced expression of idiotypic Ab-3 molecules rather than causing suppression. The idiotype expressed by Ab-3 was highly similar to Ab-1 in all model systems. In several systems, specific binding activity for the MHC antigen recognized by the original idiotype (Ab-1) was induced by therapy with anti-idiotypic Ab-2. In other systems, antigen-specific antibody was induced only in a proportion of animals, and the majority (90%) of Ab-3 molecules did not bind to MHC antigen. These results suggest that Ab-3 can be divided into two classes: (1) Ab-3 can have antigen-binding specificity and idiotype similar to Ab-1, or (2) Ab-3 molecules can have unrelated antigen specificity yet share idiotypic determinants with Ab-1 (57).

Administration of xenogeneic anti-idiotypic antiserum to recipient mice before challenge with an allogeneic skin graft caused increased expression of idiotype in the subse-

quent humoral immune response. The proportion of anti-MHC antibodies that expressed idiotype was increased from 0% to 22% in control mice to 30% to 78% in mice that received anti-idiotypic antiserum before skin grafting. Thus, anti-idiotypic antibody therapy caused a profound shift in the repertoire of anti-H-2 antibodies. Therapy with anti-idiotypic antibody did not cause idiotype suppression or prolongation of skin graft survival in these model systems (21).

B. Idiotypes on Murine Anti-HLA–Specific Monoclonal Antibodies

Ferrone and colleagues have studied extensively the idiotypic diversity and variable gene usage in the cascade of mouse syngeneic Ab-1, Ab-2, and Ab-3 responses to xenogeneic HLA-DR and HLA-DQ MHC antigens. A mouse anti-HLA-DR1,4,w6,w8,w9 reactive monoclonal antibody (Ab-1) designated AC1.59 (92) was used to immunize BALB/c mice to produce a panel of six syngeneic anti-idiotypic (Ab-2) monoclonal antibodies (mAbs) (93). The six anti-idiotypic mAbs recognized private idiotopes expressed on AC1.59 but did not recognize idiotopes expressed on a large panel of anti-class I or anti-class II MHC-reactive mAbs. All of the monoclonal anti-idiotypic antibodies inhibited the binding of AC1.59 (Ab-1) to target cells. In addition, the monoclonal Ab-2s cross-blocked each other in binding to Ab-1, suggesting recognition of spatially close idiotopes located within the antigen-combining site of AC1.59 (93). Interestingly, human alloantisera have been identified which recognize a specificity similar to the polymorphic determinant on HLA-DR antigens recognized by AC1.59 (94). Therefore, this model system may provide information relevant to idiotype cascades that are generated during human immune responses to alloantigens.

To characterize the polyclonal anti-anti-idiotypic (Ab-3) responses induced by anti-idiotypic mAb-2s in vivo, syngeneic BALB/c mice were immunized with the six monoclonal anti-idiotypic (mAb-2s) antibodies. The specificity of the Ab-3 responses elicited after immunization with individual mAb-2s was not identical. While each Ab-3 antiserum inhibited the binding of the corresponding monoclonal Ab-2 to AC1.59 (Ab-1), at least one Ab-3 antiserum did not competitively inhibit the binding of the other five anti-idiotypic (Ab-2) mAbs to AC1.59, indicating recognition of a different idiotype. Furthermore, only two of the six mAb-2s designated F5-444 and F5-830 induced Ab-3 antisera that mimicked the specificity of AC1.59 for HLA-DR1,4 or DRw8 antigens (93). These studies demonstrate that a subset of syngeneic anti-idiotypic (Ab-2) antibodies bears an internal image of MHC antigen. This subset of Ab-2s can stimulate a humoral immune response against the original HLA-DR antigens used for immunization.

Subsequently, a panel of monoclonal Ab-3 antibodies with specificity for HLA-DR was derived by immunization of BALB/c mice with anti-idiotypic monoclonal antibodies F5-830 (94) or F5-444 (95). It was shown that these two mAb-2s, which recognize idiotopes coexpressed in the combining site of AC1.9, selectively stimulated distinct subsets of idiotope-positive Ab-3–secreting B-cell clones (96). The mAb-3s exhibited several different reactivity patterns to HLA-DR antigens expressed on a panel of HLA-type B-cell lines. Thus, the fine specificities of the Ab-3s were similar yet distinct from the reactivity pattern of AC1.9 (Ab-1). Furthermore, four different patterns of idiotope expression were identified within the panel of mAb-3s elicited by F5-830. These findings demonstrate that the mAb-3s were heterogeneous in expression of idiotopes when compared to each other and to Ab-1. In addition, no direct correlation was found between idiotope expression and the specificity of Ab-3s for HLA-DR because the idiotopes recognized by F5-830 and F5-444 also were expressed on Ab-3s which did not bind HLA-DR (94,95).

Molecular analysis of the heavy and light chain V regions of each of the six Ab-2

monoclonal antibodies elicited by AC1.59 revealed considerable heterogeneity in V-gene usage (97). There was no correlation between V-gene usage and idiotope expression or ability to induce antigen-binding mAb-3s in vivo. For example, mAbs F5-830 and F5-444 elicited anti-HLA-DR–reactive Ab-3s in BALB/c mice, yet these anti-idiotypic mAbs utilize unrelated V genes. Conversely, mAb F5-444, which elicits Ab-3 antibodies reactive with HLA-DR, differs in only two CDR residues and five framework residues from an anti-idiotypic mAb designated F5-1126, which does not induce HLA-DR–specific Ab-3 antibodies. It is likely that F5-444 and F5-1126 were derived by somatic mutation of V genes expressed by a common parental B-cell clone. Thus, somatic mutation of V genes encoding anti-idiotypic antibodies appears to increase the complexity of the idiotypic cascade and may have profound effects on the nature of the Ab-3 response. The authors suggest that anti-idiotypic antibody responses may be a mechanism for expansion of the diversity of anti-HLA antibodies produced during the course of an immune response to mismatched HLA alloantigens (93,94).

In a second model system, a panel of five syngeneic mouse anti-idiotypic mAbs was generated against KS13, a mouse anti-HLA-DQw3–reactive mAb (98). Four of the mAb-2s (KO3-34, KO3-256, KO3-335, and R1-38) recognized spatially close private idiotopes expressed uniquely within the antigen-combining site of KS13. All of the mAb-2s cross-blocked each other in binding to Ab-1 and inhibited KS13 binding to cells expressing HLA-DQw3. These mAb-2s did not recognize idiotopes expressed on a panel of anti-class I and anti-class II MHC-specific mAbs, including several different anti-HLA-DQw3–reactive mAbs, which cross-blocked the binding of KS13 to target cells (98,99). However, a fifth mAb-2, designated R18-9, was shown to recognize a spatially distant cross-reacting public idiotope located outside of the KS13 antigen-combining site. The idiotope recognized by R18-9 was expressed on both anti-class I (anti-HLA-B7, anti-HLA-A2,B17), and anti-class II (anti-HLA-DQw3, anti-HLA-DR,DP) MHC-reactive mAbs (98). Only one of the five syngeneic mAb-2s elicited by KS13, designated KO3-34, was capable of inducing anti-HLA-DQw3–specific mAb-3s in BALB/c mice.

KO3-34, which recognizes an idiotope within the antigen-binding site of KS13, was used in vivo to elicit mAb S2B154, a syngeneic anti-HLA-DQw3–reactive mAb-3. KO3-34 specifically inhibited binding of either S2B154 (mAb-3) or KS13 (mAb-1) to B-cell lines expressing HLA-DQw3. In addition, both S2B154 (Ab-3) and KS13 (Ab-1) displayed similar reactivity with the panel of anti-idiotypic mAb-2s (KO3-34, KO3-256, KO3-335, and R1-38) which recognize idiotopes located within the antigen-binding site of KS13 (Ab-1). In contrast, S2B154 (mAb-3) did not express the idiotope defined by mAb R18-9, which recognizes an idiotope located outside of the antigen-binding site of the immunizing mAb-1, KS13. Thus, KS13 (Ab-1) and S2B154 (Ab-3) have identical antigen specificity and display very similar yet distinct idiotypic profiles based on reactivity with the panel of mAb-2s (100).

To determine the structural basis for idiotype expression, the V-region gene sequences of KS13 (mAb-1), the panel of five anti-idiotypic mAbs (mAb-2s), and S2B154 (mAb-3) were determined (101). Among the five mAb-2s, R1-38 and R18-9 used multiple V-gene segments, which were different from the V genes used by KO3-34, KO3-256, and KO3-335, indicating that multiple V-gene combinations can confer anti-idiotypic specificity for anti-DQw3 mAbs. In contrast, KO3-34, KO3-256, and KO3-335 probably arose from the same parental B-cell clone, because they express the same heavy and light chain V-, D-, and J-gene segments, which differ from each other only in point mutations. These results confirm that V-gene point mutations can have dramatic effects on antigen mimicry and idiotypic interac-

tions (46), because only KO3-34 induced antigen-specific Ab-3 in syngeneic or xenogeneic hosts (101).

The V_H and V_L regions of KS13 (mAb-1) and S2B154 (mAb-3) were encoded by the same V_H, V_K, and J_H genes, and the sequences in these regions differed only in a few amino acid substitutions. However, these two mAbs used different germ line D- and J_K-gene segments. Thus, the differential reactivity of R18-9 with KS13 but not S2B154 may reflect V-gene mutation events or the use of different D- or J_K-gene segments. These findings demonstrate a high degree of structural homology between an Ab-1, which was induced by nominal MHC antigen, compared to an Ab-3, which was elicited by anti-idiotypic antibody bearing an internal image of antigen. No amino acid homology was observed between mAb-2s, which express an internal image of class II MHC antigens and the α_1 or β_1 domains of HLA-DQ3 or HLA-DR molecules. Therefore, the ability of these mAb-2s to mimic MHC antigens appears to depend on the three-dimensional conformation of the anti-idiotypic antibody rather than on primary sequence homology (99,101).

Interestingly, R1-38, a mAb-2 that recognizes an idiotope within the antigen-combining site of KS13, was shown to inhibit KS13 plus complement-mediated or antibody-dependent cell-mediated cytotoxicity (ADCC) of human target cells. In contrast, R18-9, a mAb-2 that recognizes an idiotope mapping outside of the antigen-combining site of KS13, markedly enhanced ADCC, suggesting that anti-idiotypic mAbs can modulate the lytic activity of Ab-1 (98). The potential for beneficial effects of Ab-2 on kidney transplant survival after blood transfusions or in sensitized recipients (102) is discussed further in Sec. VIII.

V. NETWORK INTERACTIONS IN IMMUNOLOGICAL TOLERANCE

A. Immunological Tolerance to Self-Antigens

Immunological tolerance is the process that limits or prevents the immune response to self-antigens (103). Recent evidence suggests that self-tolerance is achieved by a combination of four basic mechanisms: (1) sequestration of self-antigens from immunocompetent lymphocytes, (2) clonal abortion or deletion of anti-self–reactive lymphocytes from the repertoire, (3) clonal anergy or delivery of a negative signal to lymphocytes specific for self-antigens, and (4) active suppression of self-reactive lymphocytes (reviewed in 103). Several mechanisms may contribute to sequestration of self-antigens; autoimmunity may be prevented by limitation of the number of antigen presenting cells (APCs), suboptimal expression of MHC antigens, or failure of accessory cells to process or bind an immunogenic peptide (103–106). However, the primary targets for tolerance induction appear to be the T- and B-lymphocyte populations rather than the accessory cells (103).

Clonal abortion results from the cellular death of high-affinity autoreactive T-lymphocyte clones after contact with self-antigens at an immature developmental stage in the thymus. In contrast, clonal deletion implies inactivation of a mature previously immunocompetent lymphocyte. These irreversible mechanisms of clonal inactivation are distinct from potentially reversible tolerogenic events such as T-cell modulation of the CD3–TCR complex following exposure to high concentrations of antigen (107,108). Self-reactive B lymphocytes appear to be downregulated by induction of unresponsiveness without cell death after encounter with self-antigens. Alternatively, B- or T-cell unresponsiveness may be secondary to a requirement for signaling events provided by cytokines or lymphokines from collaborating accessory cells or helper T cells (103).

Functional clonal inactivation has been reported for neonatally induced tolerance to

nonself allogeneic MHC antigens based on limiting dilution analysis of antigen-reactive cells (109–112). These findings do not distinguish between mechanisms involving clonal inactivation versus active suppression by anti-idiotypic responses. Considerable evidence supports the idea that clonal abortion is a principal mechanism for tolerance induction to self-MHC and minor antigens before T-cell entry into the peripheral lymphocyte pool (49,62,113). However, it is difficult to account for induction of tolerance to all self-antigens exclusively by intrathymic presentation of self-antigens. Self-antigens may migrate to the thymus directly in the circulation after shedding from cells or after acquisition by recirculating APCs. However, many self-antigens would not be expected to traffic from the periphery to the thymus. Therefore, additional mechanisms must operate in the periphery to downregulate autoreactive T cells that escape thymic selection or arise from somatic mutation of T- and B-cell receptor genes in the adult. Thus, the mature recirculating lymphocyte pool may include T and B cells that express surface receptors specific for self-antigens. These lymphocytes may be unresponsive to self-antigens as a result of network regulation by coexisting anti-idiotypic T and B cells.

B. Maintenance of Self-Tolerance by Idiotypic Interactions

Evidence from two model systems for graft versus host disease (GVHD) described initially by Glazier et al. (11) and Wilson (12) supports the idea that self-tolerance is maintained in the periphery by active suppression involving idiotypic interactions. Cyclosporine A (CsA) induces a lethal GVHD syndrome in irradiated rats (11,114) or mice (115–117) after transplantation of syngeneic or autologous bone marrow. Symptoms of autoimmune GVHD develop in reconstituted animals within 1 to 2 weeks after discontinuation of CsA therapy. Autoimmune disease does not develop in thymectomized animals after irradiation and CsA therapy, suggesting that the autoreactive cell population originates or matures in the thymus (114). Further studies indicated that autoimmune disease is associated with development of $CD8^+$ cytolytic T cells reactive against self–class II antigens (118); however, T-cell populations containing both $CD4^+$ and $CD8^+$ subsets (119) or enriched for only $CD4^+$ T cells may be more effective than $CD8^+$ T cells alone for adoptive transfer of CsA-induced disease (114). Recent reports suggest that CsA interferes with thymic T-cell development and clonal deletion of self-reactive T cells (61,117,120,121). Reduced expression of class II MHC molecules, which are critical for tolerance induction, together with selective atrophy of the thymic medulla after prolonged administration of CsA may contribute to generation of these autoreactive T cells (115,116).

Autoreactive T lymphocytes appear to be necessary but not sufficient to cause autoimmune GVHD after host irradiation and administration of CsA for 40 days. Autoimmune disease is transferred adoptively to irradiated but not to normal syngeneic secondary recipients by T-cell populations from diseased donors (11,115). Adoptive transfer of disease by T cells from an autoimmune animal is prevented by cotransfer of spleen cells from normal animals (114,122). Furthermore, administration of CsA to normal unirradiated animals does not cause autoimmune disease; however, autoreactive T cells can be demonstrated in these animals by adoptive transfer to irradiated secondary recipients (115). Thus, the normal host appears to contain a preexisting radiosensitive resistance mechanism that prevents self-reactive T cells from causing autoimmune disease after escape from thymic selection.

These findings are consistent with the idea that manifestation of autoimmune GVHD

involves complex mechanisms, including generation of autoreactive T cells as a result of defective clonal deletion in the thymus and perturbation of immune regulation in the periphery (123). Radiation and CsA therapy may inactivate anti-idiotypic suppressor mechanisms that control self-reactive T cells in the thymus as well as in the periphery (124). Autoreactive T cells that migrate to the periphery may become responsive to self-antigens after discontinuation of CsA therapy. Alternatively, CsA may interfere directly with the activity of thymic and peripheral suppressor T cells, which regulate autoreactive T-cell populations.

Woodland and Wilson have studied the mechanisms for resistance to GVHD in a rat model (125). Lethal GVHD develops in normal F_1 (A × B) recipient rats only after administration of large numbers (10^9) of parental A- or B-strain T lymphocytes; disease is mediated primarily by alloreactive parental $CD4^+$ T cells (126). In contrast, irradiated animals are highly susceptible to induction of disease by small numbers ($1–3 \times 10^7$) of parental strain T cells. Therefore, this model system provides additional evidence for a radiosensitive resistance mechanism analogous to the model for autoimmune GVHD. Specific resistance to GVHD is induced in normal F_1 rats after systemic administration of small numbers of parental strain T cells, which express surface receptors for host MHC antigens (125).

Resistance to GVHD appears to be mediated by host anti-idiotypic T-cell responses against donor strain (A anti-B) T-cell receptors, which recognize alloantigens expressed by the F_1 host (127). Host anti-idiotypic T lymphocytes, which recognize parental A anti-B–specific T-cell receptors, also prevent GVHD against third party C-, D-, or E-donor T-cell populations reactive against the B haplotype. These findings suggest that rat anti-MHC T-cell receptors expressed during the immune response to particular alloantigens may be highly conserved within the rat species. Regulatory T cells from a variety of rat strains appear to recognize a common idiotype expressed on T cells that recognize the B haplotype (128).

Kimura et al. have shown that resistance to GVHD is adoptively transferred to secondary recipients by either $CD4^+$ or $CD8^+$ host T cells alone; however, the $CD8^+$ subset is the most effective (126). Anti-idiotypic cytolytic T cells were demonstrated in the draining lymph nodes of host rats after induction of local GVH responses by strain A parental T cells (13). Host anti-idiotypic T cells were shown specifically to lyse A anti-B MLC blasts or third party T-cell blasts reactive to the B haplotype; T-cell blasts specific for irrelevant MHC haplotypes were not lysed by anti-idiotypic host T-cells. The authors suggest that anti-idiotypic cytolytic T-lymphocytes may suppress reactivity to host alloantigens by direct lysis of idiotypic donor T-cells. It is possible that $CD4^+$ anti-idiotypic T cells also can lyse alloantigen-specific target host T cells; alternatively, a nonlytic suppressor mechanism may be involved. The finding that resistance to GVHD is acquired within 1 day after inoculation of parental T cells into F_1 recipient rats implies activation of preexisting anti-idiotypic T-cell clones of high frequency in the normal host. These studies are consistent with the idea that maintenance of self-tolerance in the periphery involves active suppression of autoreactive T-cell clones by anti-idiotypic regulatory mechanisms.

C. Idiotype-Specific Suppression in Neonatal Tolerance to Transplant Antigens

Roser and Dorsch suggested in 1979 that idiotype-specific suppressor T cells are involved in the mechanism for neonatally induced transplantation tolerance (129). T cells from

neonatally tolerized rats were shown to transfer tolerance to secondary irradiated recipients, suggesting that tolerance to MHC antigens involved a suppressor mechanism. Large numbers of syngeneic T cells were required to abolish neonatally induced tolerance, whereas small numbers of syngeneic T cells, including alloreactive T cells from immunized animals, actually enhanced the suppressive effect on graft rejection. This resistance to reconstitution of tolerant rats with syngeneic alloantigen-reactive T cells provided further evidence for an active suppressor mechanism in addition to mechanisms of tolerance involving clonal deletion or anergy. Furthermore, negative selection protocols that deleted putative idiotype-positive T cells expressing receptors for donor alloantigens removed the ability to "boost" suppression in recipient rats. Thus, enhancement or "boosting" of suppression by these T cells required expression of receptors for the tolerated MHC antigens. These experiments are consistent with the idea that suppressor T cells in tolerant animals specifically recognize idiotypes expressed on alloreactive T-cell receptors (129,130).

D. Induction of Anti-Receptor Responses by T-Cell Vaccination or TCR-Peptide Immunization

I. R. Cohen and colleagues have shown that administration of attenuated myelin basic protein (MBP)-specific T-cell lines or clones to naive syngeneic rats can prevent induction of experimental autoimmune encephalomyelitis (EAE). This procedure, termed T-cell vaccination, inhibited induction of autoimmune disease by subsequent immunization with MBP or challenge with virulent activated MBP-reactive effector T cells (8,131). Remarkably, vaccination with as few as 100 cloned anti-MBP–specific T cells could protect against induction of EAE by 2 million activated MBP-reactive effector T cells (8). These studies demonstrated that activated MBP-reactive T cells could induce EAE and attenuated T cells could be used to vaccinate against induction of autoimmune disease or induce remission of ongoing disease. T-cell vaccination subsequently has been shown to prevent or cure multiple T-cell–mediated autoimmune diseases, including experimental autoimmune thyroiditis, adjuvant arthritis, experimental autoimmune neuritis, collagen II arthritis, and spontaneous autoimmune diabetes (9). T-cell vaccination appears to activate naturally occurring regulatory circuits, which inhibit the response to self-antigens in the periphery (10).

Early during the course of these studies, it was shown that the antigen specificity of the T-cell vaccine determined the specificity of resistance to disease (132,133). Vaccination with T-cell lines reactive to a particular antigenic determinant of MBP resulted in epitope-specific protection against EAE (132). In addition, vaccination induced proliferative and delayed-type hypersensitivity (DTH) responses against syngeneic anti-MBP–reactive T-cell clones but not against clones expressing irrelevant antigen specificities. Resistance to EAE was transferred to naive rats by lymph node cells draining the site of T-cell vaccination (134). Lider et al. demonstrated that vaccination of rats using an MBP-specific T-cell clone rapidly induced $CD4^+$ and $CD8^+$ anticlonotypic regulatory T cells within 5 days, suggesting the presence of preexisting anti-idiotypic immunity (135). Sun et al. showed that a $CD8^+$ anticlonotypic T-cell line, which specifically recognized and lysed MBP-reactive T cells in vitro, also could inhibit induction of EAE in vivo (136). Subsequently, several groups of investigators demonstrated that MBP-specific T cells utilized a limited set of germ line TCR V-gene subfamilies, including Vβ17a, Vβ8, and Vα2/Vα4, in particular strains of rats and mice (137–141). It was suggested that T-cell vaccination induced anti-idiotypic regulatory cells that could recognize these common TCR V-gene families.

Direct evidence for an anti-idiotypic mechanism of immunoregulation was provided

by experiments in which rats were protected against EAE by vaccination with peptides synthesized from TCRβ-chain CDR2 region sequences derived from MBP-specific T-cell clones (142,143). Vandenbark et al. used peptides derived from an invariant region of the Vβ8 gene product (residues 39–59) to elicit TCR-reactive T cells and antibodies in Lewis rats (142). Vβ8-specific T-cell proliferative and DTH responses were demonstrated in rats protected against EAE after immunization with TCR peptides. Furthermore, Vβ8 peptide-specific T cells (142) or antibodies (144) were shown to transfer resistance to EAE from protected rats to naive recipients. These results indicate that both anti-TCR-Vβ-specific antibody and T-cell responses against cross-reactive or shared self-TCR peptides can contribute to immunoregulation of MBP-reactive encephalitogenic T cells.

The TCR peptide-specific T cells have been shown to express multiple surface phenotypes, MHC restriction specificities, and regulatory/effector functions. Peptide-specific T-cell lines isolated in the Lewis rat model expressed both high levels of CD4 and low levels of CD8 surface markers; however, recognition of the Vβ8 peptide was restricted only by class I MHC molecules (142,145). In contrast, Vβ8.2-specific class II–restricted $CD4^+$ T cells have been demonstrated in B10.PL mice (146,147). These regulatory T cells were induced naturally during the course of recovery from EAE. Vaccination of SJL/J mice with a Vβ17a CDR2 peptide (residues 50–68) was shown to elicit regulatory effects against EAE in vivo and to induce $CD4^-CD8^-$ regulatory T-cell lines that could interact with T cells presenting exogenous TCR fragments in the context of self-class I MHC (148). Immunization with peptide was shown to prime Vβ17a-specific T cells, which could recognize exogenous TCR-peptide presented by irradiated MBP-reactive T cells but not when presented by conventional spleen cells. Thus, Vβ17a-specific regulatory T cells can interact directly with target T cells without requirement for conventional APCs (148,149). Downregulation of encephalitogenic T cells by regulatory TCR peptide–specific T cells may involve direct cytolysis (136) or induction of anergy without T-cell deletion (150) via elaboration of antigen nonspecific soluble mediators (148).

Human T-cell lines that are specific for CDR2 or CDR3 Vβ sequences have been established from healthy individuals or patients with chronic multiple sclerosis (MS) (151,152). Interestingly, some of these TCR-reactive T-cell lines were class I (HLA-B7) or class II (HLA-DR2) MHC restricted $CD4^+CD8^{lo}$ T cells resembling the Lewis anti-Vβ peptide–reactive T-cell lines, which protect against induction of EAE in the rat model (151). Human TCRβ reactive $CD4^+$ T-cell clones, which recognize TCR-peptides in the context of HLA-DR, also have been derived by Saruhan-Direskeneli et al. (152). These studies demonstrate that the normal human T-cell repertoire contains clones reactive against autologous MBP-reactive TCR specificities. Biased expression of TCR V-region gene families has been demonstrated in certain forms of MS in humans (153). Therefore, TCR peptides derived from regulatory idiotopes specified by these shared V genes may be effective for therapy of human disease (151,154–156).

Recently, T-cell vaccination with MHC antigen–reactive T cells has been used to inhibit the allogeneic mixed lymphocyte reaction (MLR) and to prolong vascularized heterotopic cardiac allograft survival in rodent models (157,158). Vaccination of rats or mice with attenuated alloreactive T cells reduced the proliferative response against stimulating cells expressing specific alloantigens. However, the MLR against third party stimulating cells also was reduced. T cells from vaccinated animals were shown to actively inhibit the response of naive responding cells against allogeneic stimulating cells, indicating that inhibition of the MLR was mediated by active suppression.

Similarly, vaccination of rats with syngeneic activated alloantigen-primed T cells

prolonged survival of rat cardiac allografts from 8.5 days in control recipients to 29 days in the experimental group (158). Third party grafts were prolonged significantly with a mean survival time of only 13 days. This slight prolongation of third party grafts may be explained by direct cross-reactivity of individual alloantigen-specific TCRs with third party MHC antigens. Alternatively, distinct T-cell clones that recognize alloantigens expressed in different MHC haplotypes may share expression of a common TCR V-gene family (128,155,158). The form of T-cell attenuation used in these studies involves cross-linking of surface molecules on mitogen-activated bulk alloreactive T cells. T-cell attenuation by cross-linking of surface molecules on these T cells (157,158) may favor immunity to public idiotopes specified by common TCR-Vβ elements rather than recognition of private idiotypes expressed on anti-MHC–specific TCRs as described initially by Binz and Wigzell (57,159,160). Prolongation of third party allografts also has been observed after pretransplant blood transfusion before human kidney transplantation (161). Thus, Lohse et al. have suggested that $CD4^+$ T-cell vaccination may be involved in the MHC-unrestricted suppression of alloresponses following blood transfusion (157).

Several studies have demonstrated predominant TCR gene usage in T cells derived from rejecting allografts in human and animal transplant models (61,67,68,76,77,81,85–87,162) as described in Sec. IV. In particular, the immune response to the H-$2L^d$ alloantigen is mediated primarily by $V\beta8^+$ T cells in the mouse (70). Goss et al. have shown that depletion of the $V\beta8^+$ T-cell subset in vivo using anti-Vβ8–specific mAbs reduced the dm2 anti-BALB/c cytotoxic T-lymphocyte (CTL) response and specifically prolonged skin or heart allograft survival in this strain combination (70). Furthermore, vaccination of allograft recipients with TCR-peptides derived from the CDR2 domain of the Vβ8 gene product also prolonged allograft survival (69). Collectively, these studies demonstrate that immunization with alloreactive T cells (57,157–160) or TCR-peptide sequences (69) can prolong allograft survival.

In contrast to the EAE model systems, the role of anti-TCR cellular and humoral responses in regulation of the murine immune response to H-$2L^d$ in vivo remains to be characterized. Synthetic TCR peptides were shown to inhibit generation of L^d-reactive CTL in vitro, suggesting that a central mechanism was not necessary for downregulation of the response. Goss et al. have suggested that the TCR-Vβ peptide may block T-cell activation after binding to L^d expressed on the stimulating cells (69). These findings demonstrate that mechanisms in addition to anti-idiotypic regulation may contribute to prolongation of allograft survival by TCR-Vβ peptides. However, the compelling evidence for antireceptor regulation provided by the EAE models suggests that the role of anti-idiotypic responses must be considered further as a mechanism for prolongation of allograft survival by TCR peptides in vivo in these recipient mice. The presence of TCR-reactive anti-idiotypic T cells in human graft-infiltrating lymphocyte populations (163,164) suggests that the use of TCR peptides may provide a means to selectively activate these regulatory T cells (165–167), allowing further characterization of the T-cell idiotypic network and development of strategies for therapeutic downregulation of alloresponses in vivo.

VI. IDIOTYPIC NETWORKS AND PREGNANCY

Survival during pregnancy of a fetus that expresses MHC antigens foreign to the mother remains an enigma. Despite HLA incompatibilities between mother and fetus, anti-HLA antibodies against paternal HLA antigens develop in only 30% of multiparous women (168). Suciu-Foca et al. have shown that the human T-cell receptor for alloantigen can induce an autologous anti-idiotypic T-cell response in vitro (169,170). In addition, sera

from parous women react with autologous T-cell lymphoblasts specific for paternal alloantigens (168,171). These findings support the hypothesis that anti-HLA immunity is downregulated actively during pregnancy by anti-idiotypic T- or B-cell responses to autologous alloantigen-specific lymphocyte receptors.

Suciu-Foca et al. have identified anti-anti-HLA antibodies (Ab-2) in the sera of 20 of 21 primiparous women at the time of delivery (168). These anti-idiotypic antisera (Ab-2) specifically inhibited the anti-HLA activity of maternal sera reactive against paternal class I or class II alloantigens. Anti-idiotypic antibodies that block the activity of autologous Ab-1 appear in maternal blood as early as the 5th month of pregnancy. Although 20 of 21 sera exhibited anti-idiotypic activity, only seven sera had direct anti-HLA activity against paternal cells. Thus, the Ab-2 response is observed in sera with or without the presence of anti-HLA (Ab-1) antibodies. This finding suggests that serum anti-HLA activity may reflect the balance between idiotypic Ab-1 (anti-HLA) and anti-idiotypic Ab-2 (anti-anti-HLA) antibodies.

Anti-idiotypic antibodies from parous women inhibit the activity of both autologous anti-HLA-DR antibodies and homologous reference antisera from other individuals directed against paternal HLA-DR alloantigens (172). This observation suggests sharing of idiotypes for particular anti-HLA responses within the population. Thus, human anti-HLA-DR antibodies may express public idiotypes which are markers for conserved germ line V genes.

VII. ANTI-IDIOTYPIC RESPONSES AFTER BLOOD TRANSFUSION AND KIDNEY TRANSPLANTATION

A potentially beneficial effect of pretransplant blood transfusion on renal allograft survival has been recognized for more than 20 years. Opelz and Terasaki reported a beneficial effect of pregraft blood transfusion on kidney transplant survival in 1974 (173). Subsequently, donor-specific transfusion (DST) was introduced as a preconditioning regimen by Salvatierra et al. (174). Multiple independent studies have supported the initial observation that pregraft transfusion leads to prolonged renal allograft survival (175–177). However, in recent years the use of cyclosporine-based immunotherapy to improve graft survival, an increased awareness of transmissible infection in blood products, and the risk of sensitization to HLA antigens have resulted in discontinuation of blood transfusion by many transplant centers. Nonetheless, the value of such treatment regimens in the earlier clinical experience and in animal models suggests the presence of an important immunosuppressive or tolerogenic pathway. The observation that blood transfusion at the time of resection for malignancy is associated in some series with a higher rate of tumor recurrence (reviewed in 178) further supports the concept of an immunomodulating effect of transfusion therapy.

Studies in animal models and in human patients have identified several factors contributing to induction of unresponsiveness, although the precise mechanisms responsible for the transfusion effect remain to be elucidated. The beneficial effects of blood transfusion have been attributed to selection of nonresponders and to the immunological mechanisms responsible for induction and maintenance of self-tolerance. Blood transfusions may downregulate cellular immune responses by induction of veto cells (179–181) or by causing alterations in the cytokine network related to the balance of TH1 and TH2 T-cell responses (182). Chimerism, the persistence of donor cells in the recipient at times distant from transplantation, may be required to maintain unresponsiveness (183–185). In addition, partial HLA-DR matching together with HLA-incompatibility has been shown to enhance the beneficial effect of transfusion (161). Thus, the particular mechanisms of tolerance induction versus sensitization may depend on the genetic disparity between the

transfusion donor and transplant recipient (180,185–188). Activation of host anti-idiotypic T and B lymphocytes directed against the receptors of autologous alloreactive lymphocytes remains a leading mechanism that may contribute to favorable graft outcome.

The transfusion effect may be achieved in part by selection of nonresponding recipients for transplantation. Blood transfusions can stimulate vigorous anti-HLA lymphocytotoxic antibody responses, leading to sensitization of the recipient rather than unresponsiveness to donor antigens (189). In a few patients, a positive antibody cross-match develops against a high proportion of potential donors in the population. These patients are unlikely to receive a kidney transplant. In other patients, anti-HLA antibodies develop to a segment of the population, but they usually do well when a cross-match–negative donor eventually is identified. In most patients, anti-HLA antibodies do not develop after transfusion, and they do well after kidney transplantation with the standard immunosuppressive regimens. Therefore, the transfusion effect cannot be explained entirely by exclusion of the relatively small group (<20%) of vigorous anti-HLA responders.

Singal and coworkers have studied the effect of blood transfusions on induction of anti-idiotypic antibodies and regulation of cellular immune responses across a full MHC and minor antigenic disparity in a mouse model (190,191). The proliferative and cytolytic responses of spleen cells from transfused mice are reduced when compared with the responses of cells from normal control mice (192,193). Furthermore, sera from transfused mice caused specific inhibition of the proliferative response by cells from normal responders against donor MHC antigens in MLC. The inhibitory effect was specific for responding cells; preincubation of stimulating cells with serum from transfused mice had no effect on the MLR, and proliferative responses to third party alloantigens were not inhibited (194). Similarly, antibodies that inhibit cytolytic T-cell activity were demonstrated after blood transfusion (192). These findings are consistent with the idea that blood transfusions can induce anti-idiotypic antibodies that block the T-cell receptor for alloantigen leading to inhibition of the recipient response to blood donor antigens in MLC (190). In addition, suppressor cells, which inhibit cytolytic activity (192) but not proliferation (193) of normal responding cells, were demonstrated by cell-mixing experiments.

The development of Ab-2 responses after blood transfusion in MHC-disparate rats has been reported by Downey et al. (195). The optimal time after donor-specific blood transfusion for transplantation of an allogeneic kidney correlated with a peak in the production of anti-idiotypic antibodies, which was accompanied by a decline or disappearance of cytotoxic alloreactive IgM antibodies. These studies, which are consistent with earlier studies by Stuart and coworkers (196,197) in a rat model, further establish a temporal association for the presence of anti-idiotypic antibodies and enhanced allograft survival.

Anti-idiotypic antibodies that inhibit the response of recipient cells to donor alloantigens in MLC have been demonstrated in human sera after random or donor-specific blood transfusion and subsequent renal transplantation (198–200). Singal et al. have derived an Epstein-Barr virus (EBV)-transformed B-cell line from a recipient with a long-term functioning renal allograft (201). Culture supernatant containing anti-idiotypic antibodies produced by this cell line inhibited the lymphocytotoxic activity of anti-B5, anti-B8, or anti-DR3 donor-specific reference antisera. The proliferative responses of peripheral blood lymphocytes (PBL) from the recipient or from normal individuals against donor B8 and DR3 alloantigens in MLC also were inhibited. However, absorption studies suggested that these inhibitory activities were mediated by different antibody specificities; absorption of culture supernatant on columns coupled with anti-B8 or anti-DR3 antibodies removed the inhibitory activity to antibody-mediated lymphocytotoxicity but did not affect inhibition of MLC responses. Conversely, absorption of supernatant with alloantigen-reactive T-cell

blasts removed the MLC inhibitory component but did not remove inhibitory activity to anti-HLA lymphocytotoxicity. Anti-idiotypic antibodies produced by additional EBV-transformed B-cell lines were shown to inhibit proliferation of responding alloreactive T cells in MLC and to immunoprecipitate a molecule similar in molecular weight to the human T-cell receptor (202). These findings suggest that different anti-idiotypic antibodies derived from individuals with long-term surviving renal allografts can inhibit T-cell reactivity or antibody-mediated lymphocytotoxicity against donor antigens.

Numerous studies have provided evidence that the presence of anti-idiotypic antibodies in the serum of organ transplant recipients is associated with prolonged allograft survival. Reed et al. have investigated the development of anti-idiotypic (Ab-2) responses in kidney transplant recipients in whom anti-HLA antibodies did not develop after three donor-specific blood transfusions. Significant levels of anti-idiotypic antibodies (Ab-2) were detectable at the time of kidney transplantation in all of six patients in whom donor-specific anti-HLA-DR antibodies (Ab-1) did not develop after three donor-specific blood transfusions; HLA-DR mismatched kidney allografts were not rejected by these patients (203).

For many years, it was the practice of most transplant centers to rule out the use of a donor whose lymphocytes gave a positive cross-match with any serum from the intended recipient. Thus, a positive cross-match on serum that was 1 and 2 years old would rule out acceptance of a particular kidney, even though cross-match with a current serum from the intended recipient might be negative. Several years ago, however, Cardella and coworkers found that historical positive cross-matches can be ignored without jeopardizing graft survival (204). It has become standard practice to ignore historical positive cross-matches. Such an apparent paradox could be explained if these anti-donor antibodies were directed against non-HLA antigens, which are not expressed on the graft. Alternatively, the disappearance of anti-HLA antibodies (Ab-1) could be accounted for by the development of Ab-2. Reed et al. have examined sequential samples of AB-1–negative sera obtained from two patients who lost their anti-HLA antibodies 1 year before transplantation. The Ab-1 sera were tested for the capacity to inhibit earlier autologous sera that contained antibodies against the cadaveric donor. In both patients, the disappearance of Ab-1 was associated with development of Ab-2, which remained present in the serum during the year until transplantation; neither patient has rejected the graft (203).

Long-term allograft survival occurs in patients in whom anti-HLA antibodies do not develop (205–208). In contrast, chronic rejection may be mediated by antibodies against mismatched HLA antigens of the donor. These anti-HLA antibodies are detected in the sera of 57% of patients undergoing chronic rejection compared to only 5% of patients with 4-year graft survival (205). The presence of immune complexes, demonstrated by an increase in detectable anti-HLA antibodies after depletion of soluble HLA antigens, also was associated with chronic graft rejection. However, patients in whom specific anti-idiotypic Ab-2 responses developed after an initial anti-HLA Ab-1 response had prolonged graft survival, whereas failure of Ab-2 response to develop was associated with chronic graft rejection. Thus, anti-idiotypic antibody responses may delay or prevent chronic humoral rejection by blocking the cytotoxic activity of Ab-1 (205,206).

Reed et al. also have investigated the Ab-1 and Ab-2 responses in kidney transplant patients after acute and chronic rejection. In one patient with acute rejection, anti-HLA antibodies appeared after transplant nephrectomy; Ab-2 coexisted against some but not all of the anti-HLA antibodies. In a patient with chronic rejection, anti-HLA antibodies appeared before loss of all renal function, but sera showed no Ab-2 activity (203). Hardy et al. have identified three patterns of Ab-1 and Ab-2 responses associated with prolonged

renal and cardiac allograft survival: (1) Ab-1 varied cyclically in association with Ab-2, and 100% graft survival was observed at 2 years after transplant, (2) Ab-1 varied cyclically without any detectable Ab-2, and graft survival was 36% for renal and 71% for cardiac allografts, and (3) Ab-1 alone was detected in all serum samples without production of Ab-2, and graft survival in this group was 47% for renal or 56% for cardiac allografts (207).

A more recent study by this group revealed 57% renal allograft survival at 4 and 5 years in patients producing only Ab-1, compared with 83% to 100% in patients producing both Ab-1 and Ab-2 (208). Thus, the presence of Ab-2 is associated with an excellent prognosis for long-term survival in renal and cardiac recipients who have produced anti-HLA reactive Ab-1.

The effect of anti-idiotypic antibodies on transplant survival has been studied in a retrospective analysis (102). Twenty patients with a history of presensitization to donor HLA antigens were studied; 9 of 10 patients with a functioning graft had produced Ab-2 at the time of transplantation. In contrast, antibodies that potentiated rather than inhibited the cytotoxic activity of anti-HLA antibodies were detectable in 9 of 10 patients who rejected the kidney graft. The Ab-1–potentiating activity was attributed to Ab-3. Al-Muzairai et al. tested pretransplant sera for inhibitory or potentiating activity in the "short" anti-idiotypic assay in which panel cells are incubated with donor-specific anti-HLA antibodies (209). Patients with inhibitory sera containing Ab-2 had 93% 1-year kidney graft survival. However, potentiating activity did not correlate with an increased risk of graft rejection in this patient series (209,210). These findings suggest that the immune response to alloantigens in transfused and transplanted patients is downregulated by development of anti-idiotypic antibodies. Development of Ab-2 may inhibit the binding of cytotoxic Ab-1 to target HLA antigens, leading to favorable graft outcome in the sensitized recipient. However, failure of an Ab-2 response to develop or of downregulation of the Ab-2 response by Ab-3 may lead to acute or chronic humoral rejection (102,203).

Anti-idiotypic antibodies in the serum of liver transplant recipients have been characterized by Chauhan et al. (211). These anti-idiotypic antibodies blocked the cytotoxic activity of both donor-specific antisera and anti-HLA class I and class II reactive alloantisera to public or cross-reactive groups. The anti-idiotypic inhibitory activity was contained within the purified immunoglobulin fraction and was not removed by absorption of serum using anti-HLA framework monoclonal antibodies. Thus, the inhibitory effect of anti-idiotypic antibodies on the cytotoxic activity of Ab-1 could not be attributed simply to blocking by soluble serum HLA antigens in these patients.

Although many of the recent clinical studies describe a blocking effect of anti-idiotypic antibodies on the host anti-HLA antibody response, other findings suggest that a similar mechanism may act in regulation of T-cell responses against the allograft (212,213). T-cell clones were established from liver biopsy specimens obtained during a rejection episode from a liver-kidney recipient. Flow cytometric analysis showed that three of five of these clones were recognized by autologous antibodies. Recognition of the TCR–CD3 complex by these antibodies was confirmed by co-capping studies and immunoprecipitation. Specific downregulation in the frequency of alloreactive CTLp or complete absence of detectable CTLp in particular patients was demonstrated in renal transplant recipients with long-term, well-functioning grafts after blood transfusion (214). Similar depletion of specific CTLp and nonresponsiveness to donor antigens in the peripheral blood of long-term liver allograft survivors has been described by Mathew et al. (215). Donor-specific nonresponsiveness after HLA-sharing transfusions appears to correlate with a decrease in the usage of between one and three TCR-Vβ families (216), and significant perturbations

in the host TCR repertoire after blood transfusion also have been reported recently in animal models (217). These observations are consistent with a regulatory mechanism involving induction of antibodies reactive against autologous alloreactive TCR clonotypic determinants after blood transfusion. However, a direct relationship between the observed anti-idiotypic antibody responses and the mechanism leading to apparent deletion of alloreactive CTL remains to be firmly established.

Recently, van der Mast et al. (14) have described a self-protective mechanism associated with resistance to GVHD following blood transfusion in humans. Transfusion-associated GVHD is a rare but frequently fatal disease caused by the presence of donor leukocytes in transfused blood. In most transfusion recipients, the reactivity of the donor cells against host alloantigens is decreased after multiple transfusions. Host T cells in these recipients were shown to inhibit specifically the donor anti-host MLR. The active mechanism that inhibits GVHD in normal human recipients may be analogous to the anti-idiotypic mechanisms for resistance to GVHD, which were characterized in the rat model systems described in Sec. V. Further elucidation of the mechanism responsible for these effects may allow development of pretreatment strategies for induction of specific tolerance to graft alloantigens.

VIII. PROLONGATION OF ALLOGRAFT SURVIVAL USING INTRAVENOUS IMMUNOGLOBULIN PREPARATIONS CONTAINING ANTI-IDIOTYPIC ANTIBODIES

The risk of sensitization is a major contraindication for the use of blood transfusions as a pretransplant conditioning regimen. Sensitized patients frequently have a high level of panel-reactive IgG alloantibodies (PRAs) as a result of blood transfusion, pregnancy, or previous graft rejection. These patients may wait for years until a matched kidney becomes available for transplantation. Intravenous immunoglobulin (IVIg) preparations are human serum immunoglobulins pooled from a large number of healthy donors. Therapy with high doses of IVIg has been used to treat autoimmune diseases, which are mediated by self-reactive antibodies or T cells (218,219). Recent studies have demonstrated a beneficial effect of IVIg on organ transplant survival. IVIg has been shown to contain anti-idiotypic antibodies and other antibodies directed against variable and constant regions of the B-cell or T-cell antigen receptors. These naturally occurring antireceptor antibodies may contribute to the mechanism for prolongation of allograft survival.

Infusion of IVIg has been used as therapy for sensitized recipients of primary allografts (220,221) or high-risk patients retransplanted with second kidney allografts (222). Peraldi et al. have shown in a randomized study of 41 patients that a 5-day course of high-dose IVIg in combination with the use of immunosuppressive drugs is associated with reduced delay of early graft function and enhanced long-term survival of second cadaveric kidney transplants (222). The beneficial effect of IVIg on allograft survival was observed long beyond the expected serum half-life of infused immunoglobulin. These studies indicate that an immediate or early protective effect of IVIg may be followed by a long-lasting immunoregulatory mechanism, leading to prolongation of allograft survival over several years.

Intravenous immunoglobulins have been shown to recognize and inhibit anti-HLA antibodies in vitro and in vivo. Glotz et al. demonstrated that IVIg inhibited cytotoxicity and binding of anti-HLA alloantibodies present in patient sera against particular donors in a PBL panel (223). The IVIg preparations did not contain anti-HLA antibodies reactive

against the target cells. Furthermore, a decrease in PRAs has been observed after administration of IVIg to sensitized patients, suggesting that IVIg contains anti-idiotypic antibodies that can inhibit the activity and secretion of anti-HLA alloantibodies in vivo (220,223–226). These observations are consistent with earlier studies by Reed et al. in which a decline in Ab-1 together with increased serum levels of Ab-2 indicates an excellent prognosis for patients with a historical cross-match (102,172,203,208).

The idea that antireceptor antibodies may have direct effects on the B-cell clones that produce anti-HLA antibodies is supported in a series of studies by Terness and Opelz (227,228). Lewis rats were shown to produce broadly reactive IgG anti-immunoglobulin-specific antibodies after immunization with allogeneic blood. Minute concentrations of these anti-Ig-specific antibodies purified by affinity chromatography were shown to profoundly inhibit antibody production in vitro by syngeneic B cells (227). These studies demonstrate that a very low concentration (0.9 pg/million cells) of anti-Ig antibodies may have profound regulatory effects on the B-cell response. In contrast, $F(ab')_2$ fragments of anti-Ig did not inhibit B-cell responses. Thus, simultaneous binding of FcR and antigen receptor may deliver a negative signal or induce death of target B-cell clones (229). Interestingly, the presence of serum autoantibodies against constant regions of Ig has been associated with a protective effect on human kidney allograft survival, and broadly reactive antireceptor antibodies also are present in IVIg (230,231). Collectively, these studies are consistent with the idea that IVIg may prevent renal injury by inhibition of anti-HLA antibody binding to the graft and by inhibition of antibody secretion from target B-cell clones.

Antibodies against variable and constant regions of the T-cell antigen receptor also have been demonstrated in normal human serum and in IVIg preparations. Marchalonis et al. have characterized the binding of IVIg to a library of overlapping peptides derived from the human TCRβ-chain (232,233). The specificity of IgG antireceptor antibody binding was mapped to determinants in the constant region of the β-chain and to framework and clonotypic determinants in the TCR variable region. In addition, affinity purification was used to identify an anti-Vβ8-enriched antibody fraction that displayed high levels of binding to specific Vβ8 TCR peptides (232). Analysis of a three-dimensional model of the TCR predicted that the positions of the major peptide epitopes defined in this study should be accessible to self-regulatory antibodies and T cells. These studies demonstrate that naturally occurring antibodies against the TCR are produced by healthy individuals. Furthermore, the levels of these naturally occurring anti-TCR antibodies were shown to increase during autoimmune disease (232) and pregnancy (234,235). These observations are consistent with network regulation of T-cell responses by anti-idiotypic antibodies.

Intravenous immunoglobulin has been shown to inhibit the allogeneic MLR (236–238). However, multiple specificities in addition to antireceptor antibodies have been identified in polyclonal IVIg preparations, including antibodies against CD4, CD8, class I MHC antigens, lymphocyte adhesion molecules and cytokines such as IFN-γ (239) (reviewed in 6,7). It is likely that a combination of these antibody specificities is responsible for the immunomodulatory effects of IVIg on the response to alloantigens in vitro and in vivo during prolongation of transplant survival. Therefore, studies using affinity purified antibodies with defined reactivity are necessary to determine the relative contribution of antireceptor antibodies to the beneficial effect of IVIg on transplant survival.

The therapeutic effects of IVIg may be related to the natural regulatory function of anti-idiotypic antibodies in the immune network. Anti-idiotypic antibodies appear to establish, select, and maintain homeostasis of receptor repertoires against self- and nonself-antigens in normal individuals (5–7). Diverse anti-idiotypic antibodies, which are expressed under normal physiological conditions, may provide a high level of B-cell and T-cell recep-

tor connectivity (19,240). According to this view, anti-idiotypic antibodies and other anti-receptor antibodies contributed by a very large number of donors within the IVIg pool may modulate the potential B- and T-cell repertoires, leading to the suppressive effect of IVIg on reactivity to self-antigens during autoimmune disease and responses to alloantigens during organ transplant rejection.

IX. CONCLUSION

A major side effect in the use of immunosuppressive drugs for organ transplantation is the nonspecific suppression of protective immunity, leading to an increased risk of life-threatening infections and cancer. Selective immunosuppression of the immune response to donor alloantigens remains an elusive long-term goal. The effectiveness of T-cell vaccination and TCR peptide immunization strategies in treatment of autoimmune disease indicates the feasibility of highly specific receptor-based strategies for selective regulation of alloimmune responses. The limited TCR diversity in the response to self-antigens such as MBP and the preexistence of naturally occurring anti-idiotypic regulatory circuits that can be amplified by specific immunization provides an advantage for the clinical application of receptor-based strategies for therapy of autoimmune diseases. In contrast to the relatively limited receptor diversity in the response to autoantigens, the B-cell and T-cell receptor repertoires against a particular allogeneic MHC molecule may be highly diverse (64–67, 78,126). Therefore, it is unlikely that immunization with a single receptor alone would prolong allograft survival across a full or partial MHC antigen disparity. The modest effect of host immunization with donor-specific T-cell blasts on subsequent immune responses and allograft survival (57,160,241,242) may reflect the high level of T-cell receptor diversity and the antigenic complexity of allogeneic MHC molecules.

Nonspecific immunosuppression may be required to prevent rejection at early times after transplantation. However, the immunogenicity of a transplanted organ declines with time as donor-derived passenger leukocytes and dendritic cells, which express allogenic class I and class II MHC molecules, are replaced with cells of host origin (243–245). Therefore, graft adaptation may permit a gradual reduction of immunosuppressive therapy. Indirect recognition of alloantigens presented on host accessory cells in association with self-class II MHC structures appears to be the primary pathway for activation of $CD4^+$ T-cell responses after removal of passenger cells expressing allogeneic class II molecules from the graft (246,247). These $CD4^+$ helper T-lymphocytes may augment $CD8^+$ T cells, which recognize graft antigens via the direct recognition pathway, and provide helper factors for B-cell responses, which lead to antibody-mediated chronic graft rejection. Recent evidence suggests that a restricted set of T-cell receptor genes is used in the response to dominant allogeneic peptide epitopes presented in the context of self-HLA-DR molecules (248). Thus, the limited receptor diversity expressed in the indirect allorecognition pathway may provide a potential target for the use of anti-idiotypic antibodies and other antibodies directed against TCR-V-region gene families in clinical organ transplantation.

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5

Clinical Syndromes Associated with Antibody in Allografts

WILLIAM M. BALDWIN III

The Johns Hopkins University School of Medicine, Baltimore, Maryland

PHILIP F. HALLORAN

University of Alberta, Edmonton, Alberta, Canada

I. INTRODUCTION

Although alloantibodies are an inherent component of the immune response to alloantigens and irreversible transplant rejection is usually accompanied by alloantibody production (1), T-cell immunity dominates the discussion of graft rejection (2,3). Nevertheless, the contribution of antibodies to rejection is probably greater than previously appreciated. Indeed, antibody-mediated immunity is one mechanism that is indisputably able to destroy a graft: hyperacute rejection caused by isohemagglutinins to major blood group antigens, preformed antibodies to major histocompatibility complex (MHC) class I antigens, and "natural antibodies" to discordant xenograft reactions prove that point (4–8). The real debate concerns the role of alloantibody in acute and chronic rejection (9–12).

A. Why Was the Role of Alloantibody in Clinical Transplantation Underestimated?

The tendency to downplay the role of antibody has multiple origins:

1. Early experiments in rodents showed that acute allograft rejection was transferable by cells, not by serum (2,3,13). However, these serum transfer studies are of questionable relevance to modern clinical transplantation. The design of these experiments did not account for the fact that all allograft recipients are given immunosuppressive drugs, which

are directed at inhibiting immune responses by T cells. Nor did these experiments reflect the common clinical history of patients, many of whom have multiple sources of overt and covert presensitization. About 20% to 40% of the patients waiting for renal transplants have circulating alloantibodies as measured by relatively insensitive microcytotoxicity assays on panels of leukocytes, or panel reactive antibodies (PRAs). These antibodies have been related to presensitization by previous infections, transfusions, pregnancies, or transplants (14). Preformed antibodies are of disproportionate clinical concern because current immunosuppressive therapy is ineffective against preformed antibodies and memory B cells.

2. Most clinical studies include only perfunctory testing for circulating donor-specific antibodies and place little emphasis on pathological evidence of antibody-mediated injury. Only with the combination of an incisive clinical description, a committed tissue typing laboratory, and an interested pathologist can the picture of antibody-mediated injury be unraveled (15–20).

3. Conventional serological studies on alloantibodies do not define critical parameters of the antibodies, the target antigens or the recipient. Antibodies to MHC class I and II antigens cannot be defined by the conventional technique of differential lysis of T and B lymphocytes (21–23). Similarly, the prevalent use of reducing reagents to define IgM antibodies is not valid. The subclass, affinity, titer, and epitope specificity of alloantibodies need to be assessed accurately if their pathological potential are to be understood. Different classes and subclasses of antibodies have different capacities to cross-link antigens, activate complement, and stimulate Fc receptor (FcR)-bearing cells. Antibody affinity and titer determine the kinetics of antibody binding to the antigen. The epitope specificity of the antibodies and the density of antigens affect the local concentration of antibody deposits. Moreover antibodies need to be measured before and after transplantation, because all of the variables affecting antibody-mediated immunity are dynamic. Cytokines, for example, can regulate quantitative and qualitative aspects of both antibody and antigen (see Chap. 3).

4. Studies restricted to assaying antibody activity in the blood compartment measure only the excess, unbound alloantibodies and fail to detect antibodies bound to target antigens in the graft. Usually more antibodies can be eluted from rejecting transplants than can be found in the circulation (17,18,24–27).

5. Even when antibody and complement are undoubtedly pathogenic in tissue injury, they may be below the level of detection by immunofluorescence in biopsy specimens (see Sec. III.B.4). Antibody and complement can be shed from the surface of endothelial cells and complement can be enzymatically degraded by regulatory proteins, such as factor I (28). Persons expecting diffuse staining for these mediators would conclude that antibody and complement are not involved in graft rejection.

B. Renewed Appreciation of the Pathogenic Role of Alloantibody and Complement

After universal implementation of cross-matching procedures effectively eliminated classical hyperacute rejection (29), the impetus to investigate the mechanisms by which antibodies and complement cause graft injury subsided. The expanding need for organ donors has renewed interest in the use of ABO incompatible donors and discordant xenogeneic donors. As a result, research into the pathogenic effects of antibody and complement on transplants has revived. This resurgent research has broadened our appreciation of the range of effects that antibody and complement can have on the vascular components of a graft.

With this renewed research effort, many interactions between "humoral" and "cellular" immunity have been discovered. Regulation of leukocyte adhesion molecules, for

example, has been found to be particularly sensitive to antibody and complement. It has been appreciated for many years that increased leukocyte adhesion is mediated directly by the C3b split product of complement, which covalently binds to donor cells and acts as a ligand for complement receptors on neutrophils, monocytes, and macrophages (reviewed in 28). More recently, deposition of antibody and complement on endothelial cells has been shown to cause upregulation of the adhesion molecules P-selectin, E-selectin, and intracellular adhesion molecule (ICAM) (30,31).

These experimental data have been supplemented by recent clinical studies that conclude that alloantibodies are associated with vascular injury in renal allografts. The clinical studies have several common findings:

1. Flow cytometry, antibody-dependent cell-mediated cytotoxicity (ADCC) or modified microcytotoxicity assays can detect preformed antibodies that are undetectable by routine cross-match assays (15,16,32).
2. Interstitial or peritubular capillary deposition of complement is frequent (15–18, 20,33–35).
3. Intravascular platelet aggregation and fibrin deposition, and vascular infiltration by neutrophils, monocytes, and macrophages correlate with antibody-mediated injury (15–18,20,33–37).
4. Poorer graft survival is associated with circulating donor-specific antibodies or pathological evidence of antibody-mediated injury (15–19,35).
5. Rejection is usually associated with antibodies to MHC class I antigens and A or B blood group substances (17–19,32,35). Antibodies to MHC class II antigens are common but less commonly mediate severe acute or hyperacute rejection (27,38,39). Antibodies against poorly characterized endothelial antigens have been associated with some cases of severe acute or hyperacute rejection, but these cases are infrequent (15).

In short, the average rejection episode is probably T-cell mediated, but when alloantibodies are present, they can cause injury. The fact that typical acute rejection is mediated by T cells does not argue against a separate or ancillary role for antibody; both mixed antibody and T-cell reactions, as well as pure antibody-mediated rejection, can be identified in clinical situations. Antibody-mediated injury is favored when a high titer of preformed antibody or a burst of newly formed antibody rapidly binds to an antigen expressed in high density on the vascular endothelium. If the antibody concentration builds gradually or if prior exposure to antibody has reduced antigen density, the outcome becomes less predictable and the antibody may be rendered ineffective—the phenomenon known as accommodation. Thus, antibody often displays "the need for an explosive response for extensive damage to occur" (6), because the tissue has the capacity to "accommodate" to antibody under some circumstances.

II. EXPERIMENTAL MODELS OF ANTIBODY-MEDIATED REJECTION

Recent experimental data from in vivo and in vitro models have expanded current understanding of antibody- and complement-mediated graft injury. In this section, emphasis is on in vivo studies using antibody- or complement-deficient animals and on in vitro experiments using complement-deficient sera or purified complement components.

Evidence for antibody- and complement-mediated graft injury is supported by the following experimental findings:

1. Passive transfer of antibodies into immunosuppressed or immunodeficient recipients causes acute and chronic rejection of allografts (40–42).
2. Antibody responses in animals sensitized by prior transfusions predict survival (25,26,43).
3. Allografts to complement-deficient animals have prolonged survival (44).

Using rats immunosuppressed with cyclosporine A (CsA) or anti-CD4 monoclonal antibodies, Bradley and colleagues (40,41) have found that passive transfer of immune serum can cause the acute rejection of renal and cardiac allografts. In this model of antibody-mediated rejection, the donor and host shared class II MHC antigens and were only mismatched at class I MHC antigens. Serum from PVG.RT1^u rats that contained high titers of alloantibodies to the PVG.R8 donors caused acute rejection of cardiac and renal allografts when transferred to immunosuppressed recipients. In contrast, acute rejection could not be achieved with passive serum transfer to PVG recipients of MHC class I incompatible kidneys from PVG.R1 donors, a strain combination that results in low alloantibody responses. Thus, when T-cell immune responses are immunosuppressed, as occurs in the clinical setting, antibodies can be demonstrated to cause acute graft injury and rejection.

Passive transfer of antibodies to immunodeficient mice indicates that antibodies may also contribute to some forms of chronic rejection. Cardiac allografts to mice with severe combined immune deficiency (SCID) are not rejected and do not develop chronic vascular pathology. Obstructive coronary lesions characteristic of chronic rejection can be produced in cardiac allografts to SCID recipients that received repeated injections of antiserum raised against the donor mouse strain (42).

In addition to mice with spontaneously occurring immunodeficiencies, there are an increasing array of mice in which mutations have been engineered that cause specific defects in the immune system (45). These mice offer opportunities to dissect the pathogenic mechanisms underlying chronic rejection of organs transplanted over different histocompatibility barriers. "Knockout" mice have already been used to develop a model of vasculopathy in carotid artery allografts (46). In this model, carotid arteries were transplanted between B.10A(2R) (H-2^{h2}) donor mice and C57BL/6J (H-2^b) recipients and compared with arteries isografted between C57BL/6J mice. Within 7 days, the allografted carotid artery formed a neointima composed of macrophages and T lymphocytes. By 30 days, the mononuclear leukocytes were largely replaced by smooth muscle cells. Studies in knockout mice have demonstrated that this lesion is decreased most significantly when immunoglobulins are knocked out (46).

Transplant experiments in complement-deficient animals have begun to reveal the importance of different components of this series of potent inflammatory mediators to graft injury. A deficiency in C6 can delay the rejection of an MHC-disparate cardiac allograft in rats from about 1 week to more than 2 weeks (44). This single deficiency of a terminal complement component prevents assembly of the C5b-9 membrane attack complex (MAC). This does not appear to be a strain-specific effect, because acute rejection is inhibited in all of the high responder strains into which the C6 deficiency has been bred.

Recent evidence indicates that MAC causes graft injury through endothelial cell activation as well as lysis. Allogeneic endothelial cells are usually protected from MAC-induced lysis by the many regulators of complement expressed on their membranes and circulating in the plasma. Although they prevent lysis, these regulators can activate endothelial cells. CD59, which blocks the completion of MAC formation by binding C8 or C9 and preventing the insertion of additional molecules of C9 components, is a glycosylphosphatidylinositol (GPI)-anchored protein. As such, CD59 is a potential signal transducer for the

release of growth factors from endothelial cells (47). Likewise, vitronectin may have a dual effect in sites of inflammation. Vitronectin can inhibit formation of MAC by binding to C5b-7, but when vitronectin binds to C5b-9 after insertion into a cell membrane (48), it can act as a ligand for cells with vitronectin receptors such as T lymphocytes, platelets, granulocytes, and activated endothelial cells (49,50).

A second strategy of endothelial cells for avoiding lysis is exocytosis or endocytosis of MAC after insertion (51). However, even transient insertion of MAC (or just C5b-C8) through the cell membrane increases calcium permeability, which can initiate cell signaling cascades. Finally, a direct interaction between MAC and some of the intracellular signaling pathways has been recently reported (52).

As a result of endothelial cell activation, MAC causes a rapid fusion of cytoplasmic Weibel-Palade bodies with the cell membrane, exposing preformed P-selectin, von Willebrand factor, and tissue factor (30). Similarly, platelets exposed to MAC release P-selectin from their α-granules, resulting in membrane surface expression and soluble plasma levels of P-selectin (53). In addition to release of preformed mediators, MAC-activated endothelial cells synthesize P-selectin and tissue factor (54). These responses do not occur in serum deficient in a terminal component of complement.

The major histological difference in rejection caused by the C6 deficiency is at the level of the arterial endothelial cells. In C6-sufficient combinations, all of the arterial vessels have evidence of severe endothelial cell injury and extensive fibrin deposition. In contrast, the arterial endothelium in grafts between C6-deficient rats remains intact and the vessels remain patent even though there can be significant subendothelial infiltrates. The vascular infiltration in allografts to C6-deficient recipients appears to be reversible because the longer-surviving grafts have normal vascular morphology.

Allograft rejection has not been explored extensively in complement-deficient mice, even though several commercially available strains are completely deficient in C5. It has been reported that the C5 deficiency has little (55) or no effect (56) on acute skin allograft rejection by B10.D2 old mice. The effects of complement deficiency on the rejection of cardiac and renal allografts, which are more susceptible to antibody-mediated injury than skin allografts, have not been studied fully. In their survey of different strain combinations for responses to cardiac allografts, however, Peugh and coworkers (57) did note that the responsiveness of the DBA/2 recipient tends to be lower in all combinations tested than of any other recipient strain responding to the same grafts, with a similar number of H-2 and non–H-2 disparities. Although not discussed by these investigators, DBA/2 has a complete C5 deficiency and a partial C3 deficiency.

C3 knockout mice can be used to examine the role of the early complement components. This deficiency would be expected to have a greater influence on graft rejection because it eliminates both the production of the anaphylotoxins (C3a and C5a) and the ligands for complement receptors (C3b and iC3b), as well as preventing MAC formation. This area merits further research.

III. ANTIBODY-MEDIATED REJECTION SYNDROMES IN CLINICAL TRANSPLANTATION

There are several clinical situations in which antibody-mediated graft injury must be suspected: hyperacute rejection (HAR), antibody-mediated accelerated or acute rejection (AbMAR), unexplained delayed graft function that might be otherwise diagnosed as acute tubular necrosis (ATN), and possibly some cases of chronic rejection.

A. Hyperacute Rejection

Although the term HAR is used by some to describe antibody-mediated rejection (AbMR) that occurs even days after transplantation, in this text, the term HAR is restricted to its original definition of rejections that occur within hours after transplantation; later rejections are designated as antibody-mediated accelerated or acute rejection (AbMAR). Hyperacute rejection is initiated by alloantibodies present in the recipient's circulation at the time of transplantation, binding to the donor endothelium of the graft and activating complement. The two types of antibody that can certainly mediate HAR are IgG antibodies to MHC class I antigens and IgM antibodies to A or B blood group antigens. Reports of HAR associated with antibodies to "endothelial-specific" antigens and to MHC class II antigens are less frequent.

The extensive complement activation by antibodies in HAR exceeds the capacity of the circulating and membrane-bound regulators of complement to prevent endothelial cell injury. Different components of the complement cascade initiate a series of inflammatory events. Split products of the early complement components (C3a and C5a) recruit and activate neutrophils and monocytes, as well as activate endothelial cells. The tissue-bound split products of C3 (C3b and iC3b) serve as adhesion ligands for neutrophils and monocytes. As discussed, assembly of the terminal complement components into MAC on endothelial cells causes a rapid fusion of cytoplasmic Weibel-Palade bodies with the cell membrane, exposing preformed P-selectin, von Willebrand factor, and tissue factor (30). P-selection initiates adhesion of inflammatory cells to endothelium, von Willebrand factor mediates platelet adhesion and aggregation, and tissue factor activates the coagulation cascade. Membrane attack complex also downregulates thrombomodulin and antithrombin III (54,58), completing the transformation of vascular endothelium from an anticoagulant to a procoagulant surface. Finally, MAC stimulates alteration in the cytoskeleton of endothelial cells, resulting in changes of cell shape and the formation of intercellular gaps (59). Exposure of the subendothelial basement membrane augments platelet activation and coagulation. Activated platelets and endothelial cells secrete platelet activating factor (PAF), which stimulates the secretion of thromboxane and leukotrienes. Thromboxane, a potent vasoconstrictor, also induces platelet aggregation. The combined effect is a severe reduction of perfusion, leading to infarction.

The critical contribution of complement to HAR has been demonstrated in vivo with complement inhibitors or complement-deficient recipients. Complement receptor type 1 (CR1) is a potent regulator of the early components of complement: CR1 disrupts the C3 and C5 convertases by accelerating their disassociation and acting as a cofactor for the enzymatic cleavage of C4b and C3b by factor I. A recombinant soluble form of CR1 (sCR1) prevents HAR of allografts (60). As expected from the foregoing discussion, sCR1 significantly decreases endothelial damage and delays neutrophil and monocyte infiltration of these grafts. The importance of MAC to HAR has been demonstrated with C6-deficient rats. In these animals, MAC is not formed and rejection of cardiac allografts is delayed from 6 hours to almost 4 days in presensitized recipients (44). The deficiency in MAC formation was primarily associated with decreased platelet aggregation and thrombosis.

1. Pathology

The histological changes seen on biopsy depend on the timing of the biopsy and the severity of the rejection (61–63). Platelet aggregation and fibrin deposition are the earliest features of HAR. This is rapidly followed by evidence of endothelial cell injury and progressive neutrophil sequestration in the peritubular capillaries and glomeruli. This progresses to

thrombosis, hemorrhage, and cortical necrosis. Immunofluorescence stains on biopsy samples obtained early in the rejection process demonstrate linear localization of immunoglobulin and complement (6,61–65). The class of immunoglobulin depends on the antibodies that cause HAR: antibodies to MHC class I are usually IgG, whereas isohemagglutinins are IgM. IgM has been a prominent finding in the few cases of hyperacute and severe acute rejection reported in conjunction with antibodies to MHC class II (27,38,39). Staining for immunoglobulin and complement decreases as endothelial cell destruction proceeds (62,63).

2. Differential Diagnosis

With modern cross-matching techniques, early severe graft dysfunction is usually not caused by HAR. Hyperacute rejection must be distinguished from early catastrophic renal dysfunction, such as ATN, thrombosis of the renal artery or vein, or ureteric obstruction or disruption. A fever and tender kidney are common, but both are nonspecific findings in the early postoperative patient. Urine output may taper off in a kidney with ATN that is initially nonoliguric, inviting consideration of HAR. A biopsy with frozen section may be helpful, but the usual investigations should include a perfusion scan and an ultrasound with an angiogram if necessary. A repeat cross-match should be requested urgently. A decrease in the platelet count or increase in fibrinogen degradation product supports the diagnosis of HAR, but cyclosporine (CsA)- or tacrolimus-induced hemolytic-uremic syndrome (HUS) is also possible. Treatment can be switched to OKT3 or antilymphocyte globulin, to provide immunosuppression without CsA. Oral CsA may be reintroduced toward the end of therapy with OKT3.

3. Treatment

In fulminant cases of HAR, treatment is unsatisfactory. Successful reversal with intense immunosuppression, with or without plasmapheresis, has been reported, if the graft vessels do not thrombose. In the future, plasmapheresis may be combined with new agents that intervene in complement activation, such as sCR1. If the graft becomes nonviable, however, severe systemic toxicity from necrotic renal tissue and consumption coagulopathy may develop. Graft nephrectomy is therefore recommended if the diagnosis of HAR with massive renal infarction can be confirmed; delay may lead to renal rupture. Fortunately, ABO typing and current cross-matching techniques have largely eliminated this entity.

B. Alloantibody-Mediated Accelerated or Acute Rejection

The introduction of the Banff schema (66,67) to provide a standardized method for diagnosing and classifying rejection has helped to appropriately interpret and manage the various pathological entities in the renal transplant biopsy. However, this morphological schema does not reflect fully the immunological and functional realities. Alloantibody-mediated rejection may present with different clinical and pathological features. Three common presentations can be categorized as pure antibody-mediated rejection, delayed function, and mixed antibody and T cell–mediated acute rejection.

1. Pure Antibody Mediated Rejection

The kidney functions initially but then severe dysfunction develops in association with the appearance of antibodies to MHC class I antigens in the circulation. The dominant pathological feature is neutrophils in dilated peritubular capillaries. Endothelial damage is manifested in glomeruli and arteries in association with platelet and fibrin thrombi.

2. Delayed Graft Function

The incidence of delayed graft function is increased in sensitized patients, suggesting that one type of antibody-mediated injury may present as poor initial graft function or ATN. The ATN associated with antibodies to MHC class I or class II antigens has been reported to be associated with a poorer survival outcome (17,20,29). The biopsy may show some features of antibody-mediated rejection, but may pass as ATN unless these features are sought. Feucht and coworkers (34) have reported that staining for C4d is a useful indicator of antibody-mediated injury in this setting. C4d is covalently bound to the tissue and therefore has a longer half-life than antibody after deposition.

3. Mixed Antibody- and T-Cell–Mediated Acute Rejection

Cellular mechanisms have long been known to cause acute rejection, and most rejection episodes are probably T-cell mediated. The features of acute cellular rejection may coexist with more subtle findings described in rejection associated with antibody, most typically neutrophils in the peritubular capillaries. In this situation, antibodies to class I MHC antigens may mediate the microvascular lesions. The presence of both may be equated with severe injury, but with aggressive treatment, in the form of steroids and antilymphocytic therapy, these lesions may be reversible.

4. Clinical Picture of Alloantibody-Mediated Rejection in the Edmonton Renal Transplant Population

The early studies of immunological monitoring in renal transplantation established that alloantibody is frequently detected in clinical rejection episodes (68–70). Various target cells were used in these early studies, but the likelihood is that many of these responses were against class I MHC antigens. For example, donor-specific fibroblast targets have been used in culture to show cytotoxic antibody during severe graft rejection (71). Numerous studies have established that the development of donor-specific alloantibodies after transplantation is deleterious (19,72,73). Even when PRA is used as a surrogate for measuring donor-specific antibody, most studies have reported a very poor graft outcome with an increase in PRA after transplantation (74).

The emphasis on diagnosing antibody-mediated rejection in Edmonton originated in the recognition that an occasional patient has a severe renal dysfunction without biopsy evidence of severe cell-mediated rejection. We initially described seven patients who had antibodies to MHC class I antigens against donor cells after transplantation and displayed generally atypical rejection patterns (17). All seven patients were presensitized. In three patients, repeating the cross-match demonstrated low levels of antibodies to MHC class I antigens that had been missed in the initial cross-match. The antibodies to MHC class I antigens were missed initially because of poor preparation of donor cells and because stored rather than fresh serum was used. In the other four patients, the antibodies to MHC class I antigens seemed to appear after transplantation as a "memory" (anamnestic) response. The clinical picture was distinctive, with rapid loss of renal function and development of oliguria and an ATN-like picture. The kidneys did not become nonviable: circulation was detected by perfusion scan, again compatible with ATN. The key features in the biopsy were evidence of neutrophil infiltration and microvascular injury, including HUS-like changes, with relative paucity of typical rejection features (tubulitis, interstitial inflammation). Antibody deposition could not be detected by immunofluorescence, despite the presence of antibodies to MHC class I antigens in the serum.

To estimate the frequency of antibody production to MHC class I antigens and injury

caused by these antibodies, we next conducted a prospective survey for antibodies to MHC class I antigens in sera of patients after transplantation and studied the clinical features of antibody-positive versus -negative patients (18). We tested 797 sera from 64 consecutive recipients of renal transplants (59 cadaver transplants and 5 transplants from 1 haplotype-matched living related donor) by microcytotoxic testing for donor-specific antibody activity. The positive sera were screened against recipient cells to exclude reactions caused by autoantibodies or therapy with antilymphocyte globulin or OKT3. All patients in this series had negative cross-matches. Donor-specific activity against T cells (i.e., presumptive antibodies to MHC class I antigens) was identified in 13 of 64 patients. Patients with antibodies to MHC class I antigens invariably had rejection compared with a 41% incidence of rejection in the patients without antibodies to MHC class I antigens. (The overall incidence of rejection at our center in that period was 55%.) Rejection associated with antibodies to MHC class I antigens occurred earlier, was more likely to produce oliguria and to require dialysis, and more frequently led to graft loss. The pathological method of rejection associated with antibodies to MHC class I antigens was characterized by evidence of endothelial injury and neutrophils in the peritubular capillaries; some cases also had tubulitis and features of T-cell–mediated rejection. Thus, two types of rejection appeared to be associated with antibodies to MHC class I antigens: "pure" antibody-mediated rejection and mixed antibody- and T-cell–mediated rejection.

We have recently compared the pathological method of rejection occurring in the presence and absence of antibodies with MHC class I antigens in patients whose biopsies were rejection-positive by the Banff criteria (66,67). (Because of the design, this analysis did not include the patients in whom antibodies to MHC class I antigens were associated with a rejection pattern that failed to meet the Banff criteria.) A total of 44 biopsies were compared: 20 biopsies from antibody-negative rejections and 24 biopsies from antibody-positive rejections. The results are presented in Tables 1 through 5. The patient characteristics were not informative except that AbMR was uncommon in living related donors (see Table 1). The AbMRs tended to occur earlier and to require antilymphocyte antibody therapy; these rejections were significantly more likely to result in early graft loss (see Table 2). The AbMRs were more likely to occur in patients who had previous transplants (see Table 3). Patients with AbMR were not more likely to have been sensitized, as demonstrated by PRA, but the sensitized patients who had elevated PRA (>10%) followed by

Table 1 Patient Demographics

Demographics	Ab + R (n=24)	Ab − R (n=20)	*p* Value
Age	49.1 ± 2.6	45.7 ± 2.5	NS
Sex M:F	16:8	9:11	NS
CAD/LRD[a]	24:0	16:4	0.036
Early ATN[b] n (%)	11 (46%)	9 (45%)	NS
B DR mismatch avg	2.7	2.7	NS
Length of follow-up (days)			
Mean	1571	1032	
Median	1283	1695	
Range	181–2743	194–3036	

[a]The 4 LRDs (living related donors) in the Ab-R group were all haploidentical; CAD (cadaveric).
[b]ATN is defined as dialysis dependence in the 1st week after dialysis, serum creatinine >400 at day 7 or urine output <1 L in the 1st 24 hours after transplantation.

Table 2 Clinical Features of Ab+ Versus Ab– Rejection

Rejection Feature	Ab + R	Ab – R	*p* Value
Days after transplantation to first rejection	11.25 ± 9.1	18 ± 27.3	NS
Requirement for treatment with anti-lymphocytic antibody	22/24 (91.7%)	16/20 (80%)	NS
Graft loss (total)[a]	13/24 (54%)	5/20 (25%)	0.0354
Graft loss <3 months	12/24 (50%)	3/20 (15%)	0.0246

[a]Graft loss is defined as a permanent requirement for dialysis.

Table 3 Sensitization Data

Comparison of Pretransplant Status of Patients with Subsequent Ab+ Versus Ab– Rejection			
	Ab + R	Ab – R	*p* Value
Panel reactive antibody (PRA) peak average	36.4 ± 37	24.5 ± 34	NS
PRA pre-average	11.26 ± 20.6	14.65 ± 22.8	NS
Pregnancies	1.8 average	1.9 average	NS
First transplant	12/24 (50%)	18/20 (90%)	0.008
>1 transplant	12	4	
Transfusion history >10	8/24 (33.3%)	6/20 (30%)	NS
31 £10	13	9	
0	3/24 (12.5%)	5 (25%)	NS

Effect of Pretransplant Sensitization Status on Subsequent Outcomes		
Ab Status During Rejection	PRA (%)	Fraction (%) with graft loss <3 months
+	>10	9/13 (69.2%)
+	<10	3/11 (27.3%)
–	>10	0/8 (0%)
–	<10	3/12 (25%)

rejection did poorly compared with the patients who had low or negative PRA before transplantation and subsequently produced antibodies in association with rejection. This suggests that rejection associated with memory responses are more resistant to immunosuppressive therapy than rejection mediated by primary antibody responses.

Rejections that met the Banff criteria were distinguished in the antibody-positive patients from the antibody-negative patients by more neutrophils in peritubular capillaries, more glomerulitis, and more vasculitis. All but one antibody-negative rejection had moderate to severe tubulitis.

One person who was highly sensitized displayed an unusual course manifested by "permanent ATN," which never recovered, but failed to meet conventional criteria for rejection. Antibodies to MHC class I antigens appeared in the circulation within a few days of transplantation. Eventually, severe interstitial fibrosis developed in the kidney and it was

Table 4 Pathology of Ab+ Versus Ab– Rejection: Glomerular (G), Tubular (T) and Vascular Changes (V) Graded According to Banff Classification

Data	Ab + R n=24	Ab – R n=20	*p* Value
Glomerulitis	11	2	0.01
No tubulitis (T0)	5	0	0.05
Mild tubulitis (T1)	7	1	0.05
Moderate tubulitis (T2)	9	11	NS
Severe tubulitis (T3)	3	8	NS
T2 + T3	12	19	0.002
T0 + T1	12	1	0.002
No vasculitis (V0)	6	7	NS
Mild vasculitis (V1)	4	11	0.01
Moderate vasculitis (V2)	4	2	NS
Severe vasculitis (V3)	10	0	0.0009
V2 + V3	14	2	0.01
V0 + V1	10	18	0.01
Vasculitis score (mean ± SEM)	1.75 ± 0.26	0.75 ± 0.14	0.009
Mean tubulitis (mean ± SEM)	1.42 ± 0.20	2.35 ± 0.13	0.001

[a]Mann Whitney U test.
Abbreviation: SEM = standard error of the mean.

Table 5 Histopathological Features in Biopsies During Rejection Episodes

Pathologic Findings	Ab + R (n=24)	Ab – R (n=20)	*p* Value
Infarction	9	0	0.002
Fibrin thrombi (glomerular and vascular)	11	3	0.05
Fibrinoid necrosis	6	1	0.11
Dilatation of PTC	8	2	0.08
PMN in glomeruli	7	3	NS
PMN in tubules	6	3	NS
PMN in PTC	11	1	0.003
PMN in interstitium	12	13	NS

[a]>2 glomeruli, >2 tubules, >2 peritubular capillaries, and >2 nonadjacent interstitial areas affected.
Abbreviations: PTC = peritubular capillaries; PMN = polymorphonuclear leukocytes.

removed more than a year after transplantation, without ever showing significant urine output. Thus severe ATN may be a presentation of antibody-mediated rejection.

In summary, acute rejection mediated by antibodies to MHC class I antigens usually arises in patients who were cross-match negative before transplantation, but occasionally reflects weak antibodies to MHC class I antigens that have been missed because of errors in the cross-match method. Antibody responses in patients with previous evidence of sensitization appear to be more difficult to control. Clinically, the recipient may have normal initial postoperative course until there is sudden deterioration in graft function: the clinical

hallmarks are early onset and extreme loss of function. This probably reflects the multiple acute mediators that antibody can activate, including the complement and coagulation cascades previously discussed. Although rejection episodes associated with antibodies to MHC class I antigens have a worse prognosis than rejection without these antibodies, recovery of renal function can be achieved with aggressive early treatment.

5. Treatment

The treatment of accelerated or acute rejection associated with alloantibody is based on the following principles:

1. *Intense immunosuppression.* Cases in which continued antibody production is required for rejection mechanisms to be sustained may benefit from intense immunosuppression designed to stop formation of antibody. This would include immunosuppression of T cells for primary alloantibody responses, which are more dependent on helper signals from T cells than are secondary responses. OKT3 has been reported to reverse some cases of hyperacute rejection when renal perfusion is maintained (75). We have recently treated such a case with mycophenolate mofetil (which does have effects on B cells and on the development of alloantibodies [76]) with a favorable outcome. A favorable outcome is usually associated with diminished circulating antibody (18), although at least one patient seemed to recover renal function despite persistence of the antibodies to MHC class I antigens. Rapamycin is also expected to have effect on antibody-mediated rejection.

2. *Removal of existing antibody.* Attempts have been made to remove the existing alloantibody by plasma exchange or immunoabsorption to ameliorate acute rejection (77, 78). Based on our experience, we question whether plasmapheresis has much clinical effect. The compartmentalization and metabolism of alloantibodies are the limiting variables in the application of plasmapheresis to transplants. Plasmapheresis acutely removes proteins from the intravascular compartment, but almost half of the IgG is in extravascular compartments under normal conditions. Moreover, IgG leaks across the vascular barrier in sites of inflammation, and antibodies are selectively retained because they bind to their antigenic targets in the tissues. In addition, the decreases in immunoglobulin concentrations decrease their catabolism and decreases in alloantibodies causes a rebound in IgG synthesis. Perhaps in addition with other strategies this method should be reevaluated.

3. *Intravenous immunoglobulin.* Intravenous immunoglobulin (IVIg) has a number of immunomodulatory properties that could inhibit different aspects of AbMAR (79). First, it can block FcR-dependent inflammation caused by neutrophils and macrophages as well as ADCC (80,81). Second, Frank and coworkers (82,83) have demonstrated that IVIg inhibits complement-mediated damage by binding C3b and C4b and decreasing complement deposition in tissues. Third, within the immunoglobulin preparations, antibodies to cytokines have been demonstrated to modulate the production and action of cytokines, particularly interleukin (IL)-6 and interferon (IFN)-γ (84–86). Fourth, anti-idiotypic antibodies in IVIg can neutralize circulating autoantibodies. Anti-idiotypic antibodies in IVIg have also been shown to downregulate B- and T-cell clones (see Chap. 1). Finally, there is evidence that some IVIg preparations block CD4, nonpolymorphic determinants of MHC class I molecules, and adhesion molecules of T and B cells. As the result of some or all of these mechanisms, IVIg can decrease the degree of PRA when administered before transplantation and can allow successful transplantation in previously highly sensitized patients (81,87).

4. *Inhibition of complement.* Inhibitors of different components of the complement system have been tested in preclinical models of xenotransplantation. In general, these have

been found to be more effective in preventing antibody-mediated hyperacute or accelerated acute rejection than depletion or inhibition of antibody. When combined with intense immunosuppression, sCR1 has dramatic effects on antibody-mediated rejection (88); sCR1 is currently undergoing clinical trials in transplant patients.

C. The Role of Alloantibody in Chronic Rejection

The causes of "chronic rejection" are multifactorial and it remains to be determined under what conditions antibody may contribute to this type of graft failure. The association of alloantibody with obliterative arterial lesions in the early era of immunosuppression (89,90) may have been exaggerated by inadequate or toxic levels of immunosuppression. These types of early aggressive obliterative lesions are rare in current renal transplants, but vascular injury produced by antibodies to MHC class I or class II antigens probably can progress to chronic transplant nephropathy or chronic rejection. Episodes of acute rejection are associated with chronic rejection (91), and the vascular injury associated with antibodies to MHC antigens has a worse prognosis than tubulointerstitial lesions associated with cellular immunity (15).

As discussed, obliterative arterial lesions have been produced experimentally in cardiac allografts in mice by passive transfer of antisera (42). Additionally, neointimal proliferative lesions in carotid artery allografts are decreased in "Ig knockout" mice, which are deficient in antibody production (46). These lesions do not have all of the hallmarks of the "accelerated arteriosclerosis" associated with chronic rejection in humans. Most notably, the contribution of smooth muscle cells to the myointimal proliferation is usually more limited in these models than in the clinical lesions. Once again, this may reflect the reductionist nature of the models studied. Lesions more typical of accelerated arteriosclerosis can be produced experimentally when the model system includes other clinically relevant variables, such as longer periods of warm or cold ischemia (92), inadequate or toxic levels of immunosuppression (93,94), dietary or drug-associated hyperlipidemia (95), and latent or active cytomegalovirus infections (96).

Because any acute rejection is associated with an increased risk of chronic rejection and because antibodies to donor antigens are associated with acute rejection, it is not surprising that alloantibody is increased in the first year in patients in whom chronic rejection later develops. What has not been shown clinically is that antibody-positive rejections have a higher risk of chronic rejection. In our experience with acute rejection associated with alloantibodies, the recovering patients have shown no unusual incidence of chronic rejection.

IV. PREVENTING HAR AND AbMR: CROSS-MATCHING

A. Cytotoxicity Assays

The introduction of cross-matching (7) has made HAR a rare event in modern transplantation (29). In the conventional microcytotoxic cross-match test, antibodies are detected indirectly by complement-mediated lysis of donor leukocytes. Over the years, many modifications have been introduced to cross-matching (97). The following variables have been reported to affect the sensitivity and specificity of the test:

- Length and temperature of incubation periods
- Number of washes

- Pretreatment of the sera (absorption with autologous cells or donor platelets; treatment with reducing reagents or heat)
- Addition of complement-activating secondary antibodies
- Type of target cell (T and B lymphocytes, monocytes, endothelial cells)

Because sera from sensitized patients usually contain a mixture of antibodies of different subclasses with a range of affinities, interpretation of the changes in results caused by these modifications in methods is largely empirical. For example, prolonging the incubation of serum and complement with cells increases the detection of antibodies in low concentration or with low avidity (97,98); whereas, extra wash steps remove antibodies with low avidity. Reagents such as 2-mercaptoethanol, dithiothreitol (DTT), or dithioethreitol (DTE), have been used to reduce IgM from the pentameric form, which activates complement effectively, to a monomeric form, which is ineffective in activating complement (99,100). However, the use of reducing agents or other chemical or physical means, such as heating and desalting procedures, can only provide presumptive evidence for participation of IgM versus IgG in cytotoxic reactions. Complete inactivation of IgM by these reagents can affect some subclasses of IgG. Alternatively, incomplete reduction of IgM leaves monomeric IgM with partial antigen-binding and complement-activating properties.

Complement-activating secondary antibodies have been added to the cross-match assay in an attempt to detect subclasses of IgG, such as IgG2 and 4, which activate complement weakly or not at all (101). In these assays, the patient's serum is first incubated with the target cell and then an anti-immunoglobulin reagent (e.g., goat antiserum to human light chain), which can activate complement, is added. Such antiglobulin cross-match assays also can increase the detection of antibodies directed to antigens expressed in low density on target cells; goat antibodies bound to patient's antibodies on the cell surface form closely paired duplets capable of fixing complement.

Cross-match assays using separated T and B lymphocytes have been used in attempts to better define the antigenic target of the antibodies. Cross-matches that are positive with allogeneic, but not autologous, T lymphocytes are considered to most likely result from antibodies against class I MHC antigens and are therefore a contraindication to transplantation. However, positive "B-cell cross-matches" are much more difficult to interpret. B lymphocytes, in addition to expressing HLA-DR and DQ constitutively, express higher concentrations of HLA-A, -B, and -C antigens than do T lymphocytes (21,23).

When the target antigens of antibodies causing positive B-cell cross-matches have been defined clearly by blocking with monomorphic monoclonal antibodies to class I and II antigens, or by differential absorption with HLA-typed platelets, preformed IgG antibodies to class I MHC antigens are the most frequently associated with hyperacute or acute rejection. Phelan et al (22) reported that 4 of 10 patients who had platelet-absorbable IgG antibodies to HLA class I antigens on donor B cells at the time of transplantation rejected their cadaver renal grafts within 3 months. Four more patients in this group with antibodies to class I antigens required antilymphocyte globulin or OKT3 to reverse severe rejection episodes. In contrast, no graft failures were associated with positive B-cell cross-matches in four other patients caused by antibodies to class II antigens. Similarly, Karuppan and colleagues (23) found that 8 of 16 patients who had cytotoxic antibodies at the time of renal transplantation that reacted with class I antigens on donor B cells, as defined by blocking studies with a monoclonal antibody (W6/32), rejected their renal grafts within the 1st year. The two patients in their study with IgG antibodies that were blocked with monoclonal antibody to HLA-DR (L243) had functioning grafts at 1 year.

Taken together, these studies indicate that the positive "B-cell" cross-matches, which are associated with acute graft loss, are primarily those caused by class I MHC reactive antibodies. However, sporadic cases of hyperacute and acute rejection associated with anti–B-cell antibodies that have specificity for donor HLA-DR or DQ antigens have been documented (27,38,39). In some of these cases, the titers of these antibodies have been relatively high (1:32–1:512). One patient with circulating antibodies to class I and anti–class II MHC rejected a class II mismatched kidney, and antibodies to HLA-DQ1 were eluted from the nephrectomy specimen (27).

B. Flow Cytometry Assays

Flow cytometry offers the advantage of unequivocal definition of antibody class and subclass and more quantifiable results. The use of flow cytometry with monospecific antibodies to detect IgM and IgG have demonstrated that most antibodies to MHC antigens are IgG or mixtures of IgG and IgM. In general, IgM antibodies are not as uniformly deleterious to graft survival as IgG. Nonetheless, acute rejections have been associated with IgM antibodies. Karuppan and colleagues (32) reported that three of five patients with only IgM antibodies to class I MHC antigens as measured against donor B lymphocytes on flow cytometry rejected their grafts. Three of the relatively few reported cases of hyperacute or acute rejection caused by preformed antibodies to class II MHC antigens involved IgM antibodies of high titer to HLA-DR (27,38,39).

Some of the varied effects of IgM antibodies on graft survival are related to the fact that the bulk of affinity maturation occurs after the switch from IgM to IgG. The common experience that positive cross-matches resulting from IgM antibodies frequently become negative in multiple-wash cross-match techniques is thought to reflect the low avidity of most IgM antibodies. In addition, less IgM than IgG diffuses out of capillaries into interstitial fluids because of its much greater size, thereby largely confining the effects of IgM to the intravascular compartment.

The IgG antibodies to donor lymphocytes that are detected by flow cytometry, but not by microcytotoxicity testing, are usually associated with an increase in postoperative complications in renal transplant. These complications include primary nonfunction and rejection episodes (32,102). Although most of these acute complications can be treated successfully, it is possible that they may be associated with an increased frequency of chronic rejection (102).

C. Enzyme-Linked Immunosorbent Assays

Like flow cytometry, enzyme-linked immunosorbent assay (ELISA) offers the advantage of unequivocal definition of antibody class and subclass. Because ELISA cross-match technology is based on antibodies binding to purified HLA antigens rather than viable lymphocytes, this assay offers the additional potential of simplicity and specificity. Although this approach is promising, the current commercial ELISA cross-match kits require further development to improve their sensitivity and range of antigenic specificities (103,104).

V. CURRENT AREAS OF RESEARCH AND FUTURE GOALS

Application of immunosuppressive agents largely targeted at inhibiting lymphocyte function resulted in dramatic improvements in patient and allograft survival in the first 30 years

of renal transplantation. However, this improvement in graft survival has plateaued over the past decade. One of the consequences of focusing immunosuppression on cellular immunity has been that many of the current treatment failures involve ancillary mechanisms.

Newly developed murine allograft models are available to test clinically relevant variables influencing the contribution of antibody and complement to rejection. These models will be useful to test new immunosuppressive reagents for their capacity to inhibit antibody production. In addition, the number of murine strains with naturally occurring or genetically engineered deficiencies has expanded so that critical components of the humoral immune system can be evaluated in transplant models at levels of stringency not possible previously.

The expanding need for organ donors has renewed interest in the use of ABO incompatible donors and discordant xenogeneic donors. The resurgent interest in xenografts has focused attention on the multiple soluble and membrane-bound inhibitors of complement that control the extent of tissue damage at sites of inflammation (105–107). Already research on xenografts has broadened our appreciation of the range of effects that antibody and complement can have on the vascular components of a graft. Many interactions between "humoral" and "cellular" immunity have been discovered (28,30,31).

These experimental data will be supplemented by clinical advances toward clarifying the contribution of alloantibodies to vascular injury in renal allografts.

ACKNOWLEDGMENT

W. M. Baldwin is supported by NIH grant HL53245 and AI42387.

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6

Renal Injury and Preservation in Transplantation

PHILIP F. HALLORAN
University of Alberta, Edmonton, Alberta, Canada

I. INTRODUCTION

During the course of transplantation, the kidney is frequently injured. The injury manifests itself as reduced function of the transplant, often called delayed graft function (DGF) or transplant acute tubular necrosis (TxATN). This injury can occur at many stages, and has short- and long-term consequences for the organ. The reduction of injury to the transplant is an area with significant potential for progress. Indeed, the theme of renal injury and repair is becoming central in renal transplantation.

How are nonspecific injury in the graft, immunologically specific rejection, and late graft loss related? Existing clinical and basic science suggests a hypothesis to account for these relationships, in line with new thinking on cell injury and the relationship of tissue damage to graft outcomes. The key elements are as follows:

1. The nonspecific injury causes inflammation (the "injury response").
2. The injury response increases the probability of immune recognition.
3. Immunological injury also induces an injury response, which may renew immune activation.
4. This process is preventable and potentially treatable in its early stages.

This chapter reviews some sources of renal injury in transplantation (other than immunological mechanisms); the consequences of renal injury on rejection rates and graft survival; the methods of prevention, focusing on organ preservation; and the potential treatments for injury and the injury response. Useful references include reviews by Land et al. (1) and Shoskes et al. (2).

II. SOURCES OF INJURY IN CADAVERIC RENAL TRANSPLANTATION

The initial function of the renal transplant is a critical factor in its long-term outcome. The initial function is influenced by underlying processes in the donor such as hypertension and aging, which produce progressive deterioration in renal function (3). But, the main factors in the cadaver donor are the injury processes that lead to and accompany transplantation: the process of brain death; hemodynamic instability resulting from hypovolemia or vasomotor collapse; therapies and investigative studies in the donor; the trauma of removal, preservation, and implantation; and the conditions in the recipient. These types of injury are summarized in Table 1.

A. Sources of Injury in the Brain-Dead Donor

The process of brain death triggers massive disruption of control mechanisms, associated with the collapse of vasomotion and with intravascular coagulation. Disseminated intravascular coagulation (DIC) is common following cranial trauma, and increases with the severity of brain trauma (4–9). The manifestations of disseminated intravascular coagulation include thrombocytopenia and microthrombi in the tissues. The causes of disseminated intravascular coagulation are multiple and incompletely understood. Material from damaged brain has been found in the circulation, evoking speculation that brain thromboplastin actually activates the clotting mechanisms. On the other hand, the possibility that there is widespread endothelial injury and dysfunction as part of the generalized vasomotor collapse must also be considered.

Hemodynamic instability, hypovolemia, and vasodilation all accompany brain death

Table 1 Sources of Renal Injury in Cadaver Donors

Tissue underperfusion ("ischemia") caused by
Abnormal hemodynamics
Hypovolemia: hemorrhage, diabetes insipidus
Endotoxin, septicemia
Effects of brain injury:
Disseminated intravascular coagulation, endothelial dysfunction
Catechol storm
Denervation
Endocrine abnormalities:
Thyroxine?
Effects of interventions
Deliberate "drying" to protect lung
Vasopressors, including "renal dose" dopamine
Pressor effects of vasopressin?
Nephrotoxins (e.g., angiography dyes, antibiotics)

and may lead to tissue injury. These events are often complicated by severe cardiac dysfunction. Volume deficits may reflect hemorrhage, water loss resulting from diabetes insipidus (DI), and underfilling resulting from vasodilation. The instability of the donor reflects complex interplays in which cause and effect are difficult to dissect. Are DIC and thrombocytopenia caused by endothelial injury? Does DIC cause endothelial injury? Does endothelial dysfunction contribute to thrombocytopenia and vasomotor collapse because of the loss of endothelial regulation of adhesion and vasomotion? Can underperfusion resulting from vasomotor collapse explain the organ injury in the brain-dead person, or is some of the organ dysfunction a more direct consequence of the brain destruction? Recently, hormonal abnormalities have been incriminated and interventions with hormones have been advocated, but conclusive evidence of deficiencies of hormones has been elusive, although studies are in progress (10).

Some interventions in the management of the brain-dead potential donor, including pressors and angiography dyes, may damage the organs. Vasopressin is not usually considered pressor at the doses used to treat DI, but whether the abnormal vasomotion in the brain-injured person may sensitize the recipient to the effects of vasopressin should be considered. Endotoxemia and septicemia may induce vasomotor or direct toxic changes. The management of the donor may lead to difficult choices: for example, reduction of volume to avoid fluid accumulation in the lungs may aggravate vasoconstriction and underperfusion in the kidney.

As a consequence of severe vasomotor collapse or more direct injury, many organs of the severely brain-injured or brain-dead individual deteriorate. The most severe changes are often in the lungs and the heart. Lungs often become untransplantable in brain-dead individuals, severely limiting lung transplantation. In many donors, even in young individuals, the function of the heart deteriorates quickly, and it may become unusable for transplantation because it is too injured to sustain life in the recipient. In some hearts, a lesion called contraction band necrosis develops, which may be related to "catechol storm," that is, a massive release of catecholamines associated with severe brain injury. Contraction band necrosis has been produced in baboons by brain death (11). A primary abnormality in cardiac function could contribute to dysfunction in other organs. In general, much of the deterioration in the organs of brain-dead humans is poorly understood, but clearly complex.

B. Injury Resulting from Harvesting, Preservation, and Implantation

1. Harvesting Method

The process of harvesting the donor kidney can give rise to direct renal injury and to vasospasm, which itself can damage the kidney in the period following transplantation, leading to acute tubular necrosis (ATN). Tension on the renal vessels or excessive handling of the renal vessels may be responsible for some cases of postoperative acute tubular necrosis. Harvesting surgeons use techniques involving minimal manipulation of the renal vessels. The technique of "en bloc" harvesting in the multiorgan transplant patient may reduce renal trauma and vasospasm.

2. Warm Ischemic Time

Warm ischemic time (defined as the time between clamping of the renal arterial supply and core cooling of the kidney) is associated with severe renal injury if it is prolonged (12). The technique of en bloc harvesting has reduced the initial warm ischemic time to virtually zero. The technique of in situ flush with University of Wisconsin (UW) solution or other flush

solutions has been associated with improvements in the rates of initial graft function at many centers.

3. Anastomosis Time

A key determinant of initial graft function is the length of the anastomosis time (13–15). The anastomosis, or rewarm, time is the time for completion of the arterial and venous anastomosis while the kidney is lying in the recipient, unperfused and rewarming from the body heat of the recipient. Anastomosis times of 30 minutes or less are associated with excellent initial function rates. Anastomosis times of more than 45 minutes are associated with increasing problems, and anastomosis times of 60 minutes or more are frequently associated with DGF. The reason for the association of anastomosis time with DGF may be rewarming. Alternatively, the prolonged anastomosis times may reflect issues such as the need for extensive handling of the blood vessels because of difficulties in the anastomosis. Such difficulties in the anastomosis may be associated with subsequent problems such as vasospasm in the kidney, leading to ATN.

4. Interactions Among Sources of Injury

A degree of ATN is common in brain death, even before organ harvesting. Subtle renal injury may predispose the kidney to further injury during harvesting, preservation, and implantation, and may make the renal vascular tree susceptible to vasoconstriction. One plausible interaction among the injurious influences is that donor endothelial injury causes abnormal vasomotion, making the kidney more prone to vasospasm. Cold flush and preservation may further exacerbate this tendency. Even the moderate arterial handling during the anastomosis could then trigger vasospasm in the postoperative period, leading to ATN. The extent to which the interactions among such variables actually contribute to transplant ATN (TxATN) is unknown.

5. Center Effects

Centers report remarkable differences in the quality of the kidneys they harvest, which may contribute to "center effect" differences in long-term results. These differences are not easily explained. Intensive care practice in the center even before brain death may be a contributing factor. The skill of the donor maintenance team and the kidney harvesting surgeons may account for some differences. Anastomosis time is one factor, reflecting either rewarming time (warm ischemia) or the extent of handling during a difficult anastomosis. In some units, there has been an attitude that DGF is benign and reversible and thus of no importance, which could lead to differences in practice compared with other units that compulsively seek methods to improve kidney quality.

C. Pathology of TxATN May Not Be Identical to ATN in Native Kidneys

The pathology of TxATN is not identical to that of native kidney ATN (16). For example, in TxATN, there are some entire tubular cross-sections that are necrotic, unlike in native kidney ATN in which the necrosis affects only single cells. It is likely that there are differences in the underlying mechanisms, such as the presence of endothelial injury and DIC in the brain-dead donor and the exposure of the transplant kidney to cold flush and storage. One possibility for these differences is that TxATN includes injury from the effects of brain death, including possible injury to elements of the microcirculation such as the endothelium, which would be absent in most cases of native kidney ATN.

III. INFLUENCE OF INJURY ON OUTCOMES IN RENAL TRANSPLANTATION

Clinical evidence that nonspecific injury increases graft loss in renal transplantation includes many observations over 3 decades of transplantation, documenting an increased frequency of graft loss when kidneys show poor initial function over grafts with superior measures of function. (Some of the effects of DGF on survival are reviewed in 2 and 17.) The effects of ischemia and reperfusion injury have been reviewed by Land and Messmer (1).

A. Studies of the Relationship Between Injury and Poor Outcomes

From early studies, it was appreciated that DGF was associated with adverse outcomes, whereas immediate function (urine output at the time of implantation) portends better graft survival (18). With the recognition and prevention of hyperacute rejection (19–21) and other entities, the outcome of TxATN was generally noted to reduce the probability of successful outcome (22). Whittaker et al. studied the fate of kidneys with DGF and found that good initial function was associated with better graft survival (23). They suggested that much of the adverse effect of TxATN was related to the need for additional diagnostic procedures. Thoren studied kidneys from non–heart-beating donors, which included many seriously damaged kidneys, and found that initial function and graft survival were adversely affected by long warm ischemia time (12). In analyzing the experience of the New England organ bank, Cho et al. found that TxATN significantly reduced graft survival in primary cadaveric transplantation, but that TxATN was not related to the total preservation time (24). DGF also affected long-term survival in several other early studies, and the duration of TxATN affected both graft and patient survival adversely (25,26).

A pivotal observation was reported by Opelz et al. from an analysis of the thousands of North American kidney transplants in the University of California at Los Angeles database (27). They found that renal function predicted graft survival, whether assessed by dialysis dependency or particularly as assessed by serum creatinine, at all time points—1 day, 1 week, 1 month, and so on. *The relationship between serum creatinine and graft survival is probably the most fundamental determinant of graft survival in renal transplantation.*

The Southeastern Organ Procurement Foundation (SEOPF) database provided a series of important observations in studies of large numbers of transplants. MacDonald et al. showed that TxATN adversely affected survival and that total preservation time and sharing between centers did not predict TxATN (28). Sanfilippo et al. used the Cox multivariate regression analysis to show that TxATN was not only associated with graft loss and patient death but also with irreversible rejection (29,30). This observation suggests that the relationship between injury and adverse outcomes may be related to excess rejection in injured kidneys. A later analysis confirmed that DGF was associated with increased risk of irreversible rejection (31). The researchers also found that transplants with DGF that recovered and had good function at 1 month still had an increased risk of graft loss, but that, by 6 months, grafts with DGF that had recovered good function no longer carried an increased risk of graft loss, that is, the increased risk associated with DGF was lost if the graft recovered fully. Thus, the risks of DGF were "principally manifested in the first 6 months." The method of preservation did not affect the rate of graft survival and, with longer preservation times, DGF was reduced by pulsatile perfusion (32).

The cyclosporine (CsA) era produced some variations on these themes but did not alter the fundamental relationships. Canafax et al. showed in the Minnesota database (33)

that CsA did not cause DGF but slowed recovery, and that DGF was associated with reduced graft survival whether or not CsA was used. Prolonged preservation did not predict DGF. Delayed graft function was a significant risk factor for graft loss in the Canadian multicenter CsA study, in both the control and the CsA group (34). In the multivariate analysis of CsA-treated kidneys by Kahan et al. (35), DGF reduced graft survival but kidneys that recovered function after DGF carried no long-term risk of graft loss. These researchers confirmed the powerful predictive value of the 1-month serum creatinine.

Many other analyses in the 1980s generally supported the concept that injured kidneys suffered from decreased graft survival (36–38). Williams surveyed preservation methods and DGF (39) and concluded that pulsatile perfusion was associated with a reduction in DGF but that differences were small with shorter preservation times and that CsA did not adversely affect DGF or the number of kidneys that never functioned. Lennard et al. (15) suggested that prolonged anastomosis times increased DGF. Hall el al. (40) reported that patients with prolonged TxATN who were given CsA did not experience increased graft loss.

In a prospective study of kidneys treated with sequential antilymphocyte globulin (ALG) followed by CsA, the principal determinant of graft survival was reported to be DGF, which increased the relative risk of graft loss by 2.86, and the risk of graft loss persisted in those kidneys that recovered function because the serum creatinine remained higher in the DGF group overall (41). In kidneys with initial function, the 1-year graft survival was 91%. The study emphasized that, in the era of potent immunosuppressives, DGF was assuming increased importance. Again anastomosis time was a significant predictor of DGF. Merkus et al. (42) showed a strong deleterious effect of DGF and found that total preservation time was a major determinant. Analysis of the United Network of Organ Sharing (UNOS) registry (19,525 cadaver kidney transplants) (43) showed a potent effect (reduction of 20%–30% in graft survival) with all measures of DGF (urine output, dialysis dependency, discharge serum creatinine). The risks for DGF were a combination of preservation variables such as preservation time and immunological variables such as panel reactive antibodies (PRAs) and donor related (DR) mismatches. Donor cerebrovascular accidents were a risk factor for DGF. Other analyses of the UNOS database have essentially confirmed these finding (44).

Peters et al. (45) analyzed 17,937 cadaver donor renal transplants from SEOPF in three eras: 1982–1991, 1982–1989, and 1990–1991. They found that DGF significantly predicted decreased graft survival in all eras (RR 1.6, 1.5, 2.3, respectively). The preservation time was not a significant determinant of graft survival and only weakly predicted DGF, especially in the recent era. In an analysis of 495 cadaver donor kidney transplants managed with CsA, Cacciarelli et al. (46) found that prolonged DGF was associated with prolonged cold storage, high cytotoxic antibodies, black race, increased rate of acute rejection in the 1st year (67% versus 31%), and impaired 1-year survival rate (52% versus 85%). Nevertheless, those who recovered again had no long-term decrease in renal function.

B. Not All Studies Have Found an Adverse Effect of Delayed Graft Function

Not all observers have agreed on the dangers of TxATN. In one early report, Williams et al. (47) studied 101 live donor and cadaver donor transplants, and concluded that "the presence of ATN did not alter prognosis," whereas rejection episodes before 7 days and after 4 months reduced survival rates. They even suggested that TxATN may be beneficial. This result was never duplicated and probably reflected complex factors operating in the early

years of renal transplantation. Two studies from Minnesota (48,49) concluded that TxATN is "relatively innocent." It may be significant that, in their later study (49), these investigators excluded patients with "hyperacute rejection" or thromboses or obstruction, and used a matched control group rather than all of the patients in the same population. Later analyses from Minnesota contradicted their conclusion that TxATN was benign (37).

The analysis by Salmela et al. (50) showed a smaller than usual effect of DGF on graft survival and showed that the group with DGF had less rejection than the group with immediate function, the reverse of the usual finding. Barry et al. (51) examined the significance of DGF in the CsA era in Oregon. They found higher 1-month creatinine levels but no effect of DGF on graft survival at 1 year. In this study, an early decrease in graft survival in the DGF group was offset and equalized by unexplained late loss in the group with initial function. The reasons for the differences between such findings and the usual consensus is unknown.

C. Acute Rejection Episodes Impair Graft Outcome

In the study by Williams et al. (47) of 101 live donor and cadaver donor transplants, rejection episodes before 7 days and after 4 months reduced graft survival. Many subsequent studies showed that acute rejection decreases graft survival compared to grafts free from rejection. Even a single episode of acute rejection increases the probability of graft loss in some studies (52–54). Thus acute rejection is the second major event in the posttransplant period that correlates with transplant outcomes.

In some respects, acute rejection episodes are like episodes of DGF: they acutely impair function and are potentially fully reversible, yet they carry an overall increased risk of graft loss. Because acute rejection is a treatable disease, why do such rejection episodes increase the risk of graft loss? This issue deserves further study: acute rejection episodes do increase graft loss overall but not if they are completely reversed to serum creatinine less than 130 μM (55). Thus, it appears that acute rejection episodes as a group increase late graft loss because many episodes are not completely reversed, creating a chronic unresolved process in some of the injured kidneys.

D. Delayed Graft Function May Increase Graft Loss via an Increase in Early, Irreversible Rejection

Sanfilippo et al. (31) found that ATN increased irreversible graft rejection. In a Cox regression analysis, DGF was associated with a decreased risk of emerging with no rejection. In a case control study of 69 cadaveric transplants that never functioned, 88% of the never-functioning grafts were found at nephrectomy to be severely rejected. Thus, the failure of DGF kidneys to recover usually represents severe rejection in an injured transplant (14), echoing the findings of Sanfilippo et al. In the UNOS database, DGF reduced graft survival somewhat even in kidneys that were never recorded as having acute rejection (44). Nevertheless, the worst 1-year graft survival rate (51%–59%) was recorded in grafts that had both DGF and rejection versus up to 89% when DGF and rejection were both absent. These data suggest an interaction between DGF and rejection, and suggest that the effects of these injuries operate early in the transplant course.

Analyses of the Minnesota database by Troppman et al. (56) suggested how DGF and acute rejection may interact. They found that graft survival was not significantly affected by DGF in patients who recovered from DGF with no rejection. The most disadvantaged group was the group with DGF and rejection, but rejection alone also reduced the graft survival rate. Delayed graft function was associated with an increased frequency of acute

rejection. Thus, at least some of the risk associated with DGF is due to the increased frequency of rejection in DGF. This confirms previous analyses about the increase in irreversible rejection in patients with DGF.

My colleagues and I confirmed the importance of the rejection–DGF–graft loss interaction in a recent analysis of graft survival in the University of Alberta renal transplant database of 421 cadaveric renal transplant recipients from 1984 through 1995 (57,58). We found that either rejection or ATN was associated with graft loss when analyzed as a single variable. However, when graft loss and the diagnosis of biopsy-proven chronic rejection were analyzed together, only rejection emerged as significant. The association of ATN with increased graft loss was probably accounted for by the increased frequency of graft rejection in kidneys with ATN. The ATN increased the frequency of acute rejection significantly from 55% to 68%, but ATN had little effect if no rejection occurred: rejection-negative, ATN-negative and rejection-negative, ATN-positive patients had similar good long-term survival rates. However, rejection-positive, ATN-negative and rejection-positive, ATN-positive kidneys both did badly, and rejection-positive, ATN-positive status was significantly worse than rejection-positive, ATN-negative status. Thus, the effect of ATN was probably transient if ATN recovered with no rejection, but ATN was not benign if rejection was also present, reminiscent of earlier observations (14).

These results suggest that the risk factor of DGF operates in part as an immunological effect. Delayed graft function causes an increased frequency of acute rejection, and the real cause of the impaired graft survival is the rejection. Because ATN in native kidneys does not predispose the patient to end-stage renal disease and because TxATN that fully recovers with no rejection does not increase the propensity of transplanted kidneys to late graft loss, the true cause of the late graft loss is probably the rate of acute rejection and possibly of severe or refractory rejection. It may be that the grafts at risk after either ATN or rejection are those in which the injury evokes rejection and establishes a vicious circle of new injury, leaving the kidney with impaired function at 6 to 12 months, and a program for progressive deterioration.

E. Lessons from Living Donor Transplantation

Living donor kidneys with extensive human leukocyte antigen (HLA) mismatching (e.g., spouses) have excellent graft survival, perhaps lacking the injury associated with brain death and prolonged cold storage that accompanies cadaver donation (59,60). The high survival rates in spousal donors is compatible with the concept that injury powerfully affects graft outcome and that the mechanism of the effect of injury may be immunological (59). Nevertheless, some kidneys from live donors are rejected despite the absence of injury.

IV. PATHOGENESIS OF RENAL ISCHEMIC INJURY AND INFLAMMATION

Ischemia is a general model of acute renal injury and remains the main model for the injury that accompanies renal transplantation. Although the renal injury associated with brain death is complex, it remains likely that ischemia caused by vasomotor collapse and changes in the microcirculation accounts for much of the injury. Moreover, the details of how the kidney is injured in brain death and transplantation may not be crucial to understanding the response of the kidney because the response of kidney to various injuries (ischemia, nephrotoxins) seems to be relatively stereotyped. The cellular basis of ischemic renal injury is itself difficult to study because of the complexity of renal cellular organization, and some conclusions must be inferred from studies of the effect of hypoxia rather than ischemia (61).

Ischemic ATN is associated with evidence of widespread cell injury (membrane

blebbing, loss of brush border and basolateral unfoldings, and decrease in Na/K-ATPase associated with redistribution of adenosine triphosphatase [ATPase] from the basolateral membrane) as has been shown in injured cadaver kidney transplants with DGF (62). The intracellular ATP levels decrease (as has also been shown in cadaver renal transplants with prolonged warm ischemia) (63) and intracellular calcium levels increase. Calcium may play a major role in the pathogenesis of renal cell injury in ischemia-reperfusion and other situations (61). Calcium activates the enzyme calpain, a neutral cytosolic cysteine protease, and induces nitric oxide (NO) production. Calpain could play a role in the loss of cell polarity and the redistribution of Na/K-ATPase from the basolateral membrane. Nitrous oxide could promote cellular detachment by disrupting the actin cystoskeleton and promoting sloughing of brush borders. There is recent evidence for a role of NO synthase in renal reperfusion injury (64). Calcium could also activate phospholipase A2, which could injure membranes.

A. Reperfusion Injury

When ischemia ends and there is return of blood flow to the kidney, reperfusion changes develop and are superimposed on the ischemic changes. A major event in reperfusion injury is the interaction of the neutrophils of the recipient with the endothelium of the organ, mediated first by selectins, then by integrins such as LFA-1 interacting with intracellular adhesion molecule-1 (ICAM-1). During the reperfusion phase, reactive oxygen species are generated, free radicals containing an odd number of electrons and which can only be neutralized by other free radicals. These moieties participate in chain reactions, which can lead to extensive peroxidation of unsaturated fatty acids, causing membrane damage.

The classic superoxide radical is produced by reduction of one electron from O_2, leading to O_2^-; the superoxide radical is a good reductant and a fair oxidant. A major source of superoxide is xanthine oxidase (type O), which is produced by calcium-dependent enzymes under conditions of ischemia from the enzyme xanthine dehydrogenase (type D). The reaction is shown in Figure 1. Under conditions of ischemia, ATP breaks down to adenosine monophosphate (AMP), which breaks down to adenosine, which breaks down to inosine, which in turn breaks down to hypoxanthine. At the same time, calcium proteases break down xanthine dehydrogenase to xanthine oxidase. Under conditions of reperfusion, xanthine oxidase catalyzes the conversion of hypoxanthine, with the conversion of O_2 to the superoxide radical. From superoxide, the hydroxyl radical OH can be produced. Superoxide dismutase (SOD) scavenges superoxide radicals: $O_2^- + O_2 + H^+ = H_2O_2 + O_2$ (65).

It seems likely that an element of reperfusion injury occurs in renal transplant, mediated by the interactions between the neutrophil and the endothelium. This may occur intraoperatively when the clamps are opened on the blood vessels. However, this may also occur in the donor before transplantation, in periods following resuscitation, or after massive release of vasoconstrictive mediators.

The kidney responds to injury with a complex pattern of altered gene expression (66,67): DNA synthesis; loss of prepro-epidermal growth factor (ppEGF) mRNA with slow recovery over several weeks; induction of immediate early genes Fos and Jun, induced before new protein synthesis and resistant to cycloheximide; and expression of mRNA for at least two chemokines within 4 hours, JE (a C-C chemokine known as MCP-1) and KC (a C-X-C chemokine related to IL-8). Unpublished studies at my institution have shown expression of the C-C chemokines RANTES and MIP-1α. The cellular source of chemokine expression could be the thick ascending limb of the loop of Henle, which is not severely damaged by morphological criteria. The roles of chemokines in kidney disease

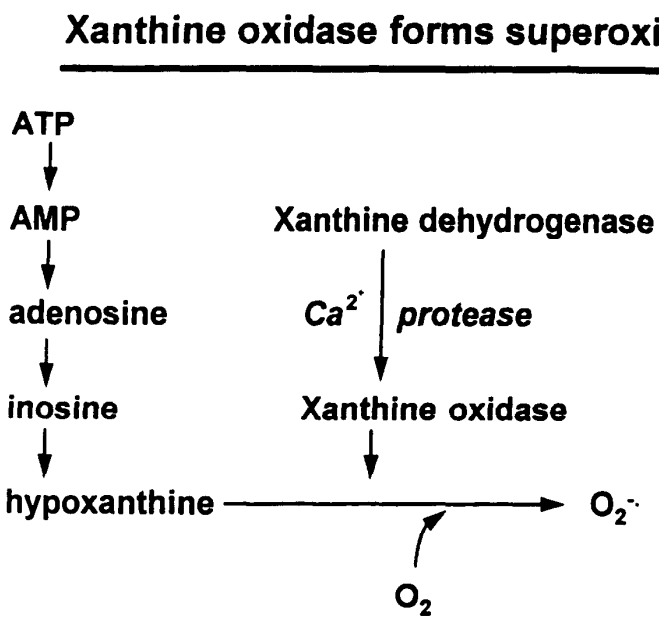

Figure 1 Xanthine oxidase is activated from xanthine hydrogenase during ischemia, probably by calcium-activated proteases. Xanthine oxidase then catalyzes the conversion of hypoxanthine to xanthine with the generation of superoxide.

have recently been reviewed (68). Overall, the injured kidney undergoes many changes including cell division, dedifferentiation, a stress response in many of the minimally injured cells, and many alterations in gene expression.

B. Stereotyped Inflammatory Response (The "Injury Response")

Renal injury of many kinds induces interstitial inflammation, including the interstitial changes that accompany glomerular disease (69) and a complex inflammatory process accompanying experimental ATN. For several years we have studied the inflammatory response to renal injury (70–74). We found that ischemic and toxic renal injury induce an inflammatory response during the phase of healing, beginning about 3 days after the injury. The main features of this response are listed in Table 2. Thus, after 3 to 5 days, the injured parenchymal cells become class I and II positive, and after 5 days there is an increase in the class II positive interstitial cells. These changes are at least partially interferon (IFN)-γ independent because they occur in mice lacking IFN-γ (IFN-γ knockout mice, or GKO), albeit somewhat reduced. Nevertheless, a host of cytokines and growth factors are increased. The response is of long duration, up to 5 weeks after a single insult. The response is basically similar whether the insult is ischemic (60 minutes of cross-clamping) or toxic (resulting from mercuric chloride, gentamicin) except that the site of injury is different: ischemia affects the deeper cortex and outer medulla, especially the thick ascending limb of the loop of Henle, where the mRNA for ppEGF is found. Thus, ppEGF is rapidly lost in ischemia but not in gentamicin injury, which affects the outer cortex. In these models, major histocompatibility complex (MHC) induction and inflammatory infiltrate are found at the area of injury.

C. The Cellular Stress Response

The pathogenic mechanisms responsible for these changes are not well understood, but factors such as epithelial cell stress or injury and detachment, and local chemokine production

Table 2 Features on Inflammation that Follow Renal Injury in Mouse Unilateral Ischemia in the Ischemic Left Kidney (Versus Control Right Kidney)

Increased MHC class I and II product, mRNA
Increased β_2 microglobulin, CIITA
Increased class II+ interstitial "dendritic" cells
Long duration: day 3 to day 35 or more
Near total loss of ppEGF mRNA (TAL injury)
Increased TGF-β mRNA
Increased IFN-γ, IL-2, IL-10, GM-CSF mRNA
Confined to injured areas

Abbreviations: MHC = major histocompatibility complex; CIITA = class II transactivator; PPEGF = prepro-epidermal growth factor; TGF = transforming growth factor; IFN = interferon; IL = interleukin; GM-CSF = granulocyte-macrophage colony-stimulating factor.

may be important. It is likely that the MHC induction reflects some effect of IFN-γ, but the main effect is likely to be independent of IFN-γ, reflecting changes in adhesion and signaling through integrin receptors. Cells that are stressed respond with complex programs, frequently involving the "stress activated protein kinases" such as Jun N terminal kinase (JNK) (75). Responses of stressed cells can lead to apoptosis, proliferation, or, in some cases, chemokine production. Bronchial epithelial cells, when subjected to detachment and deformation, express IL-8 (76), and it is possible that renal epithelial cell stress or deformation also induces chemokines. Safirstein et al. have demonstrated chemokine expression within hours of renal injury (66,67). The chemokines are likely to play many roles in inflammation in kidney, and the changes described lead us to suspect the C-C chemokines such as RANTES, MIP-1α, and MCP (68). As noted, we have recently found that RANTES and MIP-1α are increased early in ischemic mouse kidney, after 24 hours, and before the other inflammatory changes. Changes in adhesion molecules such as selectins and integrins in renal endothelium (77) could help to explain the accumulation of mononuclear inflammatory cells.

Another feature of stressed cells is often the induction of heat shock proteins. The roles of heat shock proteins in transplantation immunology are unknown but many roles are possible, as has been recently reviewed (78).

V. MECHANISMS BY WHICH INJURY PREDISPOSES THE GRAFT TO POOR OUTCOME

The key observations summarized in Table 3 suggest a theory for the events and relationships in clinical renal transplantation that determine graft outcome (79). The common denominator in many of the factors influencing long-term renal transplant survival is early injury. The principle is probably applicable to any organ transplant, but is best documented in kidney because cadaver kidneys can be compared with kidneys from live donors who experience no brain death injury. The clinical evidence documents that nonspecific injury and acute rejection are linked to early graft loss, probably by immune mechanisms, and to late graft loss (chronic rejection). Together with the experimental data, these observations suggest that injury induces a stereotyped inflammatory injury response, probably through chemokines, which promotes immune recognition and makes immunological injury more

Table 3 Top 10 Features of the Injury Hypothesis

1. Kidneys with acute tubular necrosis (ATN) have increased graft loss.
2. Kidneys with ATN have increased rejection.
3. Kidneys with ATN that recover with no rejection recover probability of survival.
4. Kidneys with rejection have poorer graft survival.
5. Kidneys with rejection that is fully reversed recover probability of success.
6. Brain injury causes poorer graft survival (living donor versus cadaver donor), not explained by human leukocyte antigen matching.
7. Severe brain injury causes injury in many organs (e.g., heart; decrease in platelets).
8. Certain types of brain injury (nontrauma, particularly cerebrovascular accidents, have poorer graft survival).
9. In experimental animals, injured tissues are inflamed.
10. Injury and inflammation promote an immune response.

likely. Immunological injury itself represents a new form of injury that can initiate a new injury response, potentially becoming self-propagating. The renal evidence for this view is as follows:

1. Initial poor graft function (often called DGF or ATN) increases the risk of both acute rejection and of late graft loss. If transplant ATN recovers to normal function with no rejection, the risk of graft loss returns to normal. Acute tubular necrosis does not strongly predispose the patient to end-stage renal disease in native kidneys and does not do so in transplant kidneys if rejection does not occur before complete recovery. Thus, the ability of transplant ATN to increase late graft loss in part reflects the risk of acute rejection. Living unrelated donor kidneys (e.g., from spouses) have excellent graft survival despite extensive HLA mismatching, perhaps because they lack the injury associated with brain death and prolonged cold storage that accompany cadaver donation (59). High survival rates in spousal donors supports the concept that injury is a powerful predisposing factor to immunological recognition and rejection (59). There is some argument for a model based on "wear and tear" for these associations (80), but this ignores the association with rejection. It is also possible that older kidneys are more susceptible to immunological injury, as suggested by the increased frequency of acute rejection in kidneys from older donors (81).

2. Although acute rejection increases the risk of late graft loss (52–54,82) recent data suggest that acute rejection episodes increase graft loss only when incompletely reversed (55). Thus, rejection behaves the same way as DGF, perhaps for the same reason—it increases the risk of immune recognition for a period after the rejection episode. Failure to resolve an acute rejection episode may caused by rejection-related injury, setting in motion a self-sustaining chronic process in some kidneys.

3. The adverse effects of transplant ATN and of the single rejection episode may operate through inducing rejection before complete recovery has occurred. In cases of early graft loss without function, the pathology suggests rejection (14), that is, transplant ATN increases the probability of severe rejection. The effect of DGF operates through acute rejection, and ATN does not adversely affect graft survival if no rejection supervenes.

A. Injury Response Hypothesis: The Injury Response Primes the Immune Response

The immune response focuses on stressed or alarmed, or injured tissue (83). The immune response can be considered as signal 1 (antigen) and signal 2 (costimulation by the antigen

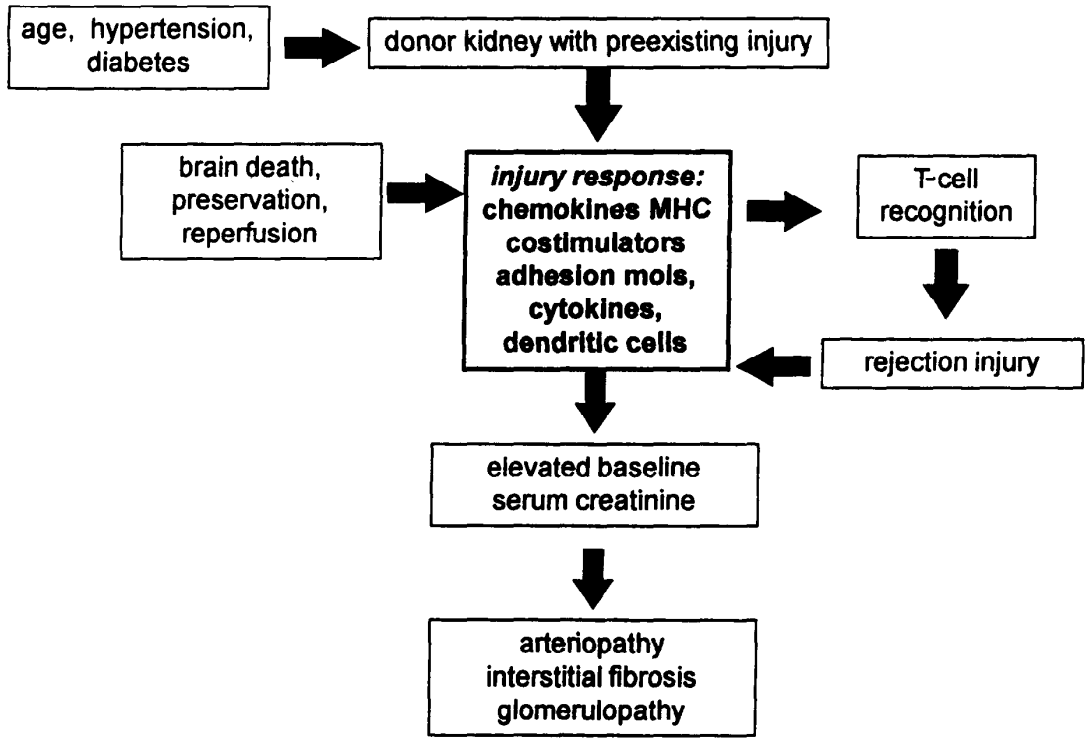

Figure 2 The central importance of the inflammatory injury response in the fate of the transplant, illustrating how injury-induced inflammation can increase T-cell recognition, which leads to rejection, which then can foster a new injury response and a self-perpetuating cycle. Without adequate treatment, this can exhaust the ability of the tissue to remodel and repair, and lead to functional impairment and the changes of chronic rejection.

presenting cells, such as B7-CD28). Injury increases MHC expression (signal 1) and probably signal 2 or costimulation by virtue of the increase in dendritic cells. Thus injury promotes the immune response. The resulting model is shown in Figure 2. The key feature of this model is that the immune response induces injury, which induces a new injury response, which promotes more immune recognition. This is the self-propagating feature that may lead to impaired baseline creatinine at 6 to 12 months. At some point, chronic rejection presumably is programmed by the impaired baseline function, even with no further immune response, but it is not known when this point occurs. However, it remains to be proven that rejection is increased by injury in experimental models, despite the human correlations.

B. Interaction Among Donor Factors, Acute Rejection, Delayed Graft Function, 6-Month Serum Creatinine, and Graft Survival

Current data suggest that the long-term survival of kidney transplants is determined by the interplay between preexisting donor disease, early injury to the kidney, the load on the kidney (size matching), and the level of function obtained at 6 months. The studies of late graft loss show that the serum creatinine is powerfully predictive at all time points. Early injury in the donor or during the transplant process predisposes the transplant toward rejection, and rejection may become self-propagating by inducing further injury and inflammation. The recovery from these injuries occurs by 6 months, and the kidneys that recover excellent function bear no long-term sequelae of ATN or rejection. However, many kidneys have impaired function at 6 to 12 months, leading to increased late graft loss.

VI. RENAL PRESERVATION, PROTECTION, AND RESUSCITATION

A number of reviews of renal preservation have appeared (84–86), as have those specialized aspects such as the role of arachidonic acid metabolism (87), the amelioration of DGF in the CsA era (88), and the role of perioperative fluid and drug therapy (89). Some possible interventions are listed in Table 4.

A. Donor Issues

It seems likely that the major determinant of initial transplant function is the quality of the tissues in the donor at the time of removal. Donor factors in cadaver donation include those antecedent to the final illness of the donor causing brain death, and those that have accompanied brain death. Both in living donors and in cadaver donors, certain principles such as volume expansion and adequate diuresis must be observed. Cadaver donation involves many measures that aim at reducing the risks of DGF, including administration of fluids and albumin, and monitoring of central pressures if possible as a guide to aggressive fluid management.

Both in living donors and in cadaver donors, changes related to aging, hypertension, and blood pressure preexist donation and adversely affect initial function and long-term outcome, but can only be reduced by selection. Very young donors also have an increased frequency of poor outcomes. The renal biopsy in the "suboptimal" donor is increasingly important in making the tough decisions about kidney use and allocation, and in establishing a baseline against which to evaluate chronic changes. Glomerulosclerosis and afferent arteriolar hyalinization probably correlate with poorer outcomes, but the probability of good outcomes should be evaluated in clinical trials to establish a solid basis for these decisions. Discarding kidneys leaves people on dialysis; excluding kidneys that could give 3 to 5 years of function with creatinine of 2.0 leaves two persons on dialysis with average renal function that is likely to be considerably worse. In view of the present donor shortage and the relentless pressure on dialysis facilities, discarding kidneys should be a last resort, and new protocols to resuscitate and stabilize suboptimal kidneys should be a priority. Rela-

Table 4 Interventions to Reduce Renal Injury in Transplantation: Selection of Optimal Donors (Ideal Not Possible in View of Donor Shortage)

Minimize injury in donor
Optimize fluid status within considerations for other organs.
Maintain perfusion with minimal pressors.
Minimize delay time before organ removal.
Minimize injury in removal/preservation/implantation
Use University of Wisconsin solution.
Use calcium channel blockers at harvest, mannitol.
Minimize warm time, cold time, and anastomosis times.
Perfuse in situ and avoid renal handling especially when warm.
Avoid hydroxyethyl starch?
Optimize condition of recipient
Apply albumin, volume.
Apply calcium channel blockers, furosemide, mannitol.
Apply antilymphocyte antibodies for induction if diuresis is poor.
Avoid calcineurin inhibitors until renal function is established?

tively poor function may be better than dialysis for 3 or 4 years, but the chance of survival of a damaged organ is not predictable. The decision not to use a potential donor kidney is difficult, and rules for predicting poor function in the recipient need to be clarified. The discarding of a suboptimal kidney is not the challenge; the ability to take suboptimal kidneys and get them to work properly is the real challenge.

To minimize renal injury in the cadaver donor, clinicians must optimize fluid status within considerations for other organs, maintain perfusion with minimal pressors, and minimize delay time before organ removal. The influences that accompany brain death may be reduced by careful donor management. Management of the donor may reduce DGF. Central pressure monitoring, aggressive fluid replacement, administration of albumin, and minimizing vasopressor influences and nephrotoxic drugs in the donor may minimize tissue injury. Nephrotoxins such as angiography dye should be avoided if possible. A recent study showing the toxicity of hydroxyethyl starch as a volume expander in brain-dead kidney transplants indicates that this agent should be avoided (90). (Hydroxyethyl starch in the flush fluid is apparently not toxic; it is a component of the UW preservation fluid widely used for kidneys.)

B. Use of Renal Dose Dopamine in the Cadaver Donor

In managing the cadaver donor after brain death, as in other patients with severe illness in intensive care, it has been popular to use so-called "renal dose" dopamine. Recently, investigators have questioned the concept of renal dose dopamine (91): they conclude that "important questions remain about the role of low-dose dopamine in critically ill patients. Until efficacy and safety are shown in controlled prospective studies, the use of dopamine as a renal sparing agent cannot be recommended. The modest improvement in urine output may come at too great a cost."

Arachidonic acid derivatives may have several roles in regulating the vasomotor and inflammatory events in transplant injury. There may be benefits from inhibitors of arachadonic acid metabolism (furegrelate, diethycarbamazine). In studies of dog kidneys, these agents were associated with lower serum creatinine in kidneys stored for 4 days in UW solution (92). Despite extensive literature on the role of eicosanoids, the enthusiasm for the development and testing of such agents seems to have waned.

Finally, lidocaine treatment of the donor has been recommended as having a potential effect in preventing DGF (93).

C. Solutions for Flush and Cold Storage: Collins and Eurocollins

Simple cold storage (CS), a primitive procedure, remains the backbone of organ preservation. The essence of organ preservation is cold storage at 4°C in a solution of electrolytes. The tradition has been to use solutions with a high potassium content because the Na/K-ATPase mechanism is inoperative in cold cells with no oxygen source, with added osmotic agents to prevent cell swelling. Attempts to preserve organs by freezing have not been successful.

Collins et al. (94) demonstrated the value of magnesium in the flush solution. Collins and Halasz demonstrated 48-hour canine kidney preservation and again emphasized the importance of magnesium. Collins et al. (94) showed advantages of magnesium in the flush solution in a cooperative trial in human kidney donors. Alternate donors were flushed with either magnesium-free Eurocollins solution or magnesium-containing Collins II solution. There was a significantly lower dialysis requirement when Collins II solution was used (33% versus 54%).

D. Efforts to Reduce the Effect of Prolonged Cold Storage or Pulsatile Perfusion Time

Cold storage time has a deleterious effect on renal quality but the effect is small per hour. In general, preservation time predicts initial kidney function poorly, and total CS time should be kept low but it should not become the overriding consideration. The prevention of cell swelling during preservation has been the subject of many studies. Coffey and Andrews did extensive studies on the value of sucrose as an osmotic agent in preserving function and ultrastructure during renal ischemia (95–97). (They also demonstrated the value of magnesium ATP [97].) Andrews and Bates showed the benefits of adding sucrose or mannitol during the flush (98).

E. University of Wisconsin Solution

For a comparison of common fluids, see Table 5. The development of UW flush solution at the University of Wisconsin has improved the preservation of liver and pancreas, and may have also helped to improve renal preservation. In some centers, UW flush is followed by machine pulsatile perfusion, but it is used for cold storage at 0°C to 5°C. The agents lactobionic acid, raffinose, and hydroxyethyl starch were included in UW solution to prevent cell swelling (85). The UW also includes an antioxidant (glutathione) to reduce oxygen-based reperfusion injury and adenosine to increase high-energy phosphate generation in the reperfusion and recovery periods. (Some concern has been expressed that glutathione is spontaneously oxidized in UW solutions, probably rendering it ineffective [99].)

Ploeg et al. in 1992 (100) demonstrated that UW improved graft survival by about 6% in a study of renal transplants. Other investigators have generally confirmed the benefits of UW in renal preservation (101,102), and other studies have confirmed benefit in dogs (103). Ploeg (104) and Barber et al. (105) have demonstrated excellent early function combining pulsatile perfusion with UW solution flush, demonstrating 95% immediate function. Howden et al. (106) have suggested some modifications to UW solution, including the elimination of hydroxyethyl starch and lowered potassium content. Not all investigators have found

Table 5 Composition of Some Flush-Cold Storage Solutions

Substance	Collins C2 Solution (86)	Eurocollins Solution (86)	UW Cold Storage Solution (24)
Lactobionate	—	—	100 mmol
Hydroxyethl starch	—	—	50g
Raffinose	—	—	30 mmol
Adenosine	—	—	5
Allopurinol	—	—	1
$H_2PO_4^-$	15	15	25 mmol
HPO_4^{2-}	42.5	42.5	—
Mg^{2+}	30	—	5
K^+	115	115	120
Na^+	10	10	30
SO_4^{2-}	30	—	5
Glucose	140	194	—
Osmolality (mOsmol/Kg)	360	375	320

that UW solution was advantageous compared to Eurocollins, as concluded by Hefty et al. (107) in a study of paired cadaver donor kidneys. Nevertheless, the population studies are convincing (108).

F. Pulsatile Perfusion

A lively debate has existed over the value of pulsatile perfusion versus simple flush and cold storage. One of the proponents of pulsatile perfusion, the late Dr. F. Belzer, had extensive success with renal preservation (84,109–111). However, Collins and others showed successful long-term preservation without pulsatile perfusion machines. Opelz and Terasaki (112) demonstrated a statistical association of improved results using cold storage versus using machine pulsatile perfusion in an early analysis of the UCLA registry. Randomized trials of cold storage versus pulsatile perfusion have sometimes shown a mild advantage with pulsatile perfusion (85). However, this advantage was not associated with any change in an important long-term outcome parameter, leading to the conclusion that pulsatile perfusion was probably not worth the extra investment. Rosenthal et al. (113) concluded that there were no important differences in outcomes between kidneys preserved with cold storage versus pulsatile perfusion, and recent population data agrees (81).

When it was demonstrated that pulsatile perfusion can harm kidneys under some circumstances (114–116), the value of the extra money devoted to pulsatile perfusion versus simple cold storage was questioned. The injury was probably mediated by poor perfusion methods, and soon ceased to be a problem in the centers that still use pulsatile perfusion. The current status of pulsatile perfusion is that it persists in a minority of centers, some of which, like Wisconsin, remain staunch advocates and report excellent initial function rates and comfort with longer preservation times. However, most centers have opted for the convenience of simple cold storage.

G. Strategies to Prevent Reperfusion Reaction

Many investigators have explored the usefulness of either SOD (a free radical scavenger) or allopurinol (an inhibitor of xanthine oxidase) for preventing free radical damage in the reperfusion phase. Raytch and Bulkley (117) demonstrated that more than half of so-called "ischemic injury" could be ablated by SOD and allopurinol. Hoshino et al. (118) showed improvement in renal function in pig kidneys from non–heart-beating donors with the use of SOD and allopurinol. Several laboratories (119–121) have explored the use of oxygen free radical scavengers in renal preservation, with some evidence of benefit. Bennett et al. (122) showed a beneficial effect of SOD in perfused kidneys, and, recently, benefits of SOD in human transplantation have been reported (123), although this result needs confirmation in a larger study. SOD or polyethylene glycol–linked SOD also protect against warm ischemia in the rat (124). Overall, the role of SOD and of allopurinol in reperfusion has been suggested but not proven to deliver benefits in a context of clinical kidney transplantation. One difficulty is the apparent lack of interest of the pharmaceutical industry in bringing SOD and related strategies to phase III trials.

The pathogenesis of ischemia reperfusion injury involves polymorphs adhering to the endothelium (125). Accordingly, strategies involving selectins, integrins (LFA-1, CD11a/CD18), ICAM-1(CD54), and chemokines may be advantageous. The ICAM-1–deficient mice are protected against renal ischemia (126). P selectin may mediate intestinal ischemic injury by enhancing complement deposition (127). Studies in the rabbit ear suggested an

effect of anti CD18 in preventing reperfusion injury (128), and reduced injury was seen with either immediate or delayed administration. Studies in lung reperfusion injury showed an effect of a monoclonal antibody against chemokine IL-8 in preventing lung reperfusion injury in rabbits (129). The use of anti-LFA-1 or of anti-ICAM-1 to reduce DGF is undergoing evaluation in animal models and in humans (130–132).

Among the other agents with potential protective value against ischemia or reperfusion injury is a C1 esterase inhibitor (133).

H. Recipient Management to Reduce Renal Injury

Luciani et al. (134) have shown the value of fluid loading in the recipient with the monitoring of pulmonary arterial pressure during the surgical period. Many investigators have shown that aggressive hydration and monitoring of central pressures, combined with albumin, were associated with improvements in the rates of DGF (134–138). Several groups (137,139,140) have recommended aggressive use of albumin, mannitol, and hydration, and have shown that intraoperative albumin is associated with improved outcomes in cadaver donor transplantation. Taylor et al. (141) have also advocated the value of aggressive intraoperative management. Mannitol has also been recommended, but whereas Weimer et al. (142) showed some effect of mannitol, there was no substantial change in the rate of transplant ATN.

Many investigators have suggested that calcium channel blockers given to the recipient or donor or both can help to prevent transplant ATN. Ferguson et al. (143) showed in a retrospective study an association between the use of calcium channel blockers and the prevention of transplant DGF. Oppenheimer et al. (144) also showed some value of calcium channel blockers in preventing DGF. Neumayer and Wagner (145,146) showed a reduction in DGF using diltiazem.

Trifluoperazine, or TFP, has been recommended for addition to preservation solution because of its action as a calmodulin inhibitor, potentially preventing the effects of high intracellular calcium on injured tissues (147). Other studies of trifluoperazine in dog kidneys showing a protective effect have also been reported (147,148). There is a possibility that calcineurin could mediate some forms a calcium-induced cellular injury, because the inhibitors CsA and FK506 prevent some experimental cell injury in brain (149), but this suggestion is not yet borne out in studies in kidney.

In mice, renal ischemic injury can be reduced by the use of N-acetylcysteine (R. Safirstein, personal communication), perhaps via inhibition of protein kinases. The use of N-acetylcysteine in organ preservation fluid has been discussed (150).

I. Interventions with Growth Factors

The expression of growth factors changes during recovery from renal injury (reviewed in 151). The rationale for the use of growth factors such as insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) is that these factors stimulate and, for HGF and EGF, are mitogenic for renal epithelial cells in vitro and are essential for growth of metanephric kidney in vitro. Levels of ppEGF mRNA decrease precipitously in acute renal failure, suggesting a deficiency state, but some evidence suggests that the production of mature product is temporarily enhanced. A protective role for TGF-β in myocardial ischemia has also been suggested (152).

The use of growth factors to resuscitate injured renal tissue is in its infancy but has considerable promise. The principal candidates have been EGF, HGF, and IGF-1 (151).

Expression of EGF is rapidly lost in ischemic injury (153). Epidermal growth factor can accelerate recovery from ischemic ATN (154,155) and from mercuric chloride–induced ATN (156). However, it has been disappointing in pig ATN (157). Hepatocyte growth factor has also shown some promise in models of rat renal injury (158).

A leading candidate for restoring injured kidneys is IGF-1, which is vasodilatory and enhances glomerular filtration, stimulates DNA synthesis in proximal tubule cells, and is anabolic (158–160). The IGF-1 enhances recovery from ischemic ATN (161), even when given up to 24 hours after surgery. We have shown that IGF given to mice reduces the injury response when given 2 hours after ischemia-reperfusion, probably by reducing the degree of injury (162). Recombinant human IGF-1 (rhIGF-1) has been evaluated in trials of acute renal failure in ATN in normal kidneys and has reportedly not been effective in the protocol used, in which administration was often long delayed after the onset of the injury. The role of rhIGF-1 in renal transplantation is uncertain. Insulin-like growth factor-1 has also been suggested to have some value in myocardial ischemic injury (163).

Thyroxine has been suggested as a renal growth factor, possibly by inducing production of mature EGF from its precursor (151). There are probably other candidates and combinations of candidate growth factors that could be explored in this setting.

J. Interventions Directed at the Inflammatory Response

If the injury-induced inflammatory response is an important player in the outcome of transplants, then it may be efficacious for preventing or treating this response. Some intervention strategies are outlined in Figure 3. Prevention of injury may be accomplished in many ways. Better donor management as well as new measures to reduce reperfusion injury, such as antibodies against C-C chemokines (e.g., RANTES) and adhesion molecules (e.g., LFA-1 and anti-ICAM-1) have been considered. Effective immunosuppression to minimize

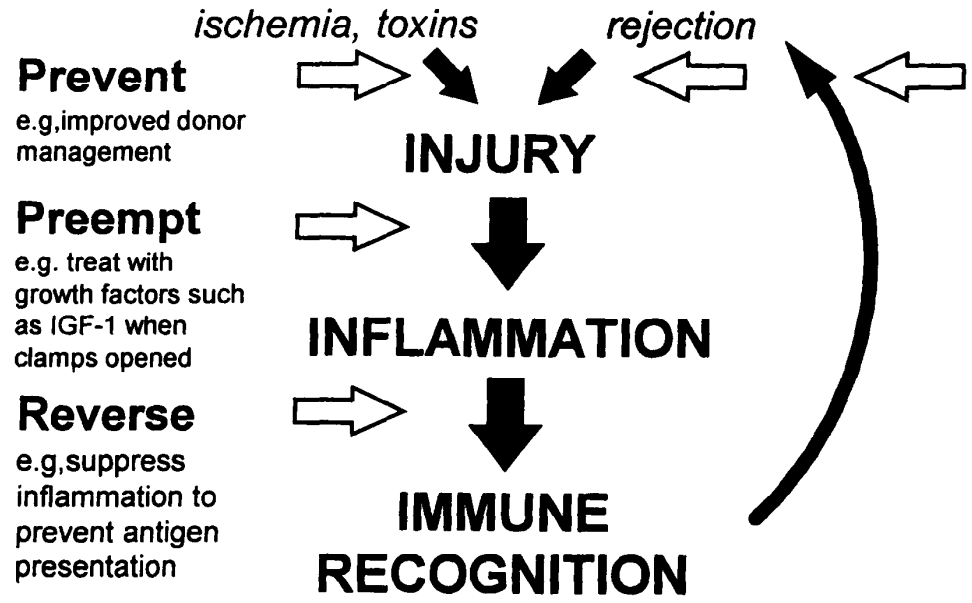

Figure 3 Opportunities for clinical intervention in the injury response include prevention by reducing ischemic injury and rejection injury, preemption by treating with growth factors, which reduce the effect of ischemia, and reversal, by suppressing injury-induced inflammation before it can lead to rejection.

acute rejection should also pay off in reducing injury and injury responses. Preemption of the injury response by intervening after the injury is a novel strategy that should be explored. For example, we have used rhIGF-1 after ischemia and 2 hours of reperfusion to reverse epithelial injury and reduce the injury response in a mouse model (162). Directly interrupting the development of the injury response after the injury has appeal because it would avoid the risk of rejection and new injury. Suppression of the established injury response by antiinflammatory strategies may also work. Some of the existing agents may work in this regard; for example, CsA can reduce some features of the injury response in mice. Perhaps some of the activity that has been termed immunosuppressive is, in fact, acting on the injury response not the immune response.

Thus, it is time to consider a larger view of the inflammatory processes in transplanted tissue. The concept that there are two highly structured processes, the immune response and the injury response, may be useful in directing research toward both better understanding and effective intervention in transplantation.

VII. CONCLUSION

Nonspecific injury to the kidney is a major determinant of the fate of the kidney, including immediate and long-term function, acute rejection, chronic rejection, and graft survival. Injury is a powerful predictor of transplant outcome, and it interacts with acute rejection. Injury triggers a remarkably complex series of events that have been termed the "injury response," analogous to the highly structured events in the immune response. The injury response can influence the likelihood of a specific immune response. It is likely that this pattern of response is common to many forms of renal injury, possibly including rejection, and it is also likely that these changes increase the probability of acute rejection. Thus, the injury response, with its chemokine, cytokine, and inflammatory elements, forms a potential self-perpetuating cycle that could contribute to progressive renal injury, including recurrent rejection. This, in turn, determines the baseline function that programs long-term survival.

Reduction of renal injury associated with transplantation represents a mandate for intervention to improve transplant outcomes. The potential lessons could have some general significance in understanding the more general rules governing the initiation of the immune response. The result should be an improvement in the quality of stable organ function (e.g., at 6–12 months) and a corresponding increase in long-term graft survival.

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7

Mechanism of Chronic Rejection

LEENDERT C. PAUL and NEIL FERGUSON
University of Toronto at St. Michael's Hospital, Toronto, Ontario, Canada

KHALID AL-FAKEEH
University of Toronto and Hospital for Sick Children, Toronto, Ontario, Canada

HALLGRIMUR BENEDIKTSSON
University of Calgary and Foothills Hospital, Calgary, Alberta, Canada

I. INTRODUCTION

Since the inception of kidney transplant programs, the short-term patient and graft survival results have progressively improved, principally because of advancements in organ preservation, better management of acute rejection episodes, and decreased mortality from infectious complications. In contrast, patient mortality and graft attrition rate after the first year has not changed significantly (1). One-half to three-fourths of graft losses after the first year are accounted for by patient deaths, whereas chronic rejection accounts for most graft failures when non–renal-related deaths are censored from the analysis. In this chapter, the risk factors and manifestations of chronic rejection are reviewed, followed by an overview of the immunological and nonimmunological mechanisms involved in its pathogenesis.

II. RISK FACTORS

Renal transplant registries have shown that human leukocyte antigen (HLA) mismatching, acute rejection episodes, and primary graft nonfunction correlate with graft loss in the early posttransplantation months as well as later on (2,3). Long-term graft survival studies often use the so-called half-life concept, the time it takes after the first posttransplantion year for

half of the grafts to fail. Although this approach allows study of long-term survival regardless of early loss, it is important to realize that many half-life analyses are based on projected survival data based on a limited follow-up period and that the rate of late graft loss is assumed to be linear, which is probably incorrect (4).

Transplants from HLA-identical sibling donors have a half-life of more than 20 years compared with less than 12 years for grafts from a one haplotype-matched sibling donor and 7 to 8 years for a completely mismatched cadaveric graft (5). Analyses of the Eurotransplant database have confirmed that HLA-matching is associated with improved long-term survival; zero mismatched cadaveric grafts have a 79% 5-year survival rate and a half-life of 13.2 years compared with 51% and 7.8 years, respectively, for completely mismatched grafts (2). Similar results have been reported by the Collaborative Transplant Study Group (6). Because acute rejection episodes correlate strongly with chronic rejection (4,7–10), it is unclear whether the matching effect is a direct effect or whether it results from a decreased incidence of acute rejection episodes. The United Network of Organ Sharing (UNOS) data, for example, show no beneficial effect of HLA-matching on graft survival in patients who have experienced one or more acute rejection episodes (11).

A single rejection episode in the first year after transplantation reduces the average half-life of first transplants from 45 ± 11 years to 25 ± 8 years, whereas multiple rejection episodes or a first rejection episode after the first year decreases the half-life to 5 ± 11 and 3 ± 1 years, respectively (9). Other studies have corroborated and extended these observations and reported that the frequency and severity of acute rejection episodes are independent risk factors for chronic rejection (4,10). One recent study reported a correlation between the duration and intensity of acute rejection episodes, as reflected in the "area under the curve" of serum creatinine over time, and chronic rejection (12). A similar correlation has been reported in a rat chronic rejection model (13). However, it remains to be seen how this correlates with the histopathology of acute rejection, because a recent biopsy study of patients with clinically diagnosed acute rejection episodes has shown that the histological type of rejection rather than the clinical severity of rejection correlates with the medium-term prognosis: only acute vascular rejection episodes seem to correlate with a poor prognosis, whereas acute interstitial rejections do not influence the long-term outcome (14).

Low doses of maintenance cyclosporine medication have also been correlated with late graft dysfunction and chronic rejection in some studies (15) but not in others (16). Conversely, in vitro donor specific hyporesponsiveness in cellular immune assays (17) or intense immunosuppression regimens (18) are associated with freedom from chronic rejection.

Nonimmune risk factors associated with chronic rejection include delayed graft function (3,19), posttransplantation infections (20), hyperlipidemia (21), obesity (22), the use of kidneys from very young, very old, female, or black donors (23), posttransplantation proteinuria (10), or impaired graft function (24).

III. CLINICAL AND HISTOPATHOLOGICAL MANIFESTATIONS OF CHRONIC REJECTION

Chronic renal transplant rejection is characterized by a slow but progressive decline in glomerular filtration rate, often in association with proteinuria and arterial hypertension. It is usually diagnosed after the initial 3 or 6 posttransplantation months (25) and, contrary to the graft dysfunction of most acute rejection episodes, it does not respond to increased doses of steroids or other forms of immunosuppression (26,27).

The histopathological picture shows mild or moderate tissue infiltration with mononuclear cells in conjunction with variable structural lesions of the vessels, the glomeruli, and the tubulointerstitial compartment. Phenotypic analysis of graft-invading cells has shown that most of them react with antibodies against macrophages, T lymphocytes, plasma cells, or natural killer (NK) cells (28), and eosinophils may also be prominently present (29). The vascular lesions are in the early stages characterized by subendothelial accumulation of macrophages and lymphocytes (30), followed later by intimal thickening as a result of proliferation of smooth muscle–like cells and fibroblasts and net deposition of extracellular matrix proteins (Figs. 1 and 2). The peritubular capillaries may show thickening and multi-

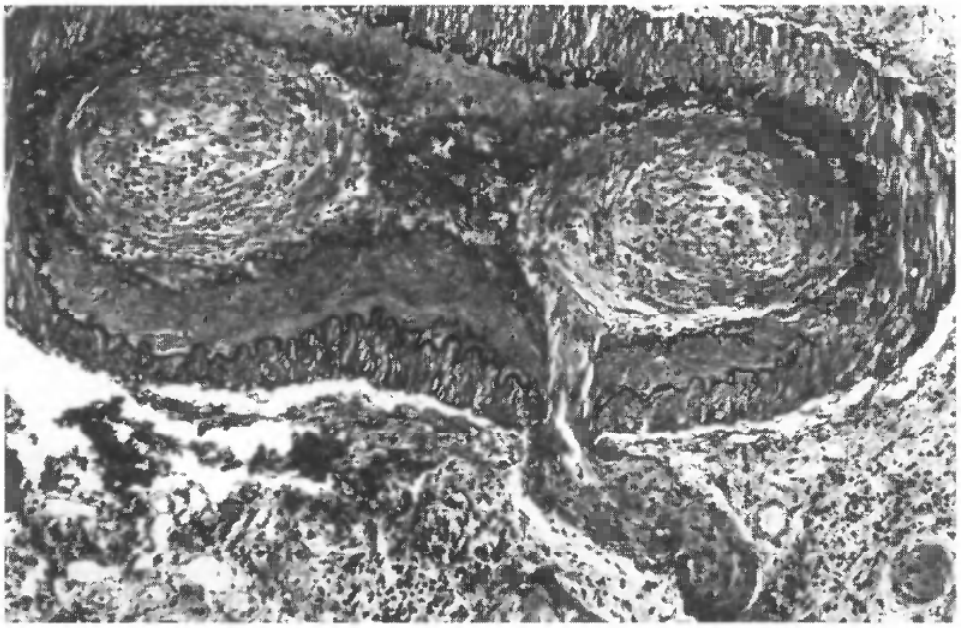

Figure 1 Photomicrograph of a vessel in a human renal transplant with chronic rejection. There is marked intimal thickening and an active inflammatory infiltrate in the intima, media, and the tissue surrounding the vessel.

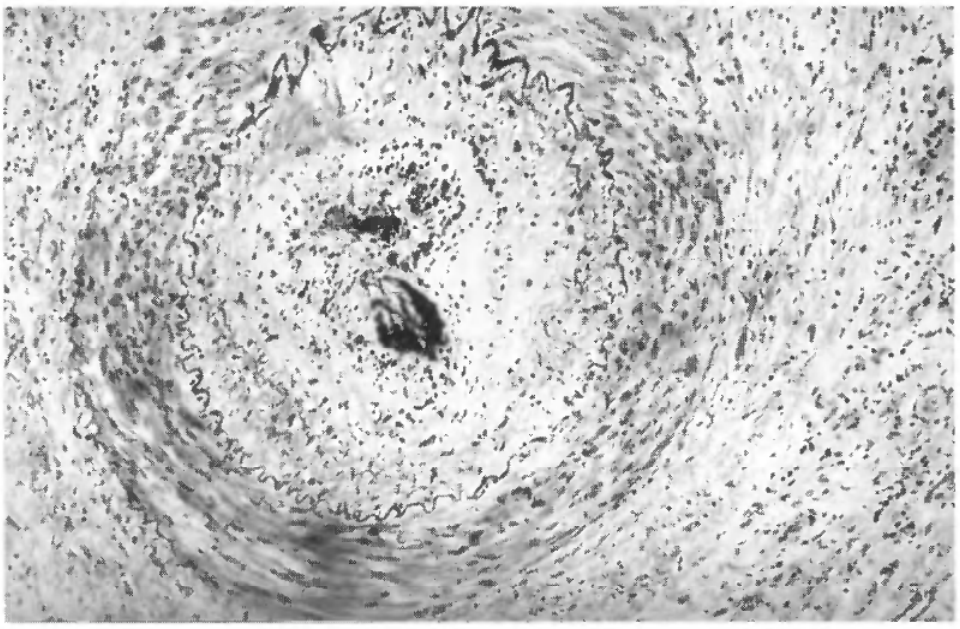

Figure 2 Photomicrograph of a vessel in a human renal transplant with chronic rejection. There is intimal thickening resulting in a significant luminal occlusion.

layering of their basement membrane (31,32), which is characteristic of chronic rejection. Interstitial fibrosis and tubular atrophy are almost always present, as in other forms of chronic renal inflammation.

The glomeruli usually show a variety of abnormalities, including wrinkling and collapse of the tuft (Fig. 3), hypertrophy (33), mesangial matrix expansion, or focal glomerulosclerosis (Fig. 4) (34,35). Hamburger et al. were the first to describe rejection glomeru-

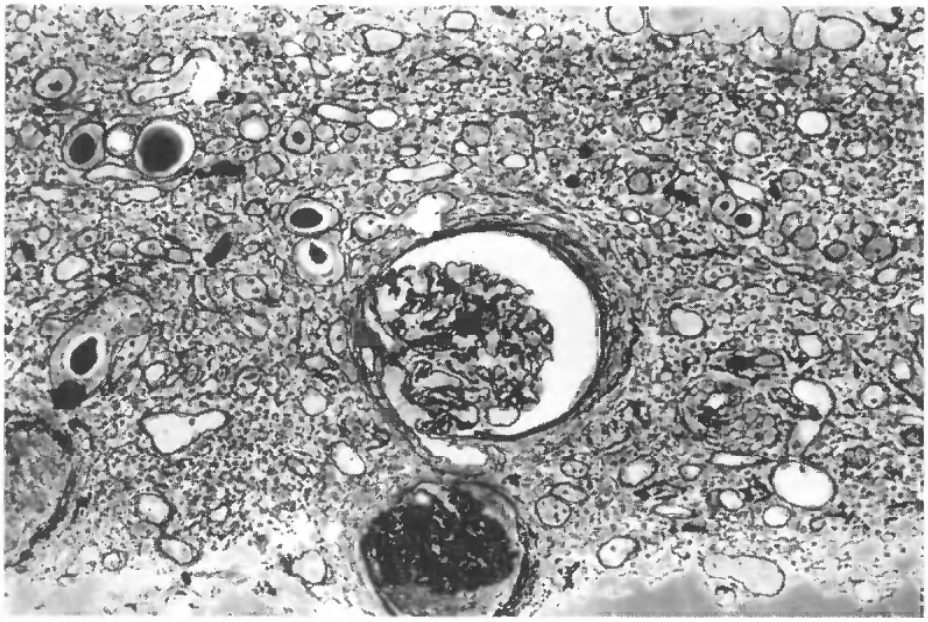

Figure 3 Photomicrograph of a glomerulus in a human renal transplant with chronic rejection. There is extensive interstitial fibrosis, tubular atrophy, and wrinkling and collapse of the glomerular tuft. The glomerular lesion is consistent with ischemia.

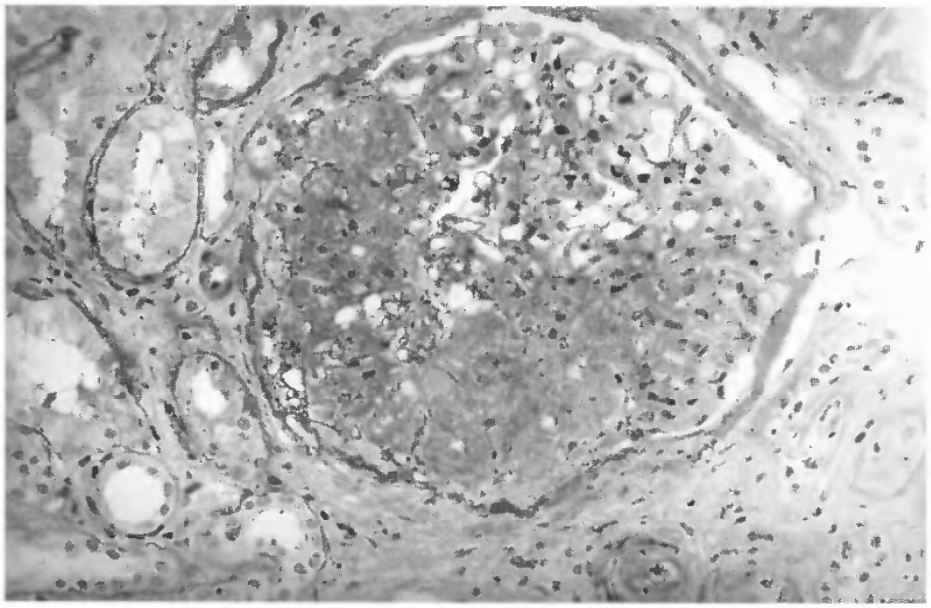

Figure 4 Photomicrograph of a glomerulus in a human renal transplant with chronic rejection. There is segmental glomerulosclerosis and hyaline deposition in the glomerular tuft.

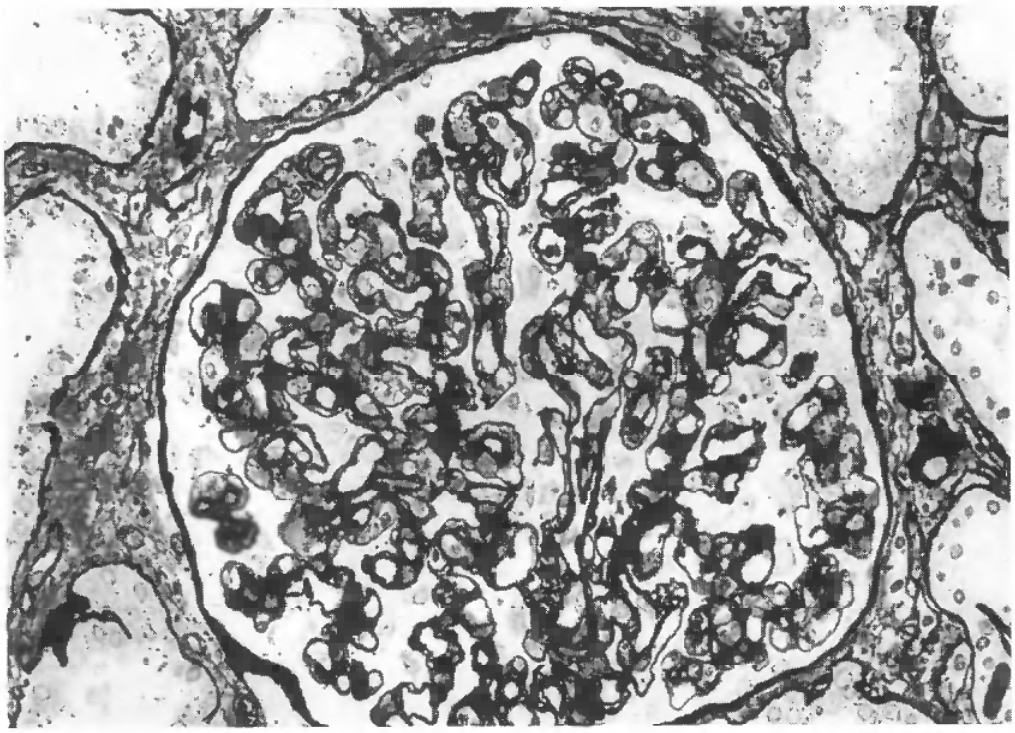

Figure 5 Photomicrograph of a glomerulus in a human renal transplant with chronic rejection. There is duplication of the glomerular basement membrane, characteristic of chronic rejection.

lonephritis (36), a lesion that has also been named rejection nephropathy, transplant glomerular disease, rejection transplant glomerulopathy, transplant glomerulopathy, or allograft glomerulopathy. Transplant glomerulopathy, like the splitting of the peritubular capillary basement membrane, is characteristic of chronic rejection and both lesions often occur together. The early lesions of transplant glomerulopathy are typified by glomerular enlargement, mesangiolysis, and glomerular hypercellularity; later, there is only mild hypercellularity, a spongy appearing expanded mesangial area containing swollen mesangial cells, and widespread reduplication of the glomerular basement membrane (Fig. 5) with peripheral deposition of collagen type IV and fibronectin (37,38).

The overall histopathological chronic rejection score, reflecting the extent and severity of lesions in all compartments of the graft, correlates with long-term function and prognosis (39,40), regardless of whether the tissue sample was obtained for the diagnostic workup of graft dysfunction or as part of a graft surveillance protocol without regard to clinical graft function (18,41).

IV. PATHOPHYSIOLOGY OF CHRONIC REJECTION: IMMUNE VERSUS NONIMMUNE MECHANISMS

Although the designation "rejection" implies the participation of immune mechanisms in the demise of graft structure and function, it has been difficult to define their role because other mechanisms such as infections or drug toxicity may cause similar lesions. Moreover, the protracted time course over which rejection evolves leaves ample opportunities for tissue repair mechanisms and physiological adaptations within the graft to develop in response to progressive damage and loss of renal mass, which further complicates attempts to understand its pathophysiology (42). Whereas several of the risk factors identified support the hypothesis that immunological mechanisms play a pivotal role, other risk factors

suggest that nonimmune factors are also involved. Inference of mechanisms is further complicated by the fact that different stimuli or forms of tissue damage result in very similar patterns of tissue activation, upregulation of cell adhesion molecules, influx of monocytes and macrophages, and local activation of a cytokine and growth factor cascade. For example, events as diverse as T-lymphocyte activation, chronic exposure to cyclosporine, or glomerular hypertension all result in enhanced production of transforming growth factor (TGF)-β (43–45), a polypeptide with widespread effects on cell proliferation, differentiation, activation, and extracellular matrix metabolism. Because the mediators involved in tissue responses and repair following various forms of injury are very similar, the question can be raised whether "chronic rejection" exists as a separate pathophysiological entity. This question remains unanswered.

The development of chronic rejection in allogeneic grafts over periods of weeks or months is a much more rapid process than is observed in syngeneic or isogeneic grafts, in which "rejection" occurs over a much longer time interval (46), suggesting a role for immune processes in allogeneic grafts. More tangible evidence to support this hypothesis comes from experiments that have demonstrated that pretransplant immunization with donor splenocytes accelerates chronic rejection (47), whereas manipulations aimed at the induction of donor-specific immune tolerance inhibit the process (48,49). Retransplantation of allogeneic kidney grafts back into the original donor strain prevents chronic rejection, if the retransplant is done within 3 months (50), suggesting that the initial stage of chronic rejection depends on allogeneic immune mechanisms. Finally, several of the more recently developed immunosuppressive drugs inhibit chronic rejection, although their effectiveness likely depends on their immunosuppressive as well as their antiproliferative effect on vascular smooth muscle cells. Thus, immune reactions seem to play a pivotal role in chronic rejection, although it has remained unresolved whether the critical immune reaction consists of a chronic, attenuated acute rejection reaction or whether chronic rejection-specific immune reactions play a role.

V. IMMUNE REACTIONS IN CHRONIC REJECTION

Recurrent episodes of reversible acute rejection may lead to chronic rejection. Acute rejection reactions are instigated by incompatible graft antigens and executed by cytotoxic T cells and macrophages and their products (51), although antibodies may be involved in some cases (52). $CD4^+$ cells play a central role in the regulation of the posttransplantation immune response because they interact with antigen-presenting cells, precursor cytotoxic T cells, macrophages, and B lymphocytes. Based on their cytokine profile secreted after long-term stimulation in vitro, two types of $CD4^+$ T lymphocytes, Th1 and Th2 cells, have been recognized (53,54). Murine Th1 $CD4^+$ cells produce interleukin-2 (IL-2), interferon gamma (IFN-γ) and tumor necrosis factor beta (TNF-β) as their predominant cytokines, and the Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. Although initial studies of human $CD4^+$ T cells failed to show the same degree of divergence of cytokine production as observed in murine clones, more recent experiments using antigen-specific clones have shown a greater degree of Th1/Th2 polarization (55). The Th1 clones, through their production of IFN-γ and TNF-β, are well suited to induce cytotoxic T-cell– and macrophage-mediated delayed type hypersensitivity reactions, whereas the Th2 clones make products that are well adapted to inhibit Th1-like responses and to act in helping B cells develop into IgG_1-producing cells (54).

The ontogeny of helper T cells is thought to consist of the following stages. Upon engagement of the T-cell receptor with the appropriate antigen, naive or pre-Th0 cells, cells

that secrete low levels of IL-2, develop into Th0 cells that can secrete additional cytokines such as IL-3, IL-4, IL-5, and IFN-γ. Upon full antigenic stimulation with the appropriate second signals, including cytokines and costimulatory molecules, Th0 cells differentiate into either Th1 or Th2 cells. The presence of IL-2, IFN-γ, and IL-12 and the absence of IL-4 and IL-10 favors the development of the Th1 phenotype, whereas the presence of IL-4 and IL-10 and the absence of IFN-γ favors the development of the Th2 phenotype (56). Therefore, cytokines secreted by one phenotypic subset of $CD4^+$ cells tend to promote newly activated T cells to develop into that subset while inhibiting the development of the other subset. Thus, once one subset becomes predominant, it tends to maintain its dominance and direct a developing immune response along its lines.

Th1 and Th2 cells can also develop in response to signals derived from the innate immune system (reviewed in 57). Activation of tissue macrophages through cell surface pattern receptors or by CD14 to which lipopolysaccharide (LPS), an endotoxin and cell activator (58) has bound, causes the secretion of IL-12 and TNF-α. Interleukin-12 induces the differentiation of naive Th0 cells to the Th1 phenotype through its ability to maximize IFN-γ and curtail IL-4 production by stimulated naive Th cells (59). Lipopolysaccharide also causes macrophages to produce IFN-γ–inducing factor, which may have similar effects (60). By virtue of their ability to take up antigen, macrophages may also bias the development of Th1 cells. T cells responding to antigen express CD40 ligand, which may cross-link CD40 on macrophages to stimulate secretion of IL-12 and TNF-α (61). These two cytokines synergize with IL-2 from T cells, or with IL-15 from the activated macrophages themselves, to induce production of IFN-γ by NK cells. Interferon-γ in turn, augments IL-12 secretion and activity through its capacity to activate transcriptionally both the inducible p40 component of the heterodimeric IL-12 protein in macrophages and the second component of the IL-12 receptor on T and NK cells. Thus, IFN-γ and IL-12 comprise an autocrine-positive feedback system that amplifies the levels of IFN-γ for macrophage activation and IL-12 for the proliferation and activation of NK and Th1 cells (62). Members of the C-C chemokine family produced by stimulated macrophages tend to recruit Th2-like responses.

Other factors that may bias the selection of Th1 versus Th2 include factors as diverse as antigen doses, cytokines produced by CD8 cells (63) or nonlymphoid renal cells (64,65), and the hormonal milieu (66).

It has been proposed that chronic rejection is the final outcome of recurrent acute rejection episodes, that is, Th1 orchestrated cytotoxic T-cell– and macrophage-mediated tissue damage (67). Recurrent episodes of acute rejection, each treated with immunosuppressive drugs, could persist as a chronic, subclinical smoldering process that leads to protracted tissue damage. Thus, chronic rejection may result from inadequately treated acute rejection episodes. This view is difficult to reconcile with the occasional development of chronic rejection in grafts that have never experienced an acute rejection episode, but it is of interest that protocol biopsies in patients with no graft dysfunction often show focal inflammatory lesions (41,68). It is conceivable that such lesions represent a population of Th1-like cells involved in localized and therefore subclinical tissue damage, which remains initially limited because of concomitant or subsequent local activation and expansion of Th2-like cells. Th2-like cells release cytokines which inhibit the generation and activation of proinflammatory T-cell responses as well as the release of IL-1, IL-6, and TNF-α by cells of the monocyte lineage, and thus limit the rejection process. Repeated subclinical rejection episodes could ultimately result in chronic tissue damage (67). Serial histopathological and immunopathological studies of chronic vascular rejection lesions have suggested that delayed type hypersensitivity reactions in the graft vessel wall constitute the main immune mechanisms underlying chronic vascular rejection (30). Immunohistochemical

and mRNA transcript studies have shown induced or enhanced expression of various cell adhesion molecules (69), cytokines, chemokines (70), and growth factors in such grafts, consistent with but in no way specific for delayed type hypersensitivity reactions.

An alternative but not mutually exclusive hypothesis is that the immune-regulatory mechanisms that come into action to prevent acute rejection result in chronic rejection. Inhibition of Th1-like responses may lead to an ascendency of a Th2-dominated response, which inhibits the Th1-like pathway but supports IgG_1 antibody production, and may result in antibody-mediated graft damage (71). Consistent with this hypothesis is the observation that the peripheral blood leukocytes of patients with chronic rejection have an intact mitogen-stimulated immunoglobulin production, whereas this response is impaired in patients with well-functioning, long-surviving grafts (72). Clinical pathological studies have shown a correlation between anti–class I–like or anti–endothelial cell antibodies and chronic vascular rejection (73–77), although such correlations do not prove causality. Most informative are studies in mice that have demonstrated that donor-specific antibodies are sufficient to produce chronic vascular rejection lesions in recipient animals with impaired T-cell immunity (78).

Recent mRNA studies of rat aortic transplants with graft atherosclerosis have shown upregulation of the immunoglobulin J chain mRNA (79). The immunoglobulin J chain is a small molecule that forms a disulfide bond with the penultimate cysteine residues of the immunoglobulin μ or α heavy chains, leading to the polymerization of soluble IgM and IgA molecules. Thus, the differential upregulation of the immunoglobulin J chain mRNA in chronic rejection suggests the presence of IgM- and/or IgA-producing plasma cells in such grafts. An experimental study of kidney transplants with chronic rejection and transplant glomerulopathy in rats tolerant to donor class I major histocompatibility complex (MHC) antigens has shown that such animals mount an IgM antibody response against endothelial cell–specific alloantigens (80). We found IgG antibodies against a novel class of glomerular and tubular basement membrane antigens in rats with chronic kidney graft rejection (81).

VI. NONIMMUNE MECHANISMS IN CHRONIC REJECTION

Syngeneic or isogeneic kidney transplants may develop histopathological lesions that resemble those of chronic rejection (46) but the time period over which such lesions develop is usually longer than in allogeneic grafts. Experiments have shown that retransplantation of allogeneic kidney transplants back into the original donor strain prevents chronic rejection if the retransplant is done within 3 months (50), suggesting that the initial stage of chronic rejection is dependent on allogeneic immune mechanisms. After 3 or more months, the progression of the lesions is independent of allogeneic immune reactions and becomes autonomous (50). In this section, the nature and potential impact of nonimmune mechanisms of tissue damage or activation on chronic kidney graft rejection are discussed.

A. Ischemia

Subjecting a kidney to mechanical manipulations or to an ischemic insult similar to that experienced during transplantation results in functional, morphological, and immunohistological changes that are comparable to those found in allografts with chronic rejection (82,83). Syngeneic grafts exposed to 60 minutes of ischemia develop long-standing or chronic vascular, glomerular, and interstitial lesions that resemble the lesions of chronic rejection. Similar degrees of ischemic exposure in allogeneic grafts results in more inter-

stitial inflammation, vascular intimal proliferation, and mesangial matrix increase than in syngeneic grafts, but it does not result in differences in the extent or degree of interstitial fibrosis or tubular atrophy (84). Similarly, ischemia alone is sufficient to stimulate intimal smooth muscle cell proliferation and macrophage influx in syngeneic aortic transplants in the rat, although the number of T lymphocytes in such grafts is less than in allografts (85). The early lesions of ischemic renal injury consist of discrete inflammatory changes and acute tubular necrosis, followed by increased cell division to replace the damaged tubular cells by new ones. These processes are accompanied by an increased expression of MHC class I and II molecules (86,87), predominantly in tubular cells for class I and in interstitial cells for class II, together with upregulation of mRNA transcripts of various proinflammatory cytokines, including IFN-γ, IL-2, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TGF-β (65). There is also production of arachidonic acid metabolites, which in turn upregulate the expression of the CD11/CD18 adhesion molecules on leukocytes, increase the expression of intracellular adhesion molecule (ICAM)-1 and MHC on endothelial cells, and enhance the avidity of CD11/CD18 for ICAM-1. As a result, there is increased adherence of leukocytes to endothelial cells, activation of leukocytes, and increased production of additional inflammatory mediators (88). Whereas perfusion of mildly ischemic kidneys with normal neutrophils or perfusion of nonischemic kidneys with activated neutrophils do not cause renal injury, perfusion of mildly ischemic kidneys with primed neutrophils causes severe renal injury (89). The role of cell adhesion molecules in this process is illustrated by the protective effect of anti–ICAM-1 monoclonal antibodies (90).

The upregulation of incompatible transplantation antigens, cell adhesion molecules, and cytokines may increase the immunogenicity and susceptibility of the transplanted kidney to allogeneic immune injury, regulate an ongoing immune response, and enhance tissue damage. However, the exact mechanism whereby ischemia enhances chronic rejection and late graft failure remains to be established. The severity of vascular and glomerular lesions of chronic rejection correlates with the duration of the perioperative ischemia period but not with the severity of the postoperative inflammatory infiltrate (84).

B. Renal Mass

Loss of renal mass results in a myriad of biochemical, hemodynamic, and tissue changes in the remaining nephrons, including glomerular hypertrophy (91), an increase in glomerular capillary blood flow and pressure (92), increased glomerular permeability characteristics (93), and an increased metabolic rate (94,95). Whereas many of these changes are regarded as beneficial adaptations in response to increased metabolic demands on the remaining nephrons, some of the changes, such as the increased glomerular hydrostatic pressure and the glomerular hypertrophy, are deleterious in the long term and result in progressive glomerulosclerosis (96,97).

Kidneys from normal individuals exhibit considerable variation in weight, cortical volume, and nephron number, depending on age, gender, and body weight or surface area. Males tend to have larger kidneys, with up to 20% more nephrons than females of the same body weight. Similarly, blacks and Asians are believed to possess fewer nephrons than whites per unit of body mass. Such variations could result in variable degrees of mismatching between the recipient metabolic demands and the graft renal mass available to deal with these physiological demands. A recent study has shown that the 5-year graft survival rate was significantly worse in recipients with a low graft weight over recipient body

mass ratio compared with combinations with a high ratio (98). It is conceivable that the substandard long-term survival of kidneys from very young, very old, female, or black donors (23) or kidneys transplanted into obese recipients (22) could, at least in part, be explained by a mismatch between graft size, maturity, or nephron number and recipient metabolic demands (23). In addition to intrinsic differences in nephron content in kidneys from different donors, many other transplant-related factors result in loss of renal mass, including ischemia-reperfusion injury, acute rejection episodes, hypertension, and drug nephrotoxicity. Work in rats has shown that less extensive chronic rejection lesions develop in kidney grafts when transplanted into recipients with a functioning native kidney or when two kidneys are transplanted rather than one (99,100). Micropuncture studies of kidney grafts with chronic rejection have confirmed that such transplants have glomerular hypertension (101, 102). Transplanted kidneys may be especially prone to development of glomerular hypertension because of the high prevalence of systemic hypertension in the transplant population, the tendency of transplanted kidneys to undergo compensatory adaptation as a solitary kidney, and the use of corticosteroid therapy (103). We have recently shown that treatment with antihypertensive drugs that also lower the glomerular hydrostatic pressure improves graft prognosis, decreases the amount of proteinuria, and inhibits graft mesangiosclerosis and vasculopathy (104). Clinical studies have shown that dietary protein restriction (105), an intervention that lowers the glomerular capillary pressure in experimental animals, or treatment with angiotensin-converting enzyme inhibitors improves the glomerular permselectivity characteristics in patients with chronic rejection, although it remains to be shown that it improves graft prognosis (106–109).

C. Hyperlipidemia

The influence of hyperlipidemia on the course of chronic kidney graft rejection remains controversial. Low-density lipoproteins are directly toxic to endothelial cells in vitro, and elevated cholesterol concentrations and hyperlipidemia foster vascular injury, atherosclerosis, and glomerulosclerosis (110). However, it has been difficult to unequivocally establish a role for hyperlipidemia in graft atherosclerosis. Alonso et al. were the first to show accelerated cardiac allograft atherosclerosis in cholesterol-fed rabbits (111), an observation confirmed by other investigators (112). Similar experiments in rats yielded controversial results: one study found enhanced graft atherosclerosis in cholesterol-fed rats (113), but this was not confirmed in other studies (114,115). Whereas hypercholesterolemia produces marked lipid deposition in areas of intimal thickening, morphometric analysis of the lesions failed to show a difference in the smooth muscle cell proliferative response compared with that in control animals fed a standard diet (114). More recent work has shown that a diet high in cholesterol, cholic acid, and glycerol leads to accelerated graft atherosclerosis (116).

One clinical study found a correlation between pretransplant hyperlipidemia and graft loss (21,117), but two other studies found no correlation (118,119). The authors of the former study also reported higher very low density lipoprotein, low density lipoprotein, total cholesterol, and triglyceride levels in patients with chronic vascular rejection compared with control subjects (120). Although many of these abnormalities may result from impaired graft function or proteinuria, the presence of similar abnormalities in cardiac transplant patients with graft atherosclerosis (121) suggests that the hyperlipidemia may play a pathogenetic role. This prospect is further supported by the presence of apolipoproteins A1, A2, and B1 in the graft vessel wall of transplants with chronic rejection (122).

Thus, clinical and experimental evidence supports the hypothesis that hyperlipidemia contributes to the pathogenesis of chronic rejection lesions, but their impact seems less pronounced than in nontransplant atherosclerosis. We postulate that the intensity of the vessel wall damage by immunological mechanisms obscures the impact of conventional atherogenic risk factors that usually take many years to become clinically detectable.

D. Immunosuppressive Drugs

The lack of protection against chronic rejection by currently used immunosuppressive drugs may be related to lack of efficacy in suppressing the relevant type of immune responses, or be due to side effects of the drugs that may result in lesions that resemble chronic rejection. Cyclosporine may cause focal interstitial inflammation, interstitial fibrosis, vascular lesions, and glomerulosclerosis commensurate with reduced glomerular filtration rate and renal plasma flow (123). Cyclosporine inhibits the calcineurin-dependent T-cell receptor signaling pathway that results in the transcription of a host of cytokine genes, including the genes for IL-2, IL-4, TNF-α, and IFN-γ, while it enhances the transcription and secretion of TGF-β in T cells (124), non–T-cell lines (125), proximal tubular cells (126), tubulointerstitial fibroblasts (126), and juxtaglomerular cells (44). Transforming growth factor β promotes the accumulation of extracellular matrix as it enhances matrix protein synthesis (127,128), diminishes the synthesis of matrix metalloproteinase, and augments production of specific inhibitors, including the tissue inhibitor of metalloproteinases (129–131). Transforming growth factor-β also enhances the synthesis of the mesangial type IV collagenase (132). Thus, although antigenic stimulation of T cells in the presence of cyclosporine does not lead to acute rejection, that is, proliferation and clonal expansion of antigen-reactive T cells, it enhances the secretion of the fibrogenic cytokine TGF-β, which could contribute to the fibrosis observed in grafts with chronic rejection. A recent retrospective clinical study has focused on the hypothesis that nephrotoxic side effects of cyclosporine have led to dosing regimens that are insufficient to prevent chronic rejection (15); it was suggested that cyclosporine nephrotoxicity is not very prominent and that the tendency to lower the drug doses may enhance the development of chronic rejection.

FK506, a macrolide lactone structurally unrelated to cyclosporine, exerts its immunosuppressive efficacy through inhibition of the same calcineurin pathway and shows a nephrotoxicity profile that is very similar to that of cyclosporine (133). Studies in a rat liver transplant model have shown that the intrahepatic expression of TGF-β_1 mRNA is higher in animals treated with FK506 than in control subjects (134), suggesting that FK506 also enhances TGF-β synthesis.

E. Infectious Complications

Cytomegalovirus (CMV) infection increases morbidity early after transplantation and may play a role in the pathophysiology of graft atherosclerosis. In heart transplant patients, graft atherosclerosis develops earlier and more frequently in patients who have had a CMV infection (135) although the epidemiological evidence has remained controversial (136). However, endomyocardial biopsy specimens from heart transplant patients with CMV have linked infection with immune activation and subendothelial inflammation (137). The presence of circulating endothelial cells in CMV infection further supports the hypothesis that it may cause endothelial cell damage (138). Moreover, experimental studies in rats have shown that early posttransplantation CMV infection accelerates chronic rejection (139,

140). There are no clinical renal transplant data that link CMV infection with chronic rejection, although "late acute rejection" episodes may be a manifestation of CMV infection (141).

F. Common Pathway of Tissue Injury and Remodeling

Both immune and nonimmune mechanisms of tissue injury may converge along a common, stereotypical pattern of "response to injury". Graft injury results in tissue damage and activation, with coincident local deposition of complement, inflammatory mediators, and cytokines (69). Activated endothelial cells may trigger the classical complement pathway and lose membrane complement regulatory proteins (142). Antibody formation may be enhanced in a nonspecific fashion, as demonstrated in burn patients in whom increased amounts of antibodies are produced by mitogen-stimulated lymphocytes in conjunction with signs of complement activation (143). In addition, platelets may accumulate on collagen that is exposed to the blood, and the clotting cascade may be activated, resulting in fibrin deposition.

Inflammatory mediators such as thromboxane, platelet-derived growth factor (PDGF), leukotrienes, and platelet-activating factors are released by injured endothelial cells, and circulating leukocytes enter the graft interstitium after adherence to the endothelium, a process mediated by various adhesion molecules on the leukocytes and the graft endothelium (69,144). Although leukocyte infiltration may be intense early after injury, mononuclear cells, primarily lymphocytes and macrophages, may persist with or without clinical evidence of graft dysfunction. These cells produce a host of cytokines and growth factors that contribute to vessel wall or mesangial cell proliferation as well as interstitial fibrosis (46,69).

Macrophages seem particularly important in the pathogenesis of chronic rejection. Subendothelial plaques, consisting of macrophages in foamy transformation, have been noted in human kidney transplants with chronic rejection (145). Similarly, macrophages of various phenotypes have been noted in biopsies of heart grafts in cholesterol-fed rabbits (146) as well as in rat heart and kidney models of chronic rejection (69,147,148). Organ-specific differences in graft-invading macrophage phenotypes have been found (147), although it is unclear whether this affects the different histopathological patterns of chronic rejection in various organ transplants. Upregulation of macrophage products, particularly IL-1, IL-6, TNF-α, MCP-1, b-FGF, TGF-β, and PDGF, coincides closely with the development of irreversible vascular, glomerular, and interstitial lesions (148). Adhesion molecules, primarily ICAM-1 and VCAM-1, are present on the vascular endothelium as well as on extravascular structures (149–151), and LFA-1 and VLA-4, the T-lymphocyte counter-receptors for these molecules, are upregulated on graft-infiltrating cells (69,148). Furthermore, administration of LPS accelerates chronic rejection (152).

Different graft compartments may exhibit quantitatively different responses to the same type of injury. Vascular obliterative lesions have been conceptualized as developing from repetitive episodes of endothelial cell injury, followed by intimal proliferation and repair, leading to gradual luminal narrowing (153). The glomerular lesions of chronic rejection may result from increased mesangial cell proliferation and deposition of extracellular matrix proteins, orchestrated by locally produced cytokines such as IL-6, TGF-β, or others (154,155).

Not much work has been done on the pathogenesis of chronic rejection-associated interstitial lesions. Phenotypically unique cell types have been described in the interstitium

of kidney transplants with chronic rejection in the rat (147,156), but it is unknown whether these cells are specific for chronic rejection and what role they may play.

VII. CHRONIC REJECTION-SPECIFIC IMMUNE MECHANISMS

The data thus far suggest that chronic rejection is initiated by a combination of acute rejection or, in some instances, other mechanisms of tissue injury, followed by activation of tissue repair mechanisms. In a number of patients, the tissue damage results in excessive proliferation of smooth muscle cells, fibroblasts, and deposition of extracellular matrix proteins in the vascular intima, the glomerular mesangium, and/or the interstitium. We propose that the tissue repair process in allogeneic organ grafts is quantitatively or qualitatively different from that in nontransplanted organs, because some of the mediator or regulator molecules involved may be immunogenic and induce an immune response that interferes in the tissue restoration process (Fig. 6). Such responses may be enhanced by tissue activation, perceived by the immune system as a danger signal (157,158), as well as the widespread induction of class II MHC antigens on graft parenchymal cells, which may serve as antigen-presenting cells (159).

We recently found an autoimmune response against proteolytic enzymes and the small interstitial proteoglycan family molecules biglycan and decorin, molecules that are released by mesangial cells in response to tissue damage in rats with chronic rejection. The latter molecules bind TGF-β and complement proteins and are involved in the formation of the extracellular matrix. It is conceivable that antibodies against such molecules result in decreased proteolytic activity and local excess of TGF-β, which in turn may enhance fibrosis (160,161).

Another group of candidate target molecules possibly involved in chronic rejection are heat shock or stress proteins. Heat shock proteins are a highly conserved group of proteins found in all organisms. Originally identified in cells exposed to sudden elevations in temperature (162), heat shock proteins are induced by a variety of stressful stimuli including inflammation, ischemia, infection and acute rejection episodes (163–166). They play a

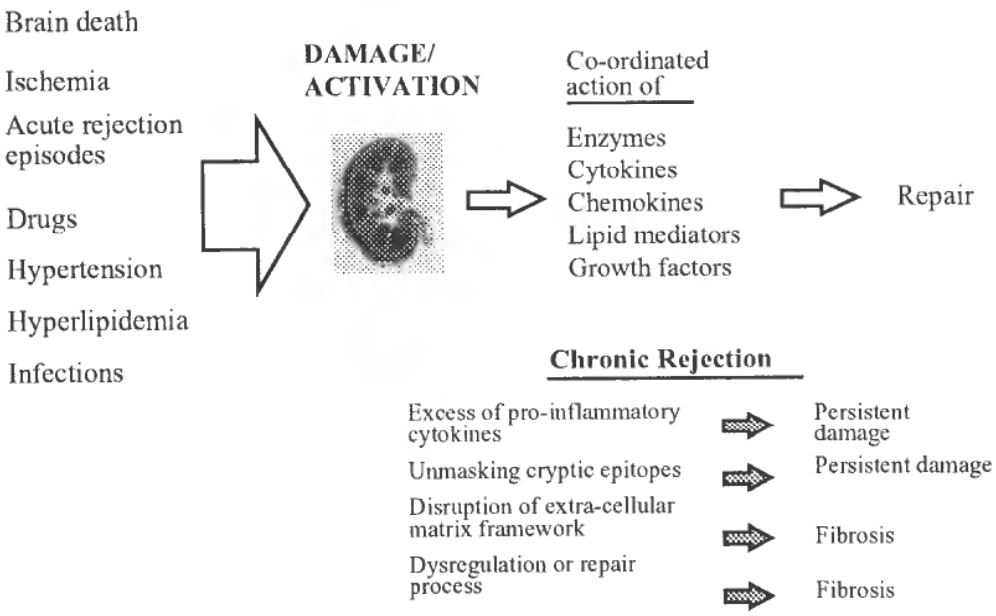

Figure 6 Proposed pathogenetic pathway to explain chronic transplant rejection.

vital role in maintaining cell function as molecular chaperones by mediating assembly, folding, and translocation of intracellular polypeptides, in protein degradation, and in interactions with various receptors (167,168). Heat shock proteins are immunogenic and have been suggested as potential targets for autoimmunity (169). Experimental studies have shown that acute rejection is accompanied by the induction of an immune response against heat shock proteins (164) and that such responses may result in further tissue damage (170). Although it remains to be established that such immunity is involved in chronic rejection, studies of nontransplant atherosclerosis have shown autoimmunity against heat shock proteins in this condition (170,171). It is therefore conceivable that immunity against molecules involved in the regulation of tissue repair processes and/or maintenance of tissue integrity is the central feature of chronic rejection.

Little attention has been given to the role of tissue factors in the development of chronic rejection. The extracellular tissue matrix is a complex three-dimensional framework whose organization and structure is important for the survival and function of cells imbedded in it. For example, adhesion of cells to their extracellular matrix is required for integrin-mediated prevention of programmed cell death (172–174). If an inflammatory response leads to disruption of the three dimensional matrix structure, as laid down during embryogenesis, the cells within the tissue undergo apoptosis, resulting in further disorganization of the tissue and progressive organ remodeling. Thus, it is conceivable that the progressive deterioration of structure and function in chronic rejection results in part from irreversible disruption of the supporting extracellular matrix structure.

VIII. CHRONIC REJECTION: HAVE RATIONAL TREATMENT OPTIONS EMERGED?

In 1992, we published an editorial under this title (27) and concluded that there is no rational treatment available to prevent or treat chronic rejection. In 1996, the clinical reality is not improved, although transplant registry data analysis suggests that FK506 improves the long-term outcome (175). Moreover, many of the new immunosuppressive drugs, currently in the preclinical stage or about to be tested clinically, may be effective in preventing chronic rejection in the clinical situation (176–184). It seems likely that their efficacy results from a combination of immunosuppressive efficacy and their ability to inhibit smooth muscle cell proliferation. A number of nonimmunosuppressive drugs such as recombinant superoxide dismutase administered at the time of transplantation (185), lipid-lowering drugs such as HMG-CoA reductase (186,187) or the somatostatin analogues (188) may provide adjunctive treatment strategies, while the role of monoclonal antibodies, fusion proteins, and novel peptides (189) remains to be determined.

IX. CONCLUSION

"Chronic rejection" is currently the main cause of graft failure after the first posttransplantation year. Its diagnosis has remained difficult because of the lack of specific tests and the insidious and protracted time period over which the lesions develop. Most chronic rejections are associated with previous acute rejection episodes, and several nonimmune factors such as ischemia, glomerular hypertension, hyperlipidemia, and perhaps infections enhance chronic rejection. We propose that chronic rejection results from an immune response against molecules produced by activated graft parenchymal cells in response to

injury and that such responses interfere with the normal tissue restoration process after injury (see Fig. 6).

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8

Xenotransplantation: Potential Opportunity Versus Resistant Challenge

WILLIAM E. BESCHORNER

Ximerex, Inc., and the University of Nebraska Medical Center, Omaha, Nebraska

THOMAS J. LOMIS

The John Wayne Cancer Institute, Santa Monica, California

I. INTRODUCTION

The development of human kidney transplantation is a fascinating study of problem definition and innovation. As the pathophysiology of complications such as acute tubular necrosis, rejection (hyperacute, acute, chronic), and infectious diseases was defined and understood, corrective and preventive actions were undertaken. Consequently, long-term survival of kidney allografts has become the norm. Transplantation not only enhances the quality of life for recipients but is also a cost-effective alternative to hemodialysis.

The success of kidney transplant survival, however, has created another problem, which so far has proved to be more challenging—a severe and growing shortage of human kidney organ donors. Whereas the number of kidney transplantations performed in the United States annually has plateaued at slightly more than 10,000, the number of high priority patients on the United Network of Organ Sharing (UNOS) waiting list is more than 50,000. If all potential renal candidates were considered, the shortage would be considerably greater.

The shortage of organs has inspired three responses. First, there are renewed efforts to find more human donors. These efforts have not, however, kept pace with the growing list of candidates. Second, artificial kidneys are under development. Because kidneys serve

multiple functions and are closely regulated, it is unknown whether a device can be developed to function as an effective long-term and cost-effective alternative. Third, intense interest has developed in the use of kidney xenografts from animals. Pigs have become a focus as renal xenograft donors because they have kidneys structurally similar to human kidneys and can be bred in large numbers.

If pig kidneys were transplanted into humans or Old World monkeys, they would be rejected within minutes by hyperacute rejection (HAR). Because of high titers of natural antibodies against pig cells, the grafts are destroyed by the binding of these antibodies to the endothelium and the subsequent fixation of complement. The predominance of natural antibodies is directed against a single epitope, and several strategies have been developed to avoid hyperacute rejection. Even when hyperacute rejection is prevented, however, vigorous acute rejection of the xenograft develops, destroying the xenograft within days to weeks.

The risks of infection with opportunistic organisms associated with xenografts differ from those associated with human allografts. There is no possibility of contracting human immunodeficiency virus (HIV) or hepatitis from a xenograft, as there is from human allografts. There is concern, however, regarding the risk of contracting infections from the animal host. Nonhuman primates can harbor latent viruses that could become a serious health risk when activated in the recipient. The risk is generally considered to be greater for nonhuman primate donors than for swine. The suggested guidelines for xenotransplants recently released by the Food and Drug Administration are devoted primarily to monitoring the patient and animal donor for potential infectious diseases.

Whether xenografts will resolve the shortage of organ donors will depend on the therapeutic ratio for the use of the xenografts as compared with alternative therapies. Although the organ shortage is probably best defined for kidney allografts, renal allografts would also require a greater benefit than transplants such as heart xenotransplants or liver xenotransplants, in which the life of the patient depends on the transplant. In general, most people currently consider xenografts to be inferior to human allografts. If that proves to be the case, then it would also be necessary to demonstrate a significant improvement over hemodialysis, and xenotransplants would be used in those recipients without human donors. If, on the other hand, xenografts prove to have significant advantages over human kidney allografts, then xenografts would become the transplant of choice.

To understand the potential problems with xenografts, it is useful to compare xenografts with allografts. To the extent that the xenograft resembles an allograft, the complications would be similar. Generally, xenografts from one primate species to another should function and be rejected in a manner similar to allografts. Surprisingly, however, pig xenografts also have similarities to primate tissues and are treated in a manner similar to allografts. For example, there is considerable homology between the major histocompatibility complex (MHC) of the pig tissues and human tissues. Indeed, there appears to be homology between some private epitopes of pig and human histocompatibility antigens.

On the other hand, xenografts also differ significantly from allografts, particularly for porcine xenografts. Numerous xenogeneic antigens are presented to the recipient immune system in a manner resembling foreign bodies. Furthermore, hosts sensitized to bacterial antigens could cross-react with some of these xenoantigens. There is the potential that some of the soluble regulatory factors or the corresponding receptors may be species specific, causing dysfunction of the graft within the recipient. Although porcine renal xenografts appear to function appropriately in primate recipients for a short term, it is unknown how the function is affected over the long term. Species-specific cytokines could also affect the

immune response to the xenograft or the development and maintenance of immune tolerance over the long term.

While focusing on the potential problems and potential solutions for xenotransplantation, however, the opportunities that come from the use of animal tissues should not be overlooked. If the use of animal tissues is efficacious, xenotransplants could satisfy the demand for kidney grafts. The use of animal donors could provide other advantages as well. For example, the transplants would be performed on an elective basis rather than an emergency basis. By harvesting the kidney at the time of transplant, cold ischemia time could be nearly eliminated. The animals can be bred, monitored, and treated under controlled conditions to minimize the risk of infectious diseases, and xenografts are naturally resistant to most serious human infections such as HIV and hepatitis. Furthermore, xenografts could be modified before transplantation. Genetically engineered pigs can be produced to decrease the risk of rejection. Inbred swine can be used, providing donors that are uniform. Hemopoietic chimerism can be induced within the animal donors with human or with the recipient patient's marrow for the induction of immune tolerance.

Because animals have not yet been used as a source of kidney xenografts for human recipients, these advantages must be considered potential rather than real. The principal obstacle to the use of kidney xenografts is their inevitable rejection. With most nonhuman primate and pig donors, the risk is considerably greater than with human allografts.

Although the obstacles to widespread xenotransplantation are substantial, the flexibility and opportunities that come from the use of animals is also substantial. With human allografts, there is relatively little that can be done with the donor beyond screening and selection. Most efforts at immune modulation have, therefore, been directed at the recipient. Generally, human recipients are older, chronically ill, or have latent or active infections. Therefore, they do not provide an ideal environment for either suppression or tolerance. In using animals, transplant conditions can be better controlled.

With unlimited possibilities in defining the ideal approach to induce xenogeneic tolerance, it seems appropriate to first design a set of immunological goals to be satisfied by the most ideal system. With this gold standard in mind, the current approaches are briefly reviewed in this chapter.

The story of xenotransplant is young, controversial, and exciting. It is far from being complete. To provide more insight, a number of preliminary studies and contradictory studies are discussed, and opinions regarding their significance are put forward, recognizing that we may be proven wrong on some of these points, and we may not give deserved credit to many of the innovations that will prove to be critical.

II. HISTORY

Possibly the first to view xenotransplantation as more of an opportunity than a problem was Daedelus (1). He engrafted wings of feathers and wax to his arms, providing endless flight. His son, Icarus, was perhaps the first to discover the limitations of xenotransplants, experiencing tragic graft failure when he approached the sun.

The first historical efforts for engrafting animal tissues into humans appeared in the mid-17th Century with the transfusion of animal blood, typically from lambs, into humans (2). A documented account describes a patient of Jean Baptiste Deny who received a transfusion of lamb blood. By historical account, the patient survived the transfusion, however, he "pist a large glass of black urine that you would have said it has been mixed with soot"

(3). The clinical significance of this early observation of the phenomenon of immune-mediated hemolysis was at the time not appreciated but would serve as an omen of future difficulties. In the early 19th century, James Blundell, an obstetrician, demonstrated the incompatibility of heterologous blood transfusions through repeated animal experiments in which dogs were transfused with blood of sheep (2). This finding was further verified by a report from Henry Gradle, who described an attempt at heterologous blood transfusion by injecting lamb blood into a tubercular patient, which resulted in a severe transfusion reaction. Thus, by the late 19th century, heterologous transfusions fell into disfavor and attempts at xenotransplants would await renewed interests generated by advancement in vascular surgical techniques (3).

In the early 20th century, Australian surgeon Edward Payr's demonstration of the first workable method of vascular suturing led to widespread interest in solid organ transplantation and renewed attempts at xenotransplantation. In 1902, the first successful organ engraftment was carried out by Emerich Ullmann at the Vienna Medical School (4). His paper described the autotransplantation of a canine kidney from its normal location to the vessels of the neck. The autotransplanted kidney produced urine and was demonstrated to the Vienna Medical Society on March 1, 1902. The successful result of this autotransplant stimulated several efforts at xenotransplantation over the ensuing years. In 1905, Princeteau inserted slices of rabbit kidney into a nephrotomy on a child with renal insufficiency (5). The report of this attempt described "excellent immediate results . . . the volume of urine increased; vomiting stopped. . . . On the 16th day the child died of pulmonary congestion."

In 1906, solid organ xenotransplantation was attempted using vascular anastomoses when Jaboulay reported transplantation of two renal xenograft into the antecubital space of man (6). One of the transplanted organs was from a pig and another from a goat. Neither graft functioned, and it was speculated that the failure was due to vascular thromboses. The first attempt at renal transplantation from a nonhuman primate into a man was reported by Unger in 1910 (7). The patient survived only 32 hours after transplantation and venous thrombosis of the graft was demonstrated at autopsy.

The trend of xenotransplantation failures attributing to venous thromboses was broken in 1923 when Neuhof reported an attempt to treat mercury bichloride poisoning with a renal xenograft (8). Because a human kidney was not available, Neuhof transplanted a lamb kidney into the patient. Even though the patient died 9 days later, autopsy did not demonstrate hemorrhage or thrombosis at the anastomosis. Neuhof was encouraged by this finding and wrote that it should renew interest in xenotransplantation. However, significant efforts at renal xenograft would await definition of the immunological basis of rejection and the development of immunosuppressive drugs and techniques.

During the period when Neuhof performed his renal xenograft, an often omitted chapter in the history of xenotransplantation was occurring. During this era, many false reports of successful xenografting of various tissues appeared in both the media and scientific literature. Many of these reports related to the xenografting of monkey testicles into aging men in an effort to restore youth and reverse declining health status. Even though the events of this period did much to discredit organ transplantation, knowledge of these historical anecdotes serves to caution against misuse of the scientific method and the premature acceptance of new techniques. An interesting account of this period is described in *The Monkey Gland Affairs* written by David Hamilton (Chatto and Windus, London, 1978).

The potential importance of immune rejection has been recognized since the beginning of the 20th century (9). Ehrlich and Morgenroth recognized that the injection of red cells from another individual led to hemolysis, whereas the injection of one's own cells did

not. They established the concept of "horror autotoxicus" to note that the immune system must ignore the host's own tissues.

Insight into the mechanism of distinguishing self from non-self came 50 years later. Ray Owen observed that cattle litter mates were tolerant to each other. He related the tolerance to the cross-circulation of blood through a shared placenta. Burnet speculated that tolerance was induced within the developmental environment. Hašek along with Billingham, Brent, and Medawar provided the key experimental support for these ideas (10,11). The process was accomplished by intravenously infusing donor splenocytes into immature mice immediately after birth. When the inoculated recipients matured, they were capable of accepting skin and other tissue transplants from the donor mouse strain but continued to reject transplants from all other mouse strains. They termed this phenomenon neonatal tolerance. In other mammals, tolerance to self is known to develop in utero, during fetal development. Thus, the term developmental tolerance is probably more appropriate.

Efforts to induce specific immune tolerance to foreign grafts would have to wait, however. In the meantime, immune suppression was necessary to prevent rejection. The initial attempts at immune suppression for organ transplants used total body irradiation and had only limited success. Chemical immunosuppression was introduced when Dameshek and Schwartz used the anticancer drug 6-mercaptopurine (6-MP) to decrease the immune response to foreign protein in rabbits (12). This approach was soon applied with some success to canine renal allografts by Calne (13) and independently by Zukosti et al. (14). However, 6-MP was found to have only limited efficacy in the prevention of rejection when used in human renal allograft. Eventually, the combination of 6-MP with prednisone improved graft survival. Subsequently, the development of a derivative of 6-MP (known as azathioprine or Imuran) demonstrated increased efficacy and decreased toxicity when compared with 6-MP. The regular use of azathioprine in combination with prednisone became the standard regimen of immune suppression after reports by Goodwin et al. (15) and Starzl et al. (16). This advancement led to remarkably good results with renal allotransplantation and a renewed optimism for efforts of xenotransplantation augmented with immunosuppression. The interest was further sparked by a human organ shortage crisis, which became evident with the routine feasibility of renal allotransplantation in the era of immunosuppression.

In 1963, Claude Hitchcock engrafted a kidney xenograft from a baboon donor into a 65-year-old woman. It functioned for 4 days (17). Reemtsma et al. and Starzl et al., working independently, carried out several renal xenotransplantations using immunosuppressive regimens (18,19). Reemtsma used chimpanzee donors while Starzl used baboons as donors. Reemtsma reported immediate graft function in six of seven transplanted kidneys. However, despite high-dose immunosuppression, acute rejection occurred. Five of the patients died of sepsis related to the immunosuppressive regimen. One graft functioned normally for 6 months. Starzl reported similar results, with initial graft function in six baboon xenografts followed by vigorous rejection. The longest surviving xenograft in that series functioned for 60 days. The six patients in the series ultimately died. In most cases, the patients died from septic complications of high-dose immune suppression. In 1964, Hume et al. also reported the results of a single chimpanzee to human xenograft (20). The Hume group also reported good initial graft function with 60 L of urine output observed in the first 24 hours after transplantation. However, the patient died of an apparent cerebral hemorrhage shortly after the procedure.

In 1970, Calne defined the terms discordant and concordant xenotransplants, to distinguish those grafts (discordant) that would be destroyed by hyperacute rejection (21).

Until hyperacute rejection could be prevented, xenotransplants were engrafted with concordant donor animals.

The next attempt at a clinical xenotransplant occurred October 26, 1984, when Bailey transplanted a baboon heart into an infant girl with hypoplastic left heart syndrome (22). This became the widely discussed Baby Fae case. It was anticipated that the infant's immune system would be sufficiently immature for immune tolerance to develop. Improved immune suppression, such as with cyclosporine (CsA), might also contribute. The child survived 20 days before succumbing to graft failure.

Neonatal or developmental tolerance would appear to have limited utility clinically, because most transplant candidates are mature individuals. However, the principles of developmental tolerance could be applied to adult recipients. Long-term acceptance of pancreatic islet xenografts was achieved in a rodent model by Ildstad and Sachs in 1984 (23). The recipients were made severely immune deficient and then received a bone marrow transplant (BMT) consisting of both donor and recipient marrow. As the recipient reconstituted, tolerance to both cell populations was established.

A symposium devoted to xenotransplantation was held in 1989 to honor the 25th anniversary of Reemtsma's transplants (24). The symposium systematically outlined the potential utility and the potential problems for xenotransplantation. Thereafter, efforts intensified to make vascular xenografts a reality.

At this meeting, Alexandre reported a series of pig kidney to baboon xenotransplants (25). Hyperacute rejection was prevented with plasmapheresis and splenectomy. Acute rejection was delayed in the recipient by immunosuppression with cyclosporine. The porcine kidney xenografts survived and functioned for up to 22 days. This was the first indication that even with the prevention of hyperacute rejection of pig xenografts, acute rejection represented a formidable challenge.

In 1993, Starzl again initiated attempts at clinical xenotransplantation when his group performed two baboon to human hepatic transplants in patients suffering from hepatic failure secondary to hepatitis B infection. One of the patients demonstrated graft function for an initial 55 days, but the second patient never demonstrated adequate graft function. Both patients died of septic complications.

In two cases, acquired immunodeficiency syndrome (AIDS) patients have been given bone marrow transplants from baboons (26,27). Baboon marrow should be resistant to infection by HIV. In both cases, however, the baboon marrow failed to engraft within the human recipient. The first patient died of progressive disease, whereas the second recipient showed clinical improvement in the absence of long-term engraftment with baboon cells.

With the proposal for the second baboon to human BMT, the Food and Drug Administration held hearings regarding the risk of zoonotic infections with xenotransplantation. Concern was expressed about the risk not only to the recipient but to the population at large. The hearings led to the first suggested guidelines for xenotransplantation (28).

The first pig to human vascular organ xenotransplants involved the temporary use of pig livers for patients with fulminant hepatitis (29,30). Blood from the patient was perfused through one or more porcine livers to remove the toxins and maintain the patient until a human liver donor was found (31,32). Diabetic patients have been engrafted with fetal pig pancreatic islets with immune suppression (33,34). Porcine C-peptide could be detected in the urine of the patients for up to 400 days.

Probably the most successful long-term pig to human xenotransplant to date is the transplantation of porcine basal ganglia cells into a patient with Parkinson's disease (35). Because fetal pig tissue was transplanted into the brain, an immune privileged site, there

was no apparent rejection. The patient experienced considerable clinical improvement. Histologically, the porcine neurons showed growth and extension of the axons.

The very early studies were performed without a thorough understanding of the immune reaction to xenografts. Not surprisingly, the results were clinically unsatisfactory. In the modern period, with a better understanding of these mechanisms, clinically successful vascular xenografts are yet to be performed. However, the barriers are better understood and approaches to prevent rejection are being developed. There are also concerns about the function of xenografts and the risk of zoonotic infections arising from the grafts. The concerns have not been realized, although the data are limited, both in terms of duration of graft survival and numbers of recipients.

III. FUNCTION AND GROWTH OF ORGAN XENOGRAFTS

Assuming that the barriers of xenograft rejection can be overcome, will kidneys from other species function normally in human recipients? Porcine kidneys have been transplanted into baboons, using plasmapheresis and splenectomy to prevent hyperacute rejection and immune suppression to delay acute rejection. Three of five such transplants functioned normally, maintaining a normal serum creatinine until the kidneys were rejected (25). Similarly, a minipig kidney transplanted into a cynomolgus monkey kept the blood urea nitrogen and serum creatinine levels near normal until the graft underwent rejection (36).

Pigs are one of the few nonprimate species with kidneys that morphologically resemble human kidneys (37). As with humans, the porcine kidneys is nonlobulated, has multiple pyramids, and separate papilla. The pyramids are smaller, however, than in humans. The physiological makeup of porcine kidneys is similar to that of human kidneys, including the renal blood flow, similar glomerular filtration rate, and a similar capacity to concentrate solutes (38). As in humans, the pig kidney has other metabolic functions, including the metabolism of vitamin D, regulation of blood pressure, and the production of erythropoietin. It is unknown how well these functions will be maintained in the xenografts over the long term.

Normal outbred swine grow quickly and their weights can exceed a thousand pounds. Will a pig xenograft grow within the xenogeneic recipient and become too large? No firm data answer this question, although initial data suggest that xenografts grow according to the growth rate of the host, not the donor. Rhesus monkey heart grafts were placed within juvenile baboons and the recipients were followed up for up to 1 year. The xenografts grew in parallel with the host and stopped growing when the growth curve of the recipient reached a plateau (39).

Using surrogate tolerogenesis, aortic grafts from 2-week-old chimeric outbred pigs were transplanted into mature sheep. Three grafts were followed up for 75 to 88 days when they were rejected. The grafts were not exposed to immune suppressive agents during this time. Two grafts did not experience rejection in the early part of their course, and the third graft had a self-limited rejection episode. During this period, the pig donors would have grown from 10 pounds to approximately 70 pounds. The aortas would have been expected to grow significantly in length and diameter, yet the grafts showed no evidence of growth.

IV. ZOONOTIC INFECTIONS

The transplant recipient is at significant risk for contracting infectious diseases from the organ donor. This has been demonstrated for human allografts, with the documented transmission of herpes viruses (including cytomegalovirus [CMV] and Epstein-Barr virus

[EBV]), HIV, hepatitis, and *Toxoplasma gondii,* among others. Precautions are taken to minimize that risk. The risk for transmission of an infectious agent from an animal donor is just as significant. Furthermore, there is concern that endogenous agents such as retroviruses may behave in a benign manner in the animal donor but act in an aggressive manner within the human host. For an individual patient, the benefit of a life-saving organ transplant would usually outweigh the risk of a possible infection. If the potential zoonotic infection were contagious, however, the public health risk must also be factored into the overall risk.

In comparing the potential use of swine and primate organs, the greatest concern has been expressed for the use of nonhuman primates as xenograft donors (40). Whereas swine can be bred rapidly and can be raised under controlled conditions, the conditions for primates cannot be as well controlled. Several viruses that are relatively benign in primates have produced catastrophic results when they infect humans, such as the Marburg virus and the Ebola virus (41,42). Although these viruses have a high mortality rate in humans, human hosts are unable to sustain the amplification for prolonged periods of time (43). Proviral DNA for endogenous retroviruses *is* found in baboons and monkeys. When human cells are cocultured with baboon cells, the virus can be detected (44).

Swine also pose some risk for zoonotic infections, although most researchers consider it to be much less than with nonhuman primates. Multiple agents can potentially be passed from humans to pigs, which, in turn, could infect xenograft recipients. These include bacterial infections such as *Salmonella, Campylobacter,* and *Yersinia,* parasites such as schistosomiasis, and viruses such as influenza (45). Swine may also carry herpes viruses, including a swine cytomegalovirus. High standards of animal husbandry, however, have made these entities more theoretical than actual. Ye et al. reported a thorough necropsy examination of 10 pigs that included 150 tests. The stool contained some parasites considered to be commensals but no agents pathogenic for humans were identified (46).

Transformed porcine cell lines produce C-type endogenous retroviruses. When two such cell lines, PK-15 and MPK, were cocultured with human cell lines, porcine virus could be identified within the human cells (47). Although the study raises the possibility of infective porcine retroviruses, it is still unknown whether normal porcine tissues in contact with normal human tissues would lead to an active virus infection and whether that infection would be pathogenic.

In response to a proposal to treat an AIDS patient with marrow transplanted from a baboon, the Food and Drug Administration has held hearings and recently issued suggested guidelines as related to infectious diseases from xenotransplantation (28). The suggestions include the formation of a registry for xenotransplantation, formation of institutional xenotransplant committees including experts in infectious diseases, the surveillance of both animal donors and human recipients, the archiving of blood and tissues from donors and recipients, and husbandry measures designed to provide pathogen-free animal donors.

Humans have already had extensive exposure to porcine tissues. At this time, there is not a great deal of concern of a public health hazard. At the slaughter houses, it is probable that pig blood frequently comes into contact with skin and open wounds of employees. Fixed porcine heart valves are routinely placed into patients. Fetal porcine islets have been transplanted into diabetics, with patients being immune suppressed to prevent rejection. Bridge transplants of porcine livers have sustained life of patients with fulminant hepatitis until human donors are located; the recipients again received immune suppression. Multiple investigators have also transplanted porcine tissues into nonhuman primates. Depend-

ing on the protocol, the primates may have received porcine blood cells, severe pretransplant immune suppression, or chronic posttransplant suppression. In modern times, no public health hazard or infectious outbreak has been reported related to these exposures. It seems unlikely that the public health risk of porcine xenografts is notably greater than with human allografts.

Much of the concern is related to the possibility that particularly aggressive immune suppression may be required to prevent xenograft rejection. Considerable progress is being made to produce more selective means of immune suppression and to induce specific immune tolerance, minimizing the need for immune suppression.

V. XENOGRAFT REJECTION

Allograft rejection involves primarily the immune reaction to polymorphic antigens that differ between the recipient and donor. These antigens include the major and minor histocompatibility antigens and blood group antigens. The type of rejection reflects the character of the immune response. Hyperacute rejection involves preformed antibodies and complement fixation. Acute rejection involves the primary sensitization of the host to the donor antigens, with resulting humoral and cellular injury. Chronic rejection is not as well understood and may involve multiple factors including humoral and cellular reactions to minor antigens, autoimmune reactions, and reparative processes. These processes are reviewed in an earlier chapter in this book.

Recipients reject xenografts by the same immune processes as with allografts. However, in addition to the polymorphic antigens seen on allografts, xenografts have numerous additional antigens reflecting the differences in species. These add to the challenge of preventing rejection. The xenoantigens can elicit a typical indirect immune response similar to that seen with an infectious agent. The recipient's immunological experience to infectious diseases may also lead to cross-reaction with some xenoantigens.

In addition to hyperacute rejection, acute rejection, and chronic rejection, a fourth type of rejection has been described: delayed xenograft rejection (DXR). It is typically observed at two or three days after transplantation in settings in which hyperacute rejection has been prevented. The timing and the associated antibodies (generally IgG) suggest that delayed xenograft rejection may involve a secondary immune response to xenograft antigens.

At this point, most efforts have been directed at preventing hyperacute rejection resulting from preformed natural antibodies and, more recently, at preventing acute xenograft rejection. The emphasis on hyperacute and acute rejection should not imply that chronic rejection is not a problem. When long-term xenograft survival becomes commonplace, most likely chronic rejection will also be described more frequently.

A. Hyperacute Rejection

For most combinations of species, the placement of a vascular graft into the recipient leads to destruction of the graft within minutes of revascularization, a process termed hyperacute rejection. Complement fixes to the endothelial cells throughout the graft, leading to adhesion of platelets, thrombosis, and ischemic necrosis of the tissue. For transplants between many close species, such as monkey to baboon transplants, HAR does not occur. Calne

termed xenografts that typically undergo HAR, such as pig to primate transplants, "discordant." Xenografts that do not undergo HAR, such as the chimpanzee to human transplants, were termed "concordant" (21).

For some discordant xenotransplants, such as the guinea pig to rat model, the fixation of complement is by the alternate pathway (48). The endothelium shows deposition of properdin, C6, and membrane attack complex. C2 and C4 are not involved. Hyperacute rejection of pig xenografts by Old World monkeys involves the immediate binding of circulating preformed antibodies to the endothelium and the classic complement pathway (49–53). The binding of complement to the endothelial cells can then lead to loss of heparin sulfate, activation with change in shape, cell death, coagulation, and adhesion of neutrophils (54). The widespread thrombosis of the vessels causes ischemic destruction of the xenograft.

In contrast to HAR of renal allografts caused by presensitization, the responsible xenophyllic antibody is IgM, not IgG. The antibody is almost entirely directed to a single epitope on the endothelial membrane, a glycoprotein with a terminal galactose α-1,3-galactose residue. This residue is expressed on most mammalian tissues. However, humans, apes, and Old World monkeys have a deficiency in the enzyme α-1,3-galactosyltransferase and therefore do not constitutively produce this epitope (55). The saccharide is generally expressed on intestinal bacteria, which most likely sensitizes hosts who do not constitutively produce it.

The expression of α-1,3-galactosyl residue is variable in different tissues (56). Whereas it is extensively expressed on porcine endothelial cells, the expression is considerably less in fetal porcine pancreatic islets (57). Thus, vascular grafts are more susceptible to HAR than pancreatic islets.

B. Delayed Xenograft Rejection

If hyperacute rejection is prevented, by either therapeutic measures or with certain concordant grafts, the xenograft may be vigorously rejected by 2 to 4 days. Immunopathological studies describe activation of the endothelial cells, deposition of IgG natural antibody and complement on the endothelium, and adherence of platelets and fibrin. Few lymphocytes are seen. The infiltrate consists of neutrophils, monocytes, and natural killer (NK) cells (58).

The antibody specificities observed in DXR differ from the IgM natural antibodies seen in HAR. They are not against the α-galactosyl transferase epitope (59). Because they are IgG, presumably the reaction is a secondary response to antigens seen previously.

In the guinea pig to rat model, DXR does not appear to require T cells because the reaction is also observed in nude rats (60,61); if mature T cells are essential for xenograft rejection, then athymic (nude) mice or rats should accept xenografts. When guinea pig hearts were transplanted into nude rats treated with cobra venom factor to prevent HAR, the grafts were rejected as quickly as in normal rat recipients. Immunohistochemistry of the rejected graft showed a dense infiltrate of macrophages and NK cells but no B cells or T cells (α/β TCR).

This model appears to define a T-cell–independent process involved in the delayed destruction of xenografts. As described in the next section, there is also compelling evidence supporting the need for a T-cell response. The apparent discrepancy may relate to differences in the models. The guinea pig to rat model is known to differ from the hamster to rat model

with respect to HAR. It may also differ from other models with respect to acute rejection. For example, whereas chemotherapy agents effective against T cells (e.g., cyclosporine, cyclophosphamide) can delay pig xenograft rejection in primates, they are relatively ineffective in the guinea pig to rat model (62,63). Therefore, the contribution of T cells may be more important in primates. It is furthermore possible that the few residual T cells remaining in the nude rats contribute to the rejection process (64,65).

C. Acute Rejection

The severe and swift destruction of discordant xenografts by HAR was initially seen as the principal barrier to xenotransplantation. It was believed that if HAR could be prevented, acute xenograft rejection could be controlled as with allografts. Xenotransplantation would then become commonplace. The success in overcoming HAR against pig organs was enabled in part because the process is basically an uncomplicated antibody response against a single nonessential antigen. It has become clear that the severity of acute xenograft rejection is just as daunting as HAR was. Furthermore, the pathophysiology of acute xenograft rejection is much more complex, involving multiple antigens and multiple mechanisms of immune destruction.

Concordant grafts between closely related species, such as Old World monkey to baboon transplants are rejected in a manner similar to allografts. Heart xenografts are rejected in less than a week without immune suppression. Although antibodies participate in the rejection, the predominant effector cells appears to be T cells. Several reports of long-term acceptance have been described. Using cyclosporine and steroids, heart xenografts survived for 77 and 94 days (mean graft survivals) (66,67). Transplantation of monkey hearts into infant baboons with suppression consisting of splenectomy, FK506, methotrexate, and steroids resulted in a mean survival of 127 days (68). Adding total lymphoid irradiation to cyclosporine and azathioprine, Roslin et al. attained a mean survival of 255 days (69).

When HAR is prevented in more disparate xenografts, including discordant grafts, the grafts are destroyed in a very narrow time frame, generally 5 to 8 days after transplantation. This is consistent with a primary immune response. The severity of the rejection, however, appears to vary with species combination and tissue.

In contrast to the primate to primate transplants, porcine organs transplanted into primates are rejected much more vigorously. The severity is reflected by the increased immune suppression required and the associated complications. Immune suppressive drugs, such as cyclosporine, which can provide for indefinite survival of allografts, provide only limited survival of pig xenografts (70). When HAR was prevented by splenectomy and plasmapheresis, pig kidneys functioned and survived within baboon recipients for up to 22 days (25). The recipients received ATG, azathioprine, cyclosporine, and steroids. Of the five recipients, irreversible vascular rejection developed in three, one died of pneumonia, and one was sacrificed with severe gastric dilatation.

In another series of pig kidney to monkey transplants, HAR was prevented by *ex vivo* perfusion through either pig livers or an immunadsorption column. Mixed chimerism was induced within the monkey to prevent acute rejection, using a nonmyeloablative protocol of whole body and thymic irradiation and antibodies to lymphocytes or subsets. One recipient survived up to 15 days without rejection, but most of the kidneys were lost to vascular rejection during the second week (71).

The experience with pig heart to primate transplants is similar. When sufficient immune suppression is administered to prevent acute rejection, there is a high rate of complications involving infections or toxicity (63,72). The difficulty is best illustrated in the experience with hDAF transgenic pigs (73). Although HAR was not observed in these studies, the grafts were acutely rejected at 5.1 days without immune suppression. Immune suppression by cyclosporine, cyclophosphamide, and steroids was given in two other groups to prevent acute rejection. With an average daily dose of cyclophosphamide of 12 mg/kg/day, the median graft survival was prolonged only to 9 days. When the cyclophosphamide was increased to 21 mg/kg/day, the median survival was prolonged to 40 days. However, 6 of the 10 animals had severe diarrhea or anemia, necessitating euthanasia.

The time of onset (Table 1) and the modest response to immune suppression suggest that acute xenograft rejection is a primary immune response, involving the sensitization of T and B cells (25,73–85). The experimental pathological studies reinforce that concept. When rats were given multiple doses of antithymocyte serum, human islet xenografts had significantly prolonged survival, suggesting that T cells were essential for xenograft survival (80). $CD4^+$ cells were shown to be particularly important for xenograft rejection. With the depletion of $CD4^+$ cells using monoclonal antibodies, fetal pig islets enjoyed prolonged survival (86). The recipients were unable to mount antibody responses to pig cells. These studies suggested that $CD4^+$ cells were important in at least the afferent limb of the immune response, particularly regarding the antibody response (87). Though the studies emphasize $CD4^+$ T cells, it is assumed that the antibodies also depleted $CD4^+$ monocytes and macrophages as well.

Although the $CD4^+$ cells certainly would contribute to the humoral response (88), the $CD4^+$ cells themselves can destroy the target cells. Adoptive transfer studies of cell populations to SCID mice showed that the transfer of $CD4^+$ cells without B cells led to rapid rejection of pig skin grafts (89).

With allograft rejection, $CD8^+$ cells are profoundly important in the tissue injury

Table 1 Xenograft Rejection in the Absence of Hyperacute Rejection

Xenotransplant	Tissue Transplanted	Prevention of Hyperacute Rejection	Graft Survival	Ref.
Hamster → Rat	Heart	Concordant	3 days	74
Pig → Monkey	Heart	Transgenic pig	5.1 days	73
Pig → Baboon	Kidney	Plasmapheresis	6 days	25
Pig → sheep	Vascular	Concordant	6 days	75
Monkey → baboon	Heart	Concordant	6 days	76
Pig → mouse	Skin	Nonvascular	10 days	77
Rat → mouse	Islets	Nonvascular	14 days	78
Rabbit → rat	Skin	Concordant	8 days	79
Human → rat	Islets	Nonvascular	3.7 days	80
Human → rat	Skin	Neonatal recipient	8 days	81
Goat → dog	Skin	Nonvascular	6 days	82
G. pig → rabbit	Skin	Nonvascular	7 days	83
Wolf → dog	Skin	Nonvascular	12 days	84
Fish → mouse	Islets	Nonvascular	6.5 days	85

phase. With xenografts, however, these cells are less important. $CD8^+$ cells are normally restricted to target cells expressing MHC class I. Knockout mice deficient in β-2 microglobulin are deficient in class I antigen and therefore class I–restricted $CD8^+$ T cells (90). Mouse pancreatic islet allografts had prolonged survival within these hosts. However, rat islet xenografts were promptly rejected, suggesting that class I–restricted cytotoxicity is not essential for xenograft rejection.

A T-lymphocyte response is essential during the sensitization phase of the xenograft response. Treatment of mice with anti-CD2 antibodies (sheep red cell receptor) leads to prolonged acceptance of islet xenografts. If the treatment is delayed, however, no prolongation is observed (91). When hamster hearts or livers are placed into rats, hematopoietic cells migrate to the spleen within 1 day, much like the migration from allografts (92). The spleen shows a prompt response with proliferation of IgM^+ B cells and $CD4^+$ T cells.

Thymus modification experiments also support the importance of a T-cell response (93). The effect of intrathymic injection of splenocytes was assessed for either rat heart allografts or hamster heart xenografts. The injection induced indefinite survival of the allografts. When combined with a pulse of cyclophosphamide, intrathymic cell injection resulted in significantly prolonged survival as compared with the thymus and cyclophosphamide controls. By itself, however, the intrathymic injection had little effect on the xenograft survival.

When fetal pig islets were transplanted into BALB/c nude mice, the grafts survived for prolonged periods (94). These nude mice had greater than 99% reduction in mature T cells. If normal mouse lymphocytes were adoptively transferred, the grafts were rejected within 7 days, indicating that the lymphocytes responsible for acute rejection of the pig islets were absent in the nude mouse. Transfer of $CD4^+$ cells led to rapid rejection whereas the transfer of $CD8^+$ cells resulted in a delayed rejection. Because the nude mice had macrophages, the transfer most likely provided T cells that were missing. The responsible cells were also radiation sensitive. Xenografts were eventually rejected with the transfer of B cells and NK cells (i.e., CD4 and CD8 depleted). The findings support the proposal that xenografts can be rejected by at least two mechanisms: a T-cell process (particularly $CD4^+$ T cells) and a non–T-cell process.

Similar results were achieved with the transfer of sensitized lymphocytes from a normal mouse rejecting a xenograft into a nude mouse containing the porcine islets (95). Immunohistochemistry of the rejecting xenograft showed predominantly macrophages in the infiltrate with a small infiltrate of $CD3^+$ T cells at the periphery of the infiltrate. However, the macrophages were from the nude mouse. The study concluded that immune competent T cells were responsible for the activation and accumulation of macrophages. T cells appear to be less important during the subsequent injury phase of xenograft rejection.

Using the guinea pig to rat heart transplant model to focus on the effector arm of xenograft rejection, sensitized splenocytes were transferred into immune competent rats at the time of xenotransplantation. The sensitized cells accelerated the rejection process from the normal 4 days to less than 2 days (96). The accelerated rejection was prevented when either macrophages or B cells were depleted from the transferred suspension. Therefore, B cells and macrophages are important in the early tissue injury phase.

In vitro assays of cellular immunology have provided insights into the relative strength and mechanisms of allograft rejection. The mixed lymphocyte reactions with some discordant xenogeneic combinations were found to be weak (97–99). It was widely believed at that time that cellular rejection of discordant xenografts would not present a major problem and that, once HAR was resolved, cellular rejection could be controlled with immune suppression as with allografts. As discussed, however, that optimistic view is no

longer warranted. Acute cellular xenograft rejection is as strong or stronger than mismatched allograft rejection (100). For the most relevant lymphocyte reactions, primate versus primate and human versus pig, the in vitro reaction is comparable in strength to human allogeneic reactions (101,102).

Proliferative reactions of mouse lymphocytes to monkey, human, and pig stimulator cells have been analyzed (103). The weak in vitro reactions correlated with weak direct interactions between the responder T-cell receptor and the stimulator MHC antigens. The adherence of accessory molecules to ligands, such as intracellular adhesion molecule-1 (ICAM-1) and lymphocyte function activator-1 (LFA-1), was also weak. In contrast, the interaction was strong in closely related species, such as between mice and rats (104,105). Pretransplant coating of human islet xenografts with antibody to ICAM-1 delayed rejection in mouse hosts from 7 to 21 days (106). Similar results were observed with a rat-to-mouse islet model. Molecular binding studies indicated that the binding of LFA-1 to ICAM-1 was not species specific.

Effective sensitization requires the costimulation provided by the binding of CD28 on T lymphocytes to B7 on the antigen presenting cells (APCs). CTLA-4, a monoclonal antibody resembling CD28, blocks T cell CD28 from binding to the APC. Infusion of CTLA-4 blocks the rejection of human islets by recipient mice (107).

Limiting dilution experiments can establish the relative number of lymphocytes reactive to xenogeneic stimulator cells (108–110). The results with xenogeneic reactions are more complex than those observed with allogeneic cells, suggesting multiple mechanisms of T-cell sensitization, including direct and indirect antigen presentation.

With allogeneic reactions, the MHC antigens on allogeneic stimulator cells serve as both the antigen presenting molecules and the antigenically distinct peptide. This is referred to as direct antigen presentation. Rather than a large number of cells presenting diverse peptides, all of the cells "present" the same antigen, leading to a much stronger reaction. The proliferation of human lymphocytes in response to pig stimulator cells has proved to involve direct antigen presentation, which is at least as strong as the reaction to allogeneic human stimulator cells (111,112). Porcine endothelial cells can directly stimulate human lymphocytes (102,112). Similarly, porcine dendritic cells directly stimulate human T lymphocytes (110). The reaction is against class II MHC antigen, specifically SLA-DR.

In a typical reaction to a foreign body, the foreign cells are engulfed and digested within the antigen presenting cells. The digested peptides are then nestled on the surface within the class I or II MHC antigens. The responder cells recognize the MHC antigen and the associated peptide in a process referred to as indirect antigen presentation. Indirect xenoantigen presentation would use the recipient's T cells and antigen presenting cells. It would not require compatibility between adhesion receptors and ligands in the donor and recipient. In the human versus pig stimulator cells mixed lymphocyte reaction, the addition of purified human antigen presenting cells (monocytes) enhances the proliferative response (113).

Pig endothelial cells stimulate the proliferation of both purified $CD4^+$ and $CD8^+$ human lymphocytes (102,112). Cytokine analysis of the mouse versus rat mixed lymphocyte reaction (MLR) indicates that the $CD4^+$ helper cells are those of Th2 subtype as compared with the usual Th1 subtype seen with allogeneic reactions (88). Whereas Th1 cells participate in delayed type hypersensitivity reactions, Th2 cells typically contribute to the humoral response.

The immunopathological reviews of rejecting xenografts suggest that the mechanism of tissue injury differs from that seen with allografts. They emphasize the participation of bound immunoglobulins and NK cells. In contrast to rejecting allografts, T cells represent a

minor population but may contribute to the tissue injury. Most of the immunopathological surveys were performed using either the hamster to rat model or the guinea pig to rat model with cobra venom factor to prevent HAR (114,115). The early pathological findings of the target organ show vascular endothelial injury and thrombosis as well as tissue necrosis. The inflammation is limited, consisting primarily of macrophages and, later, eosinophils. The number of T and B lymphocytes is variable but is generally sparse. Immunoglobulin and fibrin are bound to the endothelium. Immunoglobulins, particularly IgM and IgG_{2a}, as well as C3 were shown to be bound to the endothelial cells following the transplantation of rat hearts into mice (116). Depletion of $CD4^+$ cells from the recipient blocked antibody production and acute xenograft rejection, suggesting that the $CD4^+$ cells contributed to the humoral response.

The macrophages and NK cells are most likely recruited into the target tissue by the induced expression of monocyte chemoattractant protein (MCP-1) (117). The endothelial and vascular smooth muscle cells express MCP-1 within 12 hours of transplantation. It is believed that γ-interferon from NK cells induces the expression of MCP-1.

The immunopathological findings of acute xenograft rejection of porcine xenografts transplanted into cynomolgus monkeys and baboons has been systematically described in grafts surviving 7 to 15 days (118). Dying target cells were identified using the TUNEL (TdT-uridine-nick-end-labeling) assay for apoptosis and the mononuclear cell subtypes identified with immunohistochemistry. Endothelial cells within the glomeruli and in the peritubular capillaries showed frequent apoptosis. Microangiopathic thrombi were present, with associated infarcts. Cellular infiltrates were identified around some arteries, consisting of granulocytes, T cells, and monocytes. Tubulitis was also seen, with tubules containing inflammatory cells and apoptotic epithelial cells.

Several immunopathology studies support a significant function for T cells in xenograft rejection. T lymphocyte infiltrates are more apparent in some nonvascular grafts, such as late in the rejection of corneas or in pancreatic islets (86,120,121). When human blood were perfused through a porcine kidney, the T cells and NK cells were removed from the blood; these cells were later identified attached to the vascular endothelial cells of the perfused graft (119). In vitro cell-mediated cell lysis assays suggest that T cells and NK cells collaborate in the destruction of pig endothelial cells. Human lymphocytes spontaneously lysed labeled pig endothelial cells (122). The lysis was enhanced by interleukin-2 and inhibited by antibodies to CD2. CD2 is expressed on peripheral T lymphocytes as well as NK cells. Interleukin-2 is secreted by $CD4^+$T cells. The activation of $CD4^+$ cells, therefore, could enhance the NK or cytotoxic T-cell reaction.

Cell-mediated lysis has been correlated with acute rejection. Pig hearts transplanted into newborn baboons are not subject to HAR but are acutely rejected at 3.5 days. Immunopathological studies show the infiltrating cells to consist of mostly macrophages and NK cells ($CD2^+$, $CD16^+$). Only approximately 10% of the infiltrating cells were $CD3^+$ T cells. Circulating peripheral blood mononuclear cells from the recipient baboons lysed cultured pig endothelial cells. Interleukin-2 significantly enhanced the lysis. Antibodies against NK cells partially blocked the adhesion to pig endothelial cells (123). Although the immunopathological studies favor an NK mechanism, the in vitro studies are consistent with a mixed cell reaction of T and NK cells.

A complementary study supports the involvement of human T cells in the lysis of pig endothelial cells, as well as non–T-cell mechanisms (111). The cell lysis was partially blocked by antibodies to CD3, which is expressed on mature T cells.

Overview. As compared with acute rejection of allografts, acute xenograft rejection is a complex phenomenon. When the immune system of the transplant recipient "sees" an

allograft, the immune system focuses on the polymorphic expression of major and minor histocompatibility antigens. The T cells interact directly with the tissue and the antigen presenting cells from the donor. A humoral and cell-mediated response is mounted to rid the body of those few antigens.

When the immune system of the recipient "sees" a xenograft, however, the immune system sees a foreign body in addition to an allograft. It mounts multiple defenses to rid the body of the complex object. When the two species are relatively closely related, such as with monkey to baboon transplants or pig to baboon transplants, the recipient can mount a direct antigen response. The recipient lymphocytes recognize the polymorphisms of the donor MHC as well as similarities. The accessory receptors bind to the corresponding ligands on the donor cells, and the immune response resembles the response to an allograft. When the two species are more disparate, this mechanism is less important.

However, the xenograft also brings with it numerous antigens not related to the histocompatibility antigens. These elicit a strong indirect response, in which the xenogeneic cells are digested and the antigen presented through the recipient's antigen presenting cells. Each of these antigens elicits a corresponding humoral or cellular response. In addition, the xenograft may elicit a primitive natural immune response.

For most xenotransplants, initial T-cell sensitization is essential. In particular, $CD4^+$ T cells must be sensitized. If antibodies, drugs, or immune tolerance can block this initial step, then the destruction of the xenografts is postponed and possibly avoided. The pathological makeup of the rejected xenograft does not resemble that of T cell-mediated rejection of allografts, however. The grafts have bound immunoglobulins and complement, increased natural killer cells, and macrophages. The T cells are variable and often sparse in number. Most of the injury is due to direct antibody response and complement fixation, antibody-dependent cell cytotoxicity by natural killer cells, and direct injury by natural killer cells. In vitro studies indicate that a minor population of T cells may also contribute to the tissue injury.

Efforts taken to reverse acute xenograft rejection must recognize the multiple effector cell mechanisms. For example, immunopathological studies of tissues at a later stage of rejection tend to emphasize the T-cell component (84,120,121). If only one mechanism were prevented or reversed, the latter mechanism could still destroy the graft. The different effector mechanisms could also be interacting to enhance the strength of the rejection. For example, early cell destruction could enhance the processing of cells and sensitization of additional effector cells. Cytokines, such as interleukin-2 produced by T cells, may enhance the NK cell reaction (122). The activation of endothelium could lead to further adherence of T cells.

D. Chronic Rejection

In vascular allografts, chronic rejection is a major, still unresolved problem. Months after transplantation, the grafts may develop atherosclerosis, with diffuse intimal thickening and proliferation of the fibroblasts. In kidneys allografts, there is an associated interstitial fibrosis and generalized loss of tubules and a glomerulopathy.

With the substantial barriers of HAR and vigorous acute rejection, there has been relatively little experience until recently with long-term xenografts. Graft atherosclerosis has been observed in two baboon recipients of monkey hearts (124). The recipients died at 74 and 502 days after transplantation. The findings resembled accelerated arteriosclerosis seen with chronic allograft rejection. In the hamster to rat heart and aorta transplant models,

chronic vascular rejection has been described, also resembling chronic allograft rejection (125–127). In a systematic and quantitative study of hamster aorta xenografts, Scheringa et al. demonstrated thickening and infiltration of the adventitia during the acute rejection (14 days). This subsided and was followed during the chronic phase by a progressive thickening of the intima (at 56 days). Localized IgM deposits were seen in the arterial lesions (125).

Chronic xenograft rejection might prove to be more resistant to chemotherapy than allografts (128). Continuous cyclosporine or mycophenolate mofetil have been effective in reducing the intimal thickening seen in chronic xenograft rejection (125,126,129).

VI. OVERCOMING REJECTION

A. Prevention of Hyperacute Rejection

Although HAR is a dramatic and nearly ubiquitous outcome of xenotransplantation, the problem has been defined and is well understood. For the pig to primate xenotransplants, the problem is simplified by the observations that natural antibodies are mostly directed toward a single epitope and that complement is fixed by the classical pathway. Several successful strategies have been developed to prevent the immediate destruction of the xenograft.

1. Removal of Natural Antibodies

Plasmapheresis had proved useful in preventing hyperacute rejection in ABO mismatched recipients (130). A similar removal of the natural xenogeneic antibodies by plasmapheresis was performed and showed a modest but significant prolongation compared with that in control subjects (131). The shortcomings of plasmapheresis included incomplete removal of the antibodies and early return of the antibodies. With chronic plasmapheresis, coagulation factors were also depleted. Better removal of antibodies was achieved using double filtration plasmapheresis, with greater than 93% removal of the IgM natural antibody (132, 133). The prompt return of antibodies was delayed by either performing a splenectomy (25) or inducing immune suppression with, for example, cyclosporine (134).

Because the α-gal epitope is expressed on endothelial cells, a more selective removal of antibodies could be achieved by perfusing the recipient's blood through a porcine liver. Indeed, the specific anti-α-gal antibodies are undetectable immediately following the *ex vivo* perfusion (135,136). The antibodies can also be depleted using immune adsorption columns consisting of beads with bound antiimmunoglobulin antibodies (137) or bound antigen such as the α-gal epitope (135,138). The latter is the most specific method for antibody removal. With intermittent use of this column, pig graft survival of up to 3 weeks was achieved in a discordant baboon model. The IgM antibody returned within days of discontinuing the perfusions (138).

2. Soluble Oligosaccharides Infusion

Instead of removing the natural antibodies, soluble synthetic oligosaccharides with a terminal α-galactosyl residue can be infused into the recipient, effectively neutralizing the antibodies (139). The soluble saccharides prevented human serum from reacting with PK-15 pig tubular epithelial cells. Similarly, serum from baboons that were infused with the oligosaccharides showed decreased reactivity. Generally, larger oligosaccharides prove to be more effective than smaller oligosaccharides (140).

3. Systemic Inhibitors of Complement

Early xenotransplant models used cobra venom factor (CVF) to block HAR. Cobra venom factor effectively blocked complement fixation by inactivating C'3c (141), but it was associated with significant toxicity. Less toxic inhibitors of complement have been tested for their ability to prevent HAR. Complement receptor I is normally expressed on red cells. It contributes to the clearance of immune complexes and blocks the terminal complement fixation for both the classic and alternative pathways. Recombinant soluble complement receptor (sCR1) was synthesized and has been tested for prevention of HAR (63,142). With continuous administration, HAR was prevented in pig to monkey heart transplants. Recently a defective adenovirus vector containing sCR1 was developed (143); infected cells produced sCR1 and were protected from lysis by natural antibodies and complement.

Monoclonal antibodies against C5 and C8 have been produced (144). These antibodies have blocked HAR in a perfusion model of pig heart xenografts.

4. Xenotransplants into Newborn Recipients

Natural antibodies to the alpha gal epitope are believed to be derived from a cross-reaction against bacteria. Newborns would therefore be expected to have a deficiency in these natural antibodies. Systematic studies found that newborn baboons and humans lack the IgM natural antibody, although many had the IgG anti–α-gal antibody, presumably derived from the maternal blood (145–147). Pig to newborn baboon transplants did not result in HAR, but were rejected in 3 to 4 days. Histological study showed prominent mononuclear cell infiltrates consistent with acute rejection (147,148).

The possibility was considered that newborn pigs do not express the α-gal epitope. However, baboon serum was found to react equally with endothelial cells from newborn pigs and from mature pigs (149).

5. Genetic Engineering

The natural antibodies responsible for hyperacute rejection of porcine xenografts are primarily directed at a single epitope, the galactose-α-(1,3)-galactose epitope. The most obvious solution to this problem would be to genetically engineer a pig that fails to produce this oligosaccharide. A knockout mouse has been produced that fails to produce alpha-1,3-galactosyltransferase (150). At this time, however, a similar knockout pig has yet to be produced.

Two other genetic engineering strategies have been undertaken to eliminate the problem of HAR to pig organs. First, genetic engineering can provide pigs with the human complement inhibitors that naturally protect human endothelium from complement-dependent lysis. Second, pigs can be produced that enzymatically remodel the α-galactosyl epitope into a harmless epitope such as is seen on type O human red cells.

Two groups have succeeded in producing transgenic pigs with human complement inhibitors. These inhibitors block the terminal sequence of complement activation and the development of the membrane attack complex, which is responsible for cell lysis. The expression of human CD59 or human CD59 and human decay activating factor (hDAF) significantly delayed rejection of pig hearts in monkeys up to 69 hours, as compared to about 1 hour in the control subjects (152,152). In another study, hearts from pigs with human DAF and membrane cofactor protein (MPC) have been transplanted into monkeys. The HAR was effectively blocked and the grafts survived a median 5.1 days without any other measures taken to prevent HAR (73). This group recognized that the tissue expression of

hDAF was variable in different founder animals (153). They selected and bred the founders expressing the product in the endothelial and smooth muscle cells. Kidneys from these transgenic pigs expressing human DAF have been perfused *ex vivo* with human blood (154). Although both the control kidneys and transgenic kidneys had similar binding of xenoantibody to the vascular and glomerular endothelium, the transgenic kidneys had significantly less fixation of C3 and C9. The transgenic pigs have not shown any developmental or breeding abnormalities (155).

Rather than block the immune reaction to the pig cells, transgenic pigs have been produced with altered antigen expression (156). The enzymes α-1,3-galactosyltransferase and α-1,2-fucosyltransferase compete for the same substrate. Whereas the former produces the epitope for natural antibodies, however, the latter enzyme produces the H substance, as is seen with blood group type O. By producing transgenic pigs expressing α-1,2-fucosyltransferase, the expression of the α-galactosyl epitope is significantly reduced. Cultured pig cells containing the fucosyltransferase enzyme are more resistant to lysis by natural antibody and complement.

Overview. Hyperacute rejection, once considered a formidable obstacle to xenotransplantation of pig organs, has become a resolvable problem. Indeed, multiple strategies have proven to be successful. Technological advances were driven by the need to extend discordant xenograft survival from minutes to days. The best strategy, however, will be based on long-term success. The ideal strategy should not require repeated maneuvers for an indefinite period of time; if plasmapheresis, infusion of sCR1, or oligosaccharides are required indefinitely, the strategy would be expensive and inconvenient. The strategy should not chronically or severely suppress the host's immune response; if procedures to block complement fixation are combined with immune suppression for preventing acute rejection, the host could end up with a severe combined immune deficiency. The strategy should not adversely affect the graft function or contribute to acute or chronic rejection. Potential problems could arise from the persistent binding of immunoglobulins and early complement components or from a reaction to low-density expression of antigen.

There are three potential outcomes that could define a solution with good long-term outcome for the prevention of HAR. First, the recipient could achieve accommodation, a phenomenon in which the immunoglobulins continue to circulate and bind to the tissue, but through protective compensatory reactions by the host, the tissue is no longer injured. Second, the host could become specifically tolerant to the porcine endothelial antigens, such as α-gal; through negative selection, anergy, or suppression, the host stops making significant antibodies. Third, the responsible epitope could be permanently eliminated from the organ donor. Because tissue expression of α-gal is not essential for life, the production of a knockout pig deficient in α-gal remains an attractive goal.

B. Inhibition of Acute Rejection

Recipients of human allografts are typically treated with long-term immune suppression to prevent rejection. The protocols are relatively selective in that the patient retains some ability to fight infections and neoplasms. The rejection of allografts differs in several respects from the elimination of infectious agents. Rejection involves predominantly direct antigen presentation. Even though antibodies contribute to the reaction, it is predominantly a T-cell–dependent process.

The challenge in finding a similarly selective immune suppression protocol for xenografts is considerably greater. Compared with allografts, xenografts more closely resemble

infectious agents. In addition to direct antigen presentation, xenoantigens also are processed indirectly. The humoral response to xenografts is stronger than with allografts, and NK cells are reactive with certain xenoantigens.

Several immune suppressive drugs are being evaluated for their effectiveness with xenografts, with particular interest in their effectiveness in indirect antigen presentation, humoral reactions, and NK cell reactions (157). These agents include cyclophosphamide (63), cyclosporine (63), tacrolimus (158), leflunomide (159), deoxyspergualin (160,161), mycophenolate mofetil (162–164), rapamycin (164), brequinar (163,165), and others.

It seems unlikely at this time that either a single agent or combination agents can prevent xenograft rejection indefinitely without producing severe complications. Additional strategies are required. Most likely, immune suppression will be used in conjunction with other procedures intended to induce selective hyporesponsiveness to xenografts. Immune suppression may be used to supplement these measures. For example, Sheffield and coworkers showed that injection of xenoantigens into the thymus by itself was ineffective in preventing rejection (93). However, the combination of intrathymic injection with temporary suppression with cyclophosphamide significantly prolonged xenograft survival. Similarly, Zhao et al. used temporary immune ablation to achieve immune tolerance to xenoantigens. After the chimeric mice achieved immune competence, pig skin grafts were accepted indefinitely without additional suppression (77).

VII. STRATEGY FOR PROLONGED ACCEPTANCE OF XENOGRAFTS

The aggressive immune suppression needed to prevent pig xenograft rejection is associated with a high rate of morbidity and/or mortality resulting from toxicity and infectious complications. For xenografts to achieve their potential, then, it is necessary to use other processes that specifically minimize the immune reaction to xenografts. In theory, these processes could generate donor antigen identity with the recipient, establish accommodation, or induce immune tolerance.

Whereas the opportunities with allografts are limited to modifying the immune response of the recipient with minimal modification of the donor, the opportunities for intervention are far greater with the use of animal donors. With an increased number of possible maneuvers, choices must be made to achieve the desired results. As a guide to making these choices and as a standard for assessing efforts to prevent xenograft rejection, the following goals of an ideal system are suggested.

- The recipient should be specifically unresponsive to all antigens expressed on the xenograft, including tissue-associated antigens.
- The recipient should remain immune responsive to all other antigens.
- The unresponsiveness should include all immune mechanisms: cellular, humoral, and natural.
- Unresponsiveness should not develop against infectious organisms present in the donor or recipient.
- The recipient or donor animals should not be at increased risk for acquiring or activating an infection during the induction of immune unresponsiveness.
- The specific unresponsiveness should be permanent.
- If the recipient is chimeric, containing hematopoietic cells from the corresponding partner, the chimeric cells must be unresponsive to the recipient and not cause graft versus host disease (GVHD).

There is no system that satisfies all of these goals. Particularly challenging is the need to induce tolerance to the xenograft without increasing the risk for infection. For example, systems that use immune suppression during induction of tolerance not only increase the risk of development of an infection during the period of immune deficiency, but they also increase the risk of inducing tolerance to those infectious organisms.

The natural induction of immune tolerance in the fetal environment satisfies these goals in the induction of tolerance to self-antigens. Generally, the tolerance is complete, is very specific, and persists indefinitely. Efforts to induce immune tolerance to allografts and xenografts have therefore been aimed at simulating the fetal induction of self-tolerance. The following paragraphs briefly review the advantages of this ideal system.

1. Lymphocytes and tolerance develop relatively late in ontogeny. Most of the tissue antigens are expressed when the fetus begins to produce naive lymphocytes. Also, the nonlymphocyte immune components, such as the thymic epithelium and dendritic cells, are developed. The development of tolerance to self, including tolerance to tissue-associated minor antigens, is therefore complete.

2. The fetus is protected from opportunistic infections by nonimmune mechanisms, permitting a prolonged period of severe immune deficiency. If the successful induction of tolerance depends on an environment with a large excess of tissue antigen over lymphocytes, then the environment should go through a period of severe immune deficiency. The challenge is in providing that environment without increasing the risk of infection. The fetal environment in the preimmune state is usually free of infectious agents, free of neoplasms, and free of noninherited maternal antigens. For the most part, this is accomplished by the mechanical barrier provided by the placental membranes (166). Not only is the fetus protected from disease but also the only antigens typically seen by lymphocytes developing tolerance are those present on normal tissues. Tolerance to infectious organisms does not develop in the fetus.

3. Protective mechanisms develop to prevent autoimmune reactions from fetal lymphocytes. Recently, Ridge et al. have argued persuasively that the window for tolerance seen in the fetus or newborn is not due to any specific properties of the developing lymphocytes (167); both fetal and adult T cells can be either sensitized or tolerized, depending on the presentation of the antigen. Rather, tolerance develops in the fetus or newborn because of the overall environment and the nature of antigen presentation. As the immune system begins to develop, the relatively few naive lymphocytes are confronted with an overwhelming excess of self-antigen.

4. The tolerance is comprehensive, preventing autoimmune reactions by all mechanisms. Because immune competent individuals are stable and do not react against self, clearly the development of tolerance controls those mechanisms capable of reacting against self. However, are NK cells, generally thought to be antigen independent, specifically programmed to be tolerant to self? Or are they constitutively reactive with epitopes not normally expressed? Waterfall et al. studied hybrid resistance, a phenomenon in which F1 hybrids fail to engraft with parental type cells (168). They found that hybrid resistance depended on NK cells. The specificity of hybrid resistance and the responsible NK cells could be reprogrammed by exposing newborn mice to the parental antigens. The antigen specificity of NK cells has been demonstrated in other models and the mechanisms of unresponsiveness studied (169). Tolerance does not involve clonal deletion or anergy, but rather self-inhibition upon recognizing certain antigens. It is suggested that NK cell tolerance is most optimal when the cells are exposed to nonhematopoietic cells (170).

5. Protective mechanisms prevent graft versus host disease from mature maternal lymphocytes. Although the mechanical barriers of the placenta generally prevent maternal lymphocytes from entering the fetus, chimerism studies of newborns indicate that such cross-circulation does occur. The presence of mature fetal reactive lymphocytes in an immune deficient recipient satisfies the Billingham requisites for GVHD and should lead to the death of the fetus (171). As shown by Crombleholme et al., the fetus benefits from protection against GVHD (172). Even though a large number of xenogeneic T cells did lead to lethal GVHD, a limited number of mature lymphocytes did not destroy the fetus, but led to prolonged, stable chimerism. The mechanism for this protection has not been extensively studied. It has been noted, however, that placental proteins resemble interleukin-10, which could induce Th2 helper type cells that help to downregulate the immune response.

The ideal system of developmental tolerance to self cannot be wholly adapted for prolonged acceptance of xenografts. Only rarely are transplant candidates still in the protective preimmune environment of the uterus. Even these candidates face difficulties in inducing immune unresponsiveness to xenografts. The system is useful, however, in providing partial solutions to difficult problems faced in the postnatal world.

The fundamental immunological tools available for enhancing success of xenotransplantation include antigen identity with the recipient, accommodation, and immune tolerance.

A. Donor Organ Antigen Identity with the Recipient

Having demonstrated the insertion of human genes into pigs, it is tempting to extrapolate these methods and propose the development of a pig that is identical with the recipient or a universal donor pig that humans cannot reject. Neither prospect seems plausible. The task of deleting the numerous histocompatibility and xenogeneic antigens and then replacing them with the corresponding recipient antigens would be enormous, even with future technological advances. Because most of the cell surface antigens have functions beyond immune recognition, it is highly unlikely that such a massive reengineering would result in a viable pig with a functional organ. A more realistic proposal to achieve antigen identity would be to clone the recipient (173). However, ethical, legal, and serious logistical barriers make cloning impractical for transplantation.

Is it possible to engineer a universal donor pig? Analogous to type O red cells, the recipient's immune system would simply fail to recognize the antigens. The level of difficulty, however, would be far greater than for red cells. There are numerous relevant antigens capable of triggering rejection and most of the antigens have widely diverse polymorphisms. If efforts were successful, the resulting universal donor pig would incur two additional problems. First, if the pig had defective or absent antigen presenting or accessory molecules, it would be severely immune deficient. The pigs would need to be raised under gnotobiotic conditions, adding greatly to the expense and making it much more difficult to satisfy the demand for organs. Of greater concern, however, is the risk of infectious disease. If the transplanted organ became infected, the human immune system would not be able to react to it or defend it. Furthermore, the recipient might become tolerant to the infectious agent. Second, the recipient would experience severe graft versus host disease if the pig lymphocytes were still capable of recognizing recipient antigen presenting cells. The recipient would be incapable of rejecting the reactive pig lymphocytes.

A more reasonable goal would be to produce xenogeneic donor cells that are partially identical with the recipient. The donor cells could express class I or II MHC antigens from

the recipient or from the recipient species. This approach would need to be used in conjunction with other processes to establish accommodation or tolerance. By itself, the presence of recipient MHC antigen would make the donor cells more efficient as antigen presenting cells. At the very least, these cells would enhance indirect antigen presentation. If the engineered cells had species-specific MHC but differed from the recipient, they would also enhance direct antigen presentation.

Gene therapy could assist with the development of xenogeneic hematopoietic chimerism. Human class I antigen has been inserted into mouse hematopoietic cells using a retrovirus vector (174). After lethal irradiation and bone marrow transplantation with the modified cells, stable chimerism with cells expressing the human antigen has been observed. Furthermore, in the chimeric animals, a markedly reduced antibody response and partially inhibited cellular response to immunization developed with this antigen, but a normal response to third party antigens was retained (175,176).

An alternative approach would be to make the recipient's hematopoietic cells resemble those of the animal donor. Once tolerance is induced to the hybrid cell, tolerance should be established to the donor animal as well. Swine MHC class II antigen has been incorporated into murine and into baboon $CD34^+$ stem cells (177,178). In two baboons receiving autologous stem cells transfected with swine antigen, prolonged survival of the cells was detected. Kidney xenografts from the corresponding swine were transplanted but promptly rejected. Because the hematopoietic chimerism persisted after rejection, it is assumed that the tolerance was incomplete. A humoral response reacted with other swine antigens.

B. Accommodation

The body has a defensive mechanism against self-inflicted immune reactions, referred to as accommodation or adaptation. If tissues can survive the initial humoral or cellular reactions, with time, the tissues become resistant to injury.

Accommodation has routinely been described with minor ABO incompatible allografts. Passenger leukocytes produce IgG antibodies against the host's red cells. The IgG antibodies typically appear approximately 1 week after transplantation and cause significant hemolysis. The reaction, however, is self-limited. The hemolysis disappears in approximately 3 to 4 weeks, even though the antibodies persist (179,180).

A similar resolution of antibody-mediated rejection has been demonstrated for ABO mismatched allografts and for discordant xenografts. Fischel et al. described a porcine cardiac xenograft surviving in a rhesus monkey for 8 days. Even though circulating antibodies were bound to the endothelium, the graft had not undergone rejection (181). Hyperacute rejection was prevented in ABO mismatched cardiac allografts performed in baboons by infusion of soluble trisaccharides of the A and B antigen to neutralize the antibodies. Although the circulating antibodies persisted after discontinuing the oligosaccharides, some grafts showed prolonged survival (139).

Systemic and local effects may contribute to the protective effect of accommodation. Long-term (greater than 5 weeks) Lew rat recipients of hamster hearts have normal complement and circulating antihamster antibodies. Yet, a second fresh hamster heart graft is generally accepted (182). Rat recipients of hamster hearts have circulating antibodies to hamster by 3 days after transplantation; a second fresh hamster heart transplanted at that time is promptly rejected. The transplant of hamster hearts from rats undergoing accommodation, however, shows some protection (183). When the accommodated tissue is transplanted into a naive rat, rejection is delayed.

The mechanism of accommodation is unknown. It is not caused by the depletion of antibodies or by the replacement of donor endothelium with host endothelium within the graft. Immunohistochemistry of long-term cardiac xenografts (hamster to rat) shows deposition of IgG, IgM, C3, and C6 on the endothelium, but minimal fibrin deposition. Within the myocardium are numerous $CD4^+$ cells and macrophages; many of the lymphocytes express IL-2R. Cytokine stains reflect a Th2 pattern (IL-4, IL-5) for the $CD4^+$ cells rather than a Th1 pattern (184). The regulatory cells infiltrating the tissues and the secreted cytokines probably provide benefit by blocking the end stage of cell destruction. Terminal complement fixation does not occur. Natural killer cells and effector T lymphocytes are not recruited to the target tissues. Rosengard et al. observed that infiltrating lymphocytes extracted from tolerated allografts had suppressor cell activity with in vitro assays (185). Allografts retransplanted from tolerant recipients into naive recipients were protected from acute rejection (186).

It is unlikely that such a complex defense mechanism evolved for the purpose of transplanting mismatched organs. Possibly, it serves the purpose of limiting the injury caused by some autoimmune reactions, such as a cross-reaction between an infectious agent and the host tissue. Another potential function might be partial protection of the fetus from maternal antibodies that cross the placenta. Assuming that accommodation is a natural process, it is most likely specific and does not interfere with the host's defense against infectious agents.

The duration of accommodation toward an allograft or xenograft is not clear. Possibly it serves as a temporary protection until tolerance develops and the reactive antibodies or lymphocytes are removed. In the absence of tolerance, it is unclear whether accommodation would diminish or whether the bound immunoglobulins or reactive cells would eventually affect the involved tissues. For example, might these immune components contribute to chronic rejection?

C. Immune Tolerance

The mechanisms of immune tolerance have been thoroughly reviewed elsewhere (187). To oversimplify, specific immune unresponsiveness requires both central tolerance and peripheral tolerance. Central tolerance develops in the thymus. Immature T cells rapidly proliferate within the cortex and most of these cells become reactive to self-antigens. Those T cells are eliminated ("negatively selected") at the corticomedullary junction, an area rich in dendritic cells. The self-reactive T cells are greatly reduced in number by the time they enter the medulla and go into circulation. Peripheral tolerance includes mechanisms to protect the host from the self-reactive cells that escape negative selection. Furthermore, even though the thymus undergoes marked involution with age and disease, the host is still capable of mounting a primary immune response to new antigens. Presumably, then, the host is still capable of producing naive T cells. Central tolerance is most important during development and immune reconstitution, deleting the bulk of self-reactive T cells. Peripheral tolerance is needed to enforce tolerance by self-reactive lymphocytes escaping the thymus during development and, later in life to control T cells generated outside the thymus.

How does hematopoietic chimerism contribute to immune tolerance? The initial Medawar study clearly indicates that at least partial tolerance can be achieved through hematopoietic chimerism. The circulating cells can contribute in several ways. Antigen presentation of at least two signals can induce sensitization within naive lymphocytes, whereas presentation of only one antigen leads to anergy. In particular, B cells have been shown to

be instrumental for induction of anergy. Dendritic cells are also instrumental in negative selection within the thymus. When the dendritic cells at the corticomedullary junction are permanently depleted through cyclosporine and mediastinal irradiation, self-reactive cells enter the circulation and cause an autoimmune GVHD. The persistence of active suppressor cell mechanisms may also depend on chimerism. In a model of GVHD, when tolerant lymphocytes were deprived of tolerogenic strain leukocytes for a prolonged period, they lost their ability to suppress GVHD. With stimulation, however, the lymphocytes quickly regained their ability to suppress GVHD in a manner similar to a secondary immune response.

Whether detectable chimerism is either necessary or sufficient for long-term organ graft acceptance, however, is still debated. Starzl et al. (252) found evidence of microchimerism in patients with long-term allograft survival and in two human recipients of baboon liver grafts. Sharabi et al. showed that donor cell chimerism was often lost long before the graft was rejected. Others have demonstrated donor cell chimerism in patients whose allografts have been rejected. Even if a relationship between chimerism and organ graft acceptance is established, it would not necessarily indicate that the chimerism was responsible for the acceptance. Hematopoietic chimerism could simply be the result of immune tolerance.

A related issue is whether tolerance to hematopoietic cells is sufficient or whether tolerance to tissue-and species-associated antigens is also necessary. Initial studies of neonatal tolerance suggested that induction of tolerance to the donor's hematopoietic cells led to tolerance to peripheral tissues as well (188). Medawar was surprised by this outcome, believing that the skin would have additional antigens not present on leukocytes. Further studies with different strains and with different sources of skin did, in fact, show rejection of skin grafts. Using different techniques and strains of animals, multiple tissue-associated antigens have since been identified. Despite tolerance to the hematopoietic cells, these tissues are still rejected. Generally, rejection with a tissue-specific antigen mismatch is milder than with a major histocompatibility mismatch.

Triplett definitively demonstrated the importance of tissue-associated antigens in self-tolerance (189). He removed the pituitary glands from developing tadpoles. Later, when these glands were transplanted back, they were promptly rejected. Similarly, the thyroid gland totally removed from a fetal lamb and subsequently transplanted back into the mature sheep was also rejected. On the other hand, when the thyroid was only partially removed, the sheep later accepted the thyroid autograft (190). Tissue-associated antigens have been identified in multiple tissues, including skin, pituitary, thyroid, eye, and kidney, in autograft and allograft transplants (191). In some of these studies, recipients with donor cell hematopoietic chimerism rejected the tissue.

Although tissue-associated antigens have not been extensively studied in xenografts, they most likely, as with allografts, represent a barrier to successful engraftment. The best demonstration is by Rice et al. in which human hematopoietic stem cells were infused into fetal lambs. Even though the sheep demonstrated stable chimerism, natural antibodies to human endothelium still developed (192).

The ideal system for inducing immune tolerance to a xenograft must induce persistent tolerance to the xenograft, involving peripheral tolerance and probably central tolerance mechanisms. The tolerance must be antigen-specific and should be to tissue-associated and species-associated antigens as well as to the MHC. The tolerance must develop in a relatively immune deficient state. The patient must be protected against opportunistic infections. Finally the patient must also be protected against GVHD.

VIII. CURRENT SYSTEMS FOR PROLONGED XENOGRAFT ACCEPTANCE

Several novel systems have been proposed or developed for establishing long-term xenograft survival. The overall goal of the following systems is to prevent acute xenograft rejection with a minimal degree of toxicity from chemotherapy. Each proposal, supporting data, potential advantages and disadvantages are discussed.

A. Transplantation into Immune Privileged Sites

Antigens placed in certain anatomical locations (i.e., the brain, the testes, and the anterior chamber of the eye) are not rejected. Cultured human retinal pigment epithelial (RPE) cells have been transplanted into the retinal space of rabbits without immune suppression. The transplantation of RPE cells could potentially treat macular degeneration, a common cause of blindness. The transplanted cells became functional and showed prolonged acceptance. By 3 months, however, they showed evidence of early rejection (193). Dopaminergic neurons from a fetal pig were transplanted into the brain of a patient with Parkinson's disease (194). The transplant significantly improved the clinical course of the patient. Seven months later, the fetal pig neurons were identified. They were intact and had extended their axons. The immune reaction was minimal. Rat islet xenografts survived for a prolonged period in nonsuppressed mice, when transplanted into the testes as opposed to the liver, spleen, or kidney capsule (195). Similarly, islet allografts survive long term when transplanted into the brain (196).

Although the cell grafts escape rejection in the immune privileged sites, tolerance does not develop to the graft. When donor strain skin grafts are placed on rats with long-term intracerebral islet grafts, the islets were rejected along with the skin grafts (197).

The local protection of immune privileged sites was initially hypothesized to be the result of physical barrier related to the vasculature. That possibility is now recognized as being too simplistic. Activated T cells can gain access to these sites. However, the sites are deficient in dendritic cells and do not have lymphatic drainage to regional lymph nodes.

Protection of tissues of the immune privileged sites against inflammation may also be derived from localized tolerance. The brain, eye, and testes have increased expression of the Fas ligand (198,199). When lymphocytes carrying the Fas receptor (CD95) encounter Fas ligand–containing cells, they undergo apoptosis, effectively depleting the tissue-reactive T cells. When testicular tissue from a normal mouse was transplanted under the renal capsule of an allogeneic mouse, it survived. In contrast, testicular tissue from mice with a defective Fas ligand was promptly rejected (200). Belgrau et al. (200) have proposed that transgenic animals be produced in which the transplant tissue contains the Fas ligand. Donor tissue would then promptly induce tolerance to itself as the recipient's $CD95R^+$ lymphocytes encounter tissue cells.

Some studies have indicated, however, that the story might not be so straightforward. As expected, the lpr mouse, an autoimmune model of systemic lupus erythematosus, has a deficiency of CD95 (201). However, patients with lupus or rheumatoid arthritis actually have elevated levels of CD95 (202,203). Also contrary to expectation, rejecting allografts show induced expression of Fas on the infiltrating lymphocytes and expression of Fas ligand on the endothelial cells (204).

CD95 can be modulated by various cytokines. For example, IL-4 downregulates the expression of CD95 (205). This is particularly troublesome for a recipient of discordant xenografts. Interleukin-4 is increased in tissues demonstrating accommodation because of the numerous Th2 type helper cells. Thus, accommodation could interfere with this mech-

anism of tolerance. One report has also found that CD95-related apoptosis is blocked by cyclosporine or FK506 (206).

It is unknown why CD95 is expressed in certain tissues at different stages and is not expressed in other tissues. The immune privileged sites are relatively isolated and have limited risk of infection. If a mechanism for ready tolerance or evasion of immune response were present in other tissues, however, the host would be unable to destroy infectious agents or infected cells. A transgenic animal with widespread expression of the Fas and Fas ligand would be effectively immune deficient, making it difficult and expensive to produce. More importantly, infections acquired within the transplanted tissues, such as zoonotic viruses or host viruses, would escape the immune response and induce tolerance to the infectious organisms.

Clinical studies of CD95 ligand–expressing allografts and xenografts may have already been performed. A Chinese study reports that, following human testicular allografts, most of the recipients demonstrated improved testosterone levels, sexual function, or spermatozoa suggesting acceptance (207). In the 1920s, it was the practice of some European physicians to treat impotence with the injection of homogenized monkey or bull testes. If the bull to man transplants reflected the rodent transplants mentioned previously, these patients should have had persistent bovine chimerism. Analyzing biopsies or postmortem tissue from these patients could test this prospect.

B. Intrauterine Bone Marrow Transplantation

As a clinical application of the original Medawar model of developmental tolerance, some researcher have proposed infusing marrow from the animal donor into preimmune human fetuses. After birth, the chimeric newborn should then accept an organ from the donor animal. In parallel, in utero marrow transplantation is being developed for the cure of congenital enzyme deficiencies (208).

Harrison established allogeneic chimerism in monkeys by infusing sex mismatched rhesus monkey fetal liver cells (209). As a preclinical model for transplanting xenografts into chimeric human newborns, Zanjani et al. and Srour et al. established chimerism between discordant species by injecting human stem cells into fetal lambs (210,211). The human stem cells differentiated into erythroid, myeloid, and lymphoid cells. The lymphocytes differentiated into $CD3^+$, $CD4^+$, and $CD8^+$ cells. Xenogeneic chimerism was stable for more than 2 years. Human chimerism was established in fetal baboons when fetal liver cells were infused into first trimester fetuses (212). In general, donor cell chimerism has been limited in the recipient fetus. By purifying and injecting large numbers of stem cells, however, one patient showed greater than 90% donor cell chimerism (K. J. Blakemore, personal communication).

The intrauterine transplantation of stem cells provides several major potential advantages. Even xenogeneic chimerism appears to be stable once established. The intrauterine environment is not only conducive to the induction of tolerance, it is a relatively sterile, protected environment. Thus, the risk of infectious complications during the period of immune deficiency is minimal.

However, there are several limitations to in utero transplantation. First, the most obvious limitation is that it can be applied only to human fetuses with diagnosed congenital diseases. Second, the tolerance would only be against the hematopoietic cells transfused; tissue-associated antigens in subsequent organ transplants would still be antigenic to the chimeric patient. After allogeneic chimerism was induced in two fetal monkeys, kidney

transplantations from the donor monkey were performed 2 and 3 years after birth (213). Mixed lymphocyte reactions between the chimeric recipients and the donors were markedly reduced and the initial course of the graft was markedly better than in the control subjects. Nonetheless, the grafts eventually showed evidence of acute and chronic rejection. In the human to sheep chimeric model, antibody responses to the donor (human) endothelium were measured. Despite persistent hematopoietic chimerism, normal titers of antibodies to the human endothelium developed in the sheep (192).

Third, there is a significant risk of developing lethal graft versus host disease in the injected fetus. This system fully satisfies Billingham's requisites for GVHD. The preimmune fetus is antigenically distinct from the donor, the donor cells are immune competent, and the fetus is unable to reject the donor cells. It is remarkable that all chimeric fetuses do not develop GVHD. In looking at the role of T cells in allogeneic sheep to lamb in utero transplants, unfractionated marrow injected into fetal lambs invariably leads to GVHD (172). When the T cells were depleted, however, the resulting chimerism was limited. When the mature T cells were reduced, however, good chimerism developed without evident GVHD. Apparently, the fetus is able to downregulate a limited number of mature reactive T cells and prevent GVHD.

C. Mixed Chimerism by Bone Marrow Transplantation

Most transplant candidates are beyond the fetal stage of development and would not benefit from intrauterine marrow transplants. An alternative approach is to simulate the intrauterine environment within the xenograft recipient. As is done with bone marrow transplants for the treatment of leukemia, irradiation and aggressive chemotherapy are used to eradicate the recipient's immune system. This allows engraftment of the subsequently infused marrow. As the immune system regenerates, the naive lymphocytes would be in fetuslike environment (i.e. in an environment of great antigen excess). Multiple studies of full engraftment marrow transplants (donor to recipient) demonstrated tolerance to the donor allograft or xenograft. However, lethal GVHD often developed in the recipients or they were immune incompetent (214,215).

To avoid these complications, Ildstad and Sachs proposed that the immune deficient recipient be transplanted with *both* recipient and donor hematopoietic cells, establishing mixed chimerism (216). Mixed chimerism would lead to tolerance to the donor antigens by the donor marrow and to immune competence of the recipient. The recipient marrow provides antigen presenting cells. The T cells are depleted from both marrow populations to prevent lethal graft versus host disease by the donor marrow and donor marrow rejection by the recipient's lymphocytes. The recipient is prepared with chemotherapy or irradiation. As the two marrows repopulate the recipient, the thymus is regenerated with early thymocytes and dendritic cells from both partners donor and recipient. The differentiated lymphocytes are then negatively selected to deplete those lymphocytes reactive to donor or recipient hemopoietic cells. In the periphery, donor- or recipient-reactive lymphocytes become anergic and unreactive.

Mixed chimerism has proved successful in generating specific tolerance to the donor animal. In the rat to mouse transplant model, it has led to prolonged survival of rat skin grafts and pancreatic islet grafts in chimeric mice (217). The reconstituted recipients are tolerant to the donor but remain fully responsive to third party grafts.

Several significant improvements in the mixed chimerism system have been made to

make it more practical for clinical transplants. A less toxic preparation of the recipient without ablating the myeloid elements still provides for good chimerism (218). Antibodies to CD4 and CD8 given before and after the bone marrow transplantation lead to good engraftment of the thymus with donor cells, including dendritic cells, replacing the need for intense thymic irradiation. Finally, the graft can be placed at the time of the stem cell transplantation rather than sequentially following engraftment.

The mixed chimerism system is effective with pig allografts and has shown a partial effect with discordant pig to monkey xenograft transplants (36). The immune response is ablated with whole body and thymic irradiation followed by antilymphocyte globulin. Preformed antibodies were depleted by ex vivo perfusion of the monkey's blood through a pig liver or an immunadsorbant column. Antihuman IgM was administered to block the return of natural antibodies. Using inbred swine, porcine bone marrow was then infused and a kidney transplantation performed. Normal renal function was observed for up to 15 days. Low-level chimerism was present. Eventually, however, the grafts were rejected or the monkeys died of complications.

The value and principal advantage of mixed chimerism is the potential to provide indefinite xenograft survival without the need for chronic immune suppression. The risk of infections, both human opportunistic and zoonotic infections, would be significantly reduced. Because mixed chimerism achieves tolerance in both directions, graft versus host and host versus graft, severe graft versus host disease is prevented.

There are also real and potential disadvantages with mixed chimerism. Although the preparative therapy is significantly improved and is considerably less toxic than clinical bone marrow transplantation, the recipient still experiences a prolonged period of T-cell and NK cell deficiency. If the immune reconstitution depends on the recipient's thymus, then the immune deficiency would be considerably longer for those with thymic involution (i.e., patients who are older or patients with chronic illnesses). During this vulnerable period, the patient would not mount an effective immune response to the opportunistic infection. Indeed, tolerance could be developed to the infectious organisms.

Chronic graft rejection still occurs in both full and mixed chimeras. If the immune tolerance is established against donor hematopoietic cells, the graft could then be rejected by an immune response to tissue-associated antigens. In a fully chimeric rat to mouse model, skin grafts showed early evidence of chronic rejection at approximately 40 days, despite detectable rat hemopoietic chimerism (219). The late rejection of the graft may result from a loss of chimerism (220). In this system, it was speculated that donor-reactive T cells developed in the thymus after loss of the donor dendritic cells (217). Late rejection could also result from a reaction against tissue-associated antigens if tolerance was established only toward the hematopoietic cells.

Both of these problems could be resolved. If the delayed rejection were caused by the reaction to tissue-specific antigens, then the simultaneous transplantation of the graft with the bone marrow infusion should lead to tissue-specific tolerance.

The loss of chimerism appears to be more of a problem with xenografts than with allografts. The xenograft host could have low levels of antibodies against the recipient hemopoietic cells. The host might also lack the appropriate species-specific stroma or cytokines needed to maintain chimerism. Indeed, this was found to be the case with pig to monkey mixed chimeras. Initially, limited porcine chimerism was detectable for only 10 days (36). When the monkey was administered recombinant porcine cytokines, IL-3, and stem cell factor, however, the survival of chimeric pig cells was markedly prolonged (71).

D. Thymic Reeducation

The thymus is the primary source of tolerant mature T cells. Prethymocytes from the bone marrow enter the thymus and proliferate without apparent restraint within the cortex. Thymocytes that react with self-MHC as well as those that do not react with self are generated. Most of the autoreactive cells, however, are "filtered out" or clonally deleted at the corticomedullary junction (214,221). Most of the cells migrating into the medulla and on to the peripheral lymph nodes are not reactive with self antigens.

The corticomedullary junction is lined with dendritic cells. These antigen presenting cells define the antigen specificity of clonal deletion. This lesson was brought home when we studied the paradoxical model of GVHD induced by cyclosporine. When rats undergoing autologous marrow transplantation received both CsA and thymic irradiation, a syndrome resembling GVHD routinely developed. Pathological study of the thymus showed that CsA caused the rapid destruction of the medulla and loss of corticomedullary dendritic cells (222,223). Rats given only CsA promptly regenerated the medulla and replaced the dendritic cells. Rats that also received irradiation, however, failed to regenerate the medulla or replace these cells. Without the corticomedullary filter, the self-reactive cells migrated to the periphery and injured the tissues.

If the xenograft recipient continues to produce T cells, then central tolerance to both self and donor is necessary. To achieve that, it is necessary to reeducate the thymus, that is, to get antigen presenting cells from both the recipient and donor into the corticomedullary junction.

Three basic approaches have been proposed for thymic reeducation. First, the simplest approach is to inject these cells directly into the lobes of the thymus. Second, pharmacological manipulation can be done to initially deplete the APCs from the thymus, followed by reexpansion of the thymus and recruitment of APCs. Third, a preimmune thymus from the fetal donor animal, already populated with donor APCs, can be transplanted into the recipient.

Thymic reeducation is not sufficient to prevent rejection in a mature recipient with an established peripheral immune system. Other measures would need to be combined with it to prevent the peripheral T cells from reacting with the graft. With xenografts, additional measures would be needed for non–T-cell mechanisms of rejection.

1. Intrathymic Injection of Tolerogenic Antigen Presenting Cells

Prolonged acceptance of pancreatic islet allografts was achieved when rats received a single dose of antilymphocyte serum (ALS) and islets were injected into the thymus (224). Injection of islets under the renal capsule did not lead to prolonged acceptance. Rats receiving intrathymic injections were truly tolerant to the grafts. When islets were subsequently transplanted under the renal capsule, they were accepted for a prolonged period. In contrast, recipients that received the initial islets in an immune privileged site (testicle) promptly rejected the secondary grafts. Many successful and unsuccessful studies have been performed with this basic technique (225). The initial immune suppression as provided by ALS appears to be critical. Some studies also suggest that the thymic inoculum must contain tissue-associated antigens (226).

As with allografts, prolonged acceptance of rat xenografts in diabetic mice has been accomplished (225,227). The same methods have not led to engraftment of mouse islets

within rats, however. Sheffield found that, unlike in rat allografts, additional immune suppression with cyclophosphamide was necessary to attain prolonged survival of hamster heart xenografts (93,228). To the best of our knowledge, intrathymic injection has not yet been tested with swine xenografts.

Intrathymic injection can be an effective method for inducing long-term central tolerance. One attractive feature is that the tolerogens are well defined. The induced tolerance is limited to the thymic inoculum, helping to minimize the risk for tolerance development to an infectious organism. By itself, however, intrathymic injection has several limitations. The need to severely suppress the peripheral immune system puts the recipient at risk for infection with opportunistic organisms. Intrathymic injection has not been as successful with larger animals as it has with rodents. Most significantly, this approach requires a thymus. It might be difficult to inject recipients with thymic involution, such as those who are older or those with chronic illnesses.

2. Recruitment of Tolerogenic Antigen Presenting Cells into the Thymus

Cyclosporine initially provided an intriguing set of paradoxes with regard to immune tolerance. Whereas the initial animal studies promptly induced tolerance to allografts after a short course of CsA, clinical studies with prolonged CsA usually failed to induce tolerance (229,230). Cyclosporine destroys the thymic medulla and corticomedullary APCs, the very cells responsible for tolerance (223). As discussed, a combination of CsA and thymic irradiation leads to an autoimmune reaction resembling GVHD (222,231).

These contradictions are resolved by our alternative hypothesis that CsA does not induce tolerance directly, but rather it creates space within the thymus by inducing medullary involution. As the thymus recovers after stopping the CsA, new tolerogenic dendritic cells are recruited into the thymus, reeducating the thymus and redefining the antigen specificity of tolerance. By combining a short course of CsA with the timed infusion of allogeneic spleen cells, the thymus is repopulated during post-CsA recovery by the allogeneic dendritic cells (232). Recruitment was not observed when the rats were suppressed instead with steroids. Steroids cause cortical involution rather than medullary involution. Thymic chimerism was associated with a significant and specific prolongation of skin allograft survival (233). Indefinite survival was not achieved in these studies, but no provisions were taken to block peripheral immune reactions. When thymic reeducation by recruitment was combined with ablation of peripheral T cells and bone marrow transplantation, indefinite skin graft survival was achieved (237).

The pharmacological method of reeducation offers significant advantages over the intrathymic injection method. Most notably, it can be used in situations with thymic involution. Growth hormones or select cytokines can be administered during the post-CsA period to enhance the reexpansion and repopulation of the thymus. The enhanced growth of the thymus could also lead to heightened immune competence while inducing specific tolerance. Because the method is systemic rather than local, recruitment may repopulate extrathymic sites of T-cell generation.

This method of thymic reeducation has not yet been tested in a xenogeneic system. It is also unknown whether recruitment will induce tolerance to tissue-associated antigens. There is a real risk that tolerance to infectious agents may develop in the host by recruiting infected dendritic cells. Because the thymus recovers within 10 days, however, the vulnerable period is relatively short.

3. Transplantation of Preimmune Fetal Thymus from the Donor

Rather than repopulating the recipient thymus with donor APCs, the actual thymus from the donor animal can be placed in the recipient. Not only would the dendritic cells be of donor origin, the thymic epithelium, which might be essential, would be from the donor (238). Transplants of allogeneic thymuses depleted of hemopoietic cells lead to tolerance as defined by the antigens on epithelium. Accordingly, Zhao et al. have proposed that, in addition to performing a xenogeneic mixed chimerism bone marrow transplant, the recipient also receive a fetal thymus from the donor animal (77). As the immature donor and host T cells repopulate and mature within the transplanted donor thymus, they would become tolerant to the donor animal.

Pig grafts have survived indefinitely on thymectomized mice reconstituted with mixed pig and mouse marrow and a fetal pig thymus (77). The tolerance is specific in that the mice reject third party pig or mouse skin grafts. The mice are immune competent at the time of the skin transplant. The study constitutes a major milestone in establishing that permanent and specific tolerance can be established for grafts from a notably disparate xenogeneic donor. Besides providing porcine MHC antigen, the fetal pig thymus most likely provided an environment favorable to prolonged chimerism. Porcine class II+ cells, probable dendritic cells, were evident 30 weeks after transplantation. The V β region of T-cell receptors were analyzed, demonstrating that clonal deletion was a major component of the induced tolerance. It is unknown how well donor thymus transplants work in large animal transplant models.

Once the host has restored its immune competence, the tolerance appears to be long lasting and highly specific.

A major issue for clinical application is the prolonged period of immune deficiency resulting from the destruction of the recipient's T lymphocytes. The T cells must then be reconstituted through the fetal pig thymus. In mice that were not thymectomized, pig-reactive T cells developed. Reconstitution through the fetal pig thymus is the rate-limiting step for reconstitution. In the mouse model, full reconstitution required more than 17 weeks. Furthermore, it is apparently necessary to use a second trimester fetal pig thymus, which is very small. A larger thymus from a later fetus or newborn is normally resistant to engraftment by the recipient cells. Therefore, in larger recipients, including humans, the period of immune deficiency could be even longer. There would also be a greater risk of graft versus host disease from the immune competent mature pig thymus.

Although the pig to mouse model is discordant, it is unclear what measures were taken to prevent the fixation of antibodies and complement. In pig to human transplants, these measures would be needed for a prolonged period to preserve porcine chimerism. Because accommodation for reactive antibodies involves immune regulation by $CD4^+$ Th2 cells, the period of immune deficiency may delay the development of accommodation.

It is unclear whether T-cell maturation through a pig thymus would provide tolerance to tissue-associated antigens. Skin graft survival is considered one of the most stringent tests of immune tolerance. In this system, however, the issue of tolerance to tissue-specific antigens is avoided. The porcine fetal thymus consists primarily of epithelial cells and dendritic cells, similar to the cells constituting the split-thickness porcine skin grafts.

E. Inhibition of T-Cell Costimulation

Effective T-cell activation requires multiple signals between the T cell and the antigen presenting cell, in addition to the binding of the T-cell receptor to the corresponding antigen pre-

senting molecules. A major second signal is the CD28 receptor on the T cell (236). When it binds to the corresponding B7-1 or B7-2 ligand expressed on the APC, CD28 induces the production of interleukin-2. If the second signal fails to occur, the T cell becomes unresponsive or anergic.

CTLA4 Ig is an immunoglobulin that binds to the B7-1 and B7-2 molecules, effectively blocking the needed stimulation of the T cell CD28. Injecting CTLA4 into xenograft recipients has led to prolonged acceptance of cardiac and pancreatic islet xenografts (107,237). Graft survival was further prolonged when this was combined with antibodies to T-cell subsets. Eventually, however, the grafts were rejected by a humoral reaction. Although the CD28-B7 costimulation represents a primary second signal, it most likely is not the only second signal that could lead to sensitization.

The CD28 receptor on human T lymphocytes recognizes and binds to the corresponding B7-2 ligand on pig endothelial cells. Interruption of the costimulation with CTLA4, therefore, could contribute, in part, to preventing porcine xenograft rejection. A recognized risk of inducing anergy within the recipient is that tolerance could inadvertently be induced to infectious agents as well.

The repeated injections of antibodies like CTLA4 could lead to an immune deficiency by permanently impairing costimulation. For example, the ligand molecules on the APC could be masked or downregulated. Alternatively, an anti-idiotype antibody to CTLA4 could react with the corresponding receptor on the lymphocytes. The treated subject would then be unable to mount an effective immune response against any subsequent infection.

Inhibition of costimulation would need to be supplemented with other therapies to prevent T-cell–independent reactions against the xenograft. If the recipient continues to produce new xenoreactive T cells, continuous therapy would be necessary to prevent later rejection.

F. Peritransplant Infusion of Donor Cells (Donor-Specific Transfusions)

In reviewing the initial clinical trials of kidney allotransplants, it was unexpectedly discovered that transplant recipients with a history of multiple transfusions before transplantation experienced a longer allograft survival rate than those without transfusions (238,239). Consequently, until cyclosporine came into widespread use, candidates were intentionally transfused. The donor-specific transfusion (DST) phenomenon has been further studied in animal models (240). Transfusions are typically performed along with temporary immune suppression or antibodies to $CD4^+$ T cells.

The transfusions probably induce multiple mechanisms of tolerance. As discussed elsewhere, the resulting chimerism participates in the two-way paradigm (graft versus host and host versus graft reactions) of transplant tolerance (241). Two-way tolerance results with the resolution of these reactions. Chimerism also leads to repopulation of the tolerance-defining tissues such as the thymus (so-called "reeducation of the thymus"). The donor dendritic cells at the corticomedullary junction effectively deplete many of the donor-reactive T cells produced by the thymus.

The optimal development of DST-associated tolerance depends critically on the timing of the immune suppression and donor cells (242). The transfused donor cells have a direct effect on the peripheral immune system, inducing an antigen-specific reduction in immune responsiveness. Immune regulatory cells, including suppressor cells, are involved. Moderate prolongation of xenograft survival has been achieved when donor-specific transfusions are combined with chemotherapy or antibodies to T cells (243,244). In comparison

with allografts, however, xenogeneic transfusions are more likely to lead to sensitization than to tolerance (245).

G. Surrogate Tolerogenesis

The prevention of xenograft rejection has, in the past, concentrated on modifying the immune reactivity of the recipient *within the recipient.* We propose, instead, to induce chimerism and immune tolerance outside of the recipient, *within the xenograft donor,* a process termed "surrogate tolerogenesis" (246). Tolerant lymphocytes and corresponding xenografts would subsequently be transplanted back into the recipient. The induction of tolerance within the donor animal provides a great deal more flexibility and opportunity. Instead of selecting a subpopulation of patients suitable for tolerance, we can easily define the optimal conditions in the animal donors. For example, by conventional methods, developmental tolerance was limited to fetal or newborn patients. With surrogate tolerogenesis, however, developmental tolerance can be established within fetal donor animals and applied to patients of any age.

In this procedure (Fig. 1) bone marrow is aspirated from the intended xenograft recipient. The mature T cells are partially reduced and the cells infused into fetal pigs. After the birth of the litter, the best pig is selected. The splenocytes from the chimeric pig are harvested and infused back into the patient. If the chimeric cells provide immune tolerance to the chimeric pig, then a xenograft from the corresponding pig should be accepted.

The patient would be spared the need to eradicate the patient's immune system, because tolerance is induced outside of the patient, within the immune-deficient fetal pig.

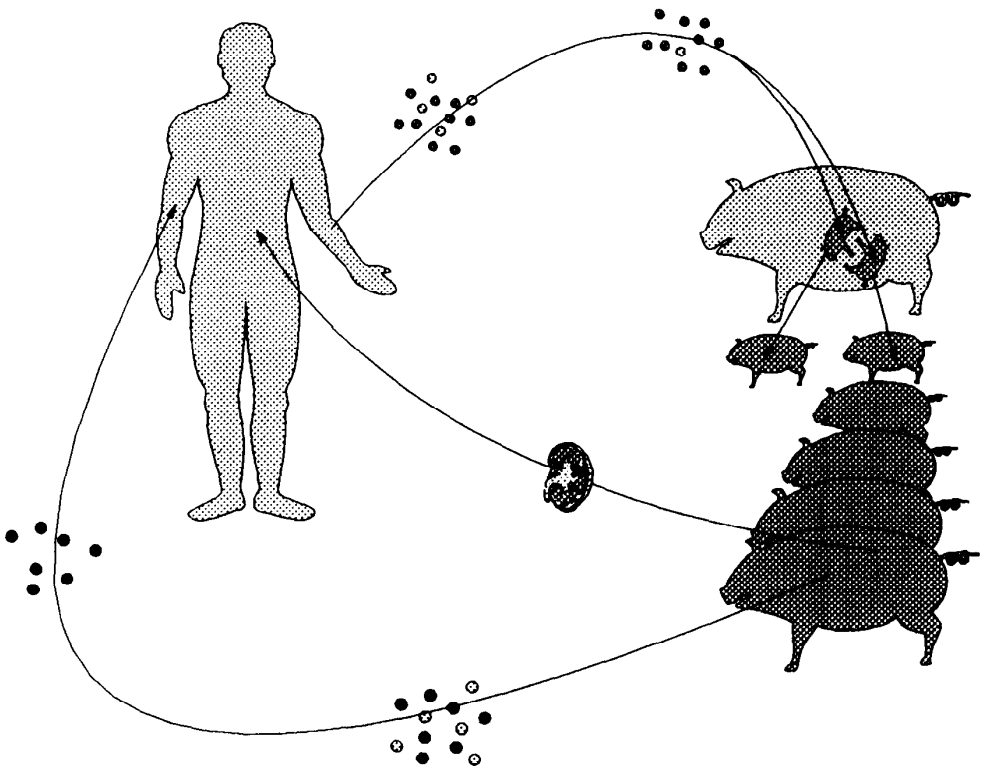

Figure 1 Surrogate tolerogenesis induces tolerance of the patient's lymphocytes to the animal donor *outside* of the patient, within the animal door.

Surrogate tolerogenesis uses not only clonal deletion and anergy but also takes advantage of the active suppressor cell mechanisms, which protect the fetus against differentiated maternal lymphocytes. The induction of antigen-specific suppressor cells outside of the recipient allows the adoptive transfer of tolerance to the recipient without the need to destroy the patient's immune system.

In contrast to induced tolerance within fetal recipient, however, the tolerance within the fetal donor would be to all tissue-associated antigens, not just to the hematopoietic cells. Because the human cells infused into fetal pigs contain B cells capable of producing natural antibodies, surrogate tolerogenesis would most likely lead to accommodation before the back transfusion or transplantation is performed.

Surrogate tolerogenesis provides advantages through multiplicity. When tolerance is attempted in the transplant recipient, the outcome of the transplant rests on the single attempt. If tolerance is induced within donor animals, however, multiple attempts can be made and the best chimeric animal can be chosen for transfer and transplant into the recipient. If tolerance does not develop in any of the transfused donor animals, the patient experiences only an irritating delay in the procedure.

Surrogate tolerogenesis provides three modes of protection against zoonotic infections. First, using fetal surrogate and donor animals, surrogate tolerogenesis leads to immune tolerance within a sterile environment. Thus, the risk of developing tolerance to infectious agents is minimized. Second, the co-culture of human lymphocytes with porcine blood and tissues takes place before transplantation, outside of the patient. The risk of activating a virus infection during lymphocyte activation is shifted to the pretransplant period. Third, the chimeric donor, including patient's lymphocytes, could be screened for zoonotic infection before transfusing the cells back to the patient.

Engraftment of the patient's cells within the donor before organ transplant could provide regional advantages. To the extent that antigen-bearing cells such as endothelial and dendritic cells are replaced with the patient's cells, the graft resembles more the patient than the animal donor. Reconstitution of regional lymphoid tissues, such as that of lung or intestine, means that these tissues would be protected from infectious complications in the early posttransplantation period.

The initial supporting studies consisted of infusing marrow from human volunteers into early second trimester fetal pigs (247). Mature T lymphocytes were depleted from 75% of the infused marrow cells. Human chimerism was detectable in the cord blood or peripheral blood of the chimeric piglets at term (up to 29%). Human lymphocytes and dendritic cells were observed by immunohistochemistry in the piglet tissues, including thymus, skin, and spleen tissue.

Mixed lymphocyte reactions (MLR) were performed, using fresh human cells from human volunteers as responder cells and irradiated lymphocytes from the respective chimeric pigs as the stimulator cells. When small numbers of viable chimeric cells were added to the MLR, the human responder cells were specifically suppressed. Whereas suppression is typically assessed with approximately equal numbers of responder cells and putative suppressor cells, these assays detected suppression with as few as one chimeric lymphocyte per 300 responder cells. The chimeric cells did not suppress the reaction to third party pig stimulator cells or the reaction of unrelated human responder cells. Neutralization experiments using monoclonal antibodies specific for human leukocytes demonstrated that human chimeric lymphocytes were essential for the observed suppression.

Although the experiments are interesting, the key issue is whether the in vitro results would translate into prolonged pig xenograft survival. In particular, can the chimeric piglet provide sufficient functional suppressor cells for a recipient the size of an adult human?

Sheep were used as the pig xenograft recipients (248). They approximate adult humans in size and acutely reject pig xenografts in a manner similar to the rejection in primates in which hyperacute rejection is prevented. Sheep do not constitutively produce natural antibodies to pig cells, but they are readily sensitized. Sheep marrow was processed and infused into early fetal pigs as with the human-to-pig studies. Because of considerable fetal mortality, newborn pigs were infused with a larger number of unfractionated sheep cells. After the fetal pigs were born, the sheep was prepared with a single dose of cyclophosphamide (Cy, 35–50 mg/kg intravenously) and 1 week later they were infused with chimeric piglet splenocytes and a vascular xenograft. In rodent models, this dose of Cy was shown to facilitate the engraftment of transferred syngeneic cells, but had no significant effect on the rejection of heart allografts. The pig xenograft consisted of a length of muscular thoracic aorta placed as a fistula between the carotid artery and jugular vein. Control groups included sheep treated with Cy and an age-related pig xenograft (Cy control) and sheep treated with Cy, normal age-related splenocytes, and a pig xenograft from the respective normal pig (DST control).

The median graft survival for the Cy group (n = 3) was 6 days and for the DST control group (n = 4) was 7.5 days. In contrast, the experimental animals (n = 4) had a median graft survival of 77 days (75 and 88 days for sheep whose cells were infused into fetal pigs, 14 and 79 days for sheep whose marrow was infused into newborn pigs).

The sheep experienced a mild transient decrease in peripheral blood mononuclear cells (PBMCs) (200 to 1200 at the time of transplantation). The counts returned to normal within 3 weeks. No precautions against opportunistic infections were considered necessary and no problems were experienced. Graft versus host disease has not developed in any of the recipient sheep either clinically or histologically (skin biopsy).

Titers of cytotoxic antibodies to pig leukocytes were assessed on a weekly basis. The control animals had a maximum titer of 256 (range 128 to 512). A maximum titer of only 16 or 32 developed in the experimental animals.

To the best of our knowledge, the vascular graft survival is the longest observed for a pig xenograft without the need for either pretransplant immune ablation or posttransplant chronic immune suppression. Nonetheless, the survival was not permanent. One possible explanation for the late rejection is that the sheep lost the porcine chimerism from a lack of porcine growth factors. A second possibility is that the transfer of tolerant cells back to the sheep established peripheral tolerance but not central, thymus-based tolerance within the sheep. Either of these problems could be corrected, the first by providing the recombinant factors or the stroma responsible for the factors. The absence of central tolerance could be resolved by transplanting a chimeric thymus from the infused pig or by reeducating the recipient thymus to recruit porcine dendritic cells as discussed previously.

A drawback of surrogate tolerogenesis is the need to wait for the xenograft. A patient requiring a life-saving heart or liver transplant would not benefit. Pigs have a short gestation period and grow rapidly after birth. Thus, the typical wait would be only about 6 months for an adult-sized organ, which is less than the average wait for a human donor. The waiting period could be further shortened to about 2 months by using the fetal pig as a source of suppressor cells and an identical mature pig, such as an inbred or cloned pig, as the source of the organ transplant.

H. Chimerism Within the Donor Graft

In a manner similar to surrogate tolerogenesis, Starzl et al. have proposed establishing human chimerism within donor animals before transplantation, or "humanization" of the

donor animal. The two-way paradigm of chimerism (GVHD, HVG) is established within the organ donor before transplantation.

Valdivia demonstrated moderate prolongation of rat xenograft survival in the mouse to rat cardiac xenograft model (249). Lethally irradiated mice were reconstituted with recipient rat marrow. The chimeric hearts were then heterotopically transplanted into the rat. Whereas the controls rejected the mouse grafts in approximately 2.5 days, the recipients of the chimeric grafts rejected the grafts in 9.4 days. FK506, which showed no effect on the control animals, prolonged the survival in the experimental animals to 20.7 days.

Starzl et al. have begun developing a herd of "humanized pigs" or "golden pigs" (250). Stored human marrow or baboon marrow has been infused into newborn pigs. Many of these recipients have shown microchimerism for human cells more than a year later. In vitro assays demonstrate reduced proliferation in response to stimulator cells from these chimeric pigs. The two-way paradigm or immunological battle taking place in the chimeric pig between the generic primate cells and the pig cells could potentially lead to partial accommodation or tolerance and eliminate the reactivity to the numerous xenogeneic antigens. The resulting organ would then resemble an allograft rather than a xenograft.

The use of chimeric organ donors would provide many of the advantages of surrogate tolerogenesis, including the advantages of transplanting multiple donors and establishing tolerance before organ transplantation. Furthermore, this approach would have the advantage over surrogate tolerogenesis in that the patient would not need to wait for the transplant. The procedure could be performed as needed. However, although the chimerism in the donor is of the same species as the recipient, it is allogeneically disparate with respect to the recipient. Whereas surrogate tolerogenesis aims to make the xenograft simulate an autograft, humanization aims to make the xenograft simulate an allograft.

To prevent the recipient from rejecting both the human allogeneic cells and the porcine cells, the recipient would receive cytoablative therapy and a marrow transplant from the chimeric pig, then the xenograft. It is unknown how well the predominantly porcine marrow would function in the recipient. Would engraftment be complete and stable? Would it be difficult to prevent graft versus host disease by the porcine or allogeneic primate cells? Would the host recover immune competence in a timely manner?

The anticipated experiments transplanting marrow and xenografts from the chimeric pigs back into the recipient baboons should resolve these questions.

IX. SUMMARY

With the steady improvements in outcomes for human kidney allografts, the population of qualified candidates who do not receive renal allografts rapidly expands. There is a great deal of interest and even pressure to develop alternative sources of kidney grafts, including the use of animals. Animal donors, particularly swine, could provide an unlimited source of xenografts.

Xenografts offer other significant advantages over human allografts, including protection from human viruses such as HIV and hepatitis, the ability to perform transplant procedures on an elective basis, and a reliable and uniform supply of healthy young organs. However, there are substantial obstacles to xenotransplantation, some hypothetical and some real. The functionality of xenografts will depend on the complexity of the organ. Porcine renal xenografts appear to function appropriately within primates, at least over the short term. The potential risk of acquiring a zoonotic viral infection is also theoretical. The risk is thought to be considerably less for xenografts from swine than from primates.

The obstacle of rejection, on the other hand, is a real problem. Rejection has proved to be far more vigorous than allograft rejection. Several years ago, most researchers believed that hyperacute rejection resulting from preformed natural antibodies and complement fixation was the primary obstacle. They believed that acute rejection would be preventable with routine immune suppression. That optimistic view proved to be false. If anything, acute xenograft rejection is even more ominous than hyperacute rejection.

The initial false sense of optimism was fortuitous for the xenotransplantation field. Had we seen that two mountain ranges had to be crossed, we might never have begun the journey. However, several techniques have since been developed that are capable of preventing hyperacute rejection of discordant swine xenografts, including the use of genetic engineering, immune adsorption, and immune regulation. Furthermore, the institutions, technology, and personnel are set up to focus on the issue of acute xenograft rejection, to understand the pathophysiology, and to develop innovative solutions.

The substantial threat of rejection is more than offset by the substantial opportunities provided by the use of animals. There is relatively little that can be done to the human allograft beyond screening, matching, and short-term organ preservation. In contrast, there are unlimited possibilities with animals, including breeding and raising them under defined conditions, genetic alterations with human genes, and generation of chimerism with recipient or human hematopoietic cells. Reduced to the essentials, the ideal xenograft system would provide prolonged acceptance of the xenograft without the risks of infection, toxicity, or graft versus host disease.

With the agents currently available, chemotherapy alone will not prevent acute xenograft rejection. The marked suppression required to prevent the pervasive immune reactions against the xenograft is invariably associated with lethal toxicity or opportunistic infections. Taking advantage of the opportunities provided by xenografts, several innovative new methods have been proposed or are being developed for the goal of prolonged xenograft survival. They use methods for achieving partial antigen identity, accommodation, and immune tolerance.

There are plenty of grounds for optimism. Currently, we know of no demonstration of indefinite survival of a vascular xenograft in a large animal recipient. However, the initial data supporting new systems for prolonged xenograft survival are most promising. It appears quite possible that prolonged xenograft survival will soon be achieved with fewer complications than are presently seen with human allografts.

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Histocompatibility and Organ Allocation

JAMES F. BURDICK
The Johns Hopkins University School of Medicine, Baltimore, Maryland

JOHN S. THOMPSON
University of Kentucky, Lexington, Kentucky

I. INTRODUCTION

The factors governing the destination of a donated cadaver kidney include those derived from underlying biological factors and those designed to serve society optimally. In this chapter, the major histocompatibility complex (MHC) underlying these rules of allocation is reviewed and the history, structure, and activities of the United Network for Organ Sharing (UNOS) in the practical application of this knowledge are summarized. The science of the human leukocyte antigen (HLA) system as it relates to clinical allotransplantation is relatively advanced; however, the effort to provide the optimal allocation of kidneys and, in particular, the dialogue on the degree to which the HLA system should impact this allocation, are areas that remain in evolution.

II. HISTOCOMPATIBILITY

Blood group antigens A, B, and O have long been recognized as major histocompatibility determinants. Incompatibility with respect to these antigens has been associated with hyperacute rejection of renal allografts (1) and markedly reduced graft survival in the absence of hyperacute rejection (1,2). Consequently, ABO compatibility is one of the prime factors considered in organ sharing. Unfortunately, these blood group antigens are not

expressed equally among the races (3); specifically, blood group B occurs in 9% of black American and 29% of Asian American donors in contrast to blood group A in 40%, 25%, and 17%, respectively. These racial differences in ABO are further complicated by the observed racial differences between the recipient and donor groups (4); approximately 80% of all donors are white, whereas approximately 31% of recipients are black American, a major factor in prolongation of the waiting time for black and Asian recipients. Individuals with AB blood type have the highest probability of a short waiting time for an ABO compatible allograft and O recipients have the lowest probability.

ABO incompatibility is also a cause of both hyperacute rejection and poor survival in cardiac and lung transplants. It does not lead to hyperacute rejection of liver transplants but it has a significant effect on long-term patient and graft survival (5).

A complex group of closely linked antigens has been identified in every mammalian species studied that together form the MHC. These have been studied most extensively in mice and humans. In humans, this complex consists of hundreds of genes that are generally separated into two classes of molecules that differ in composition, function, and location on chromosome 6. The products of these genes form the extremely polymorphic class I and class II molecules known as the HLAs.

Class I HLA molecules are composed of an invariate non-HLA 12-kD light chain, β-2 microglobulin and a 45-kD heavy chain that is encoded within the HLA complex. There are as many as 20 to 30 functionless class I pseudogenes in addition to the three loci that encode for the important HLA-A, HLA-B, and HLA-C histocompatibility determinants (6). The heavy chain, which determines the polymorphism, folds into three domains of 80 to 90 amino acids each. The α1 and 2 domains appear to contain the most hypervariable regions, and the α3 domain is the most conserved. Twenty-seven alleles have been identified by serological means at the HLA-A, 58 at the HLA-B, and 10 at HLA-C loci, and these numbers are being greatly expanded by DNA technology; for example, at least 142 HLA-B alleles have been identified by DNA analysis.

Class II molecules are heterodimers composed of a 31 to 33-kD α- and a 29-kD β-chain, both of which are encoded in the HLA complex. Three loci have been identified: HLA-DR, -DQ, and -DP. Within HLA-DR, there are nine identified β-chains associated with a single α-chain. DRβ1 is the most polymorphic with approximately 100 alleles being identified. DRβ3 (DR-52) had four alleles, DRβ4 (DR-53) 3 alleles, and DRβ5 (DR-51) five alleles as of most recent count. There is variability in both α- and β-chains of the DQ molecules, with at least 15 DQ α alleles and 26 DQ alleles. Similarly, the DP locus is highly polymorphic, with eight DP α and 59 DP β genes recognized. Whereas HLA-DR and DQ are closely linked, the locus for HLA-DP is located on the other side of the centromere of chromosome 6 and does not closely segregate with the other HLA complex antigens on the short arm of the chromosome.

In all mammalian species studied, transplant survival is predominantly influenced by the degree of MHC compatibility. If all of the antigens of each locus were inherited in a completely independent fashion from each other, there would be little possibility of finding an unrelated match for the three HLA-A, -B, and DR-β1 loci and even less if HLA-C, -DQ, and -DP were also included. Linkage disequilibrium, however, exists between alleles of these loci such that antigens at HLA- A, -B, -C, -DR, and -DQ may be inherited as a unit. Even so, the most common haplotype in whites, composed of HLA-A1, -B8, and DR3 -is found in only 3% to 6% of the population, and other combinations of these loci are more rare, particularly in the other races (7). Consequently, immunization to HLA antigens through multiple transfusions, pregnancy, and prior transplantation commonly occurs and

may result in preexisting alloantibody that can initiate hyperacute graft rejection (1,2). Sensitization to HLA is identified by reacting patient's serum with a panel of cells expressing a broad range of HLA antigens. The resulting panel reacting antibody (PRA) of patient's serum provides a good indicator of the degree of sensitization to class I HLA-A and B-antigens. Patients with high PRA are more likely than patients with low or absent PRA to have a positive cross-match with potential donor lymphocytes, hence increasing the possibility that they will wait for a longer period of time for a compatible donor. The most important factor restricting access to transplantation is the prolonged waiting time resulting from sensitization to HLA class IA and B antigens (8). In addition to its profound influence on access, HLA sensitization has a significant effect on outcome. For example, 1-year graft survival of retransplants in patients with a PRA less than 10% was 73% compared to 61% in patients with a PRA greater than 50% (9).

With the clear exception of prior sensitization to HLA antigens limiting access and graft function (8,9), the impact of HLA histocompatibility on the outcome of cadaveric kidney transplantation has been more controversial. Several reports from large single centers (10–13) have failed to observe a benefit of careful HLA matching but, in most, the number of well-matched transplants has been too small to reach statistical significance (14). Although retrospective analyses of large numbers of kidney transplants have clearly demonstrated that short-term and, particularly long-term, graft survival correlate with the degree of HLA-A, -B, and -DR compatibility (15–17), the impact of HLA matching on improved survival of kidney transplantation in toto is controversial. Laffell et al. (7) concluded that, with the exception of phenotypically identical matches, HLA matching had little effect on organ distribution because of the comparative rarity of most "private" HLA antigens plus the differences in frequency of these antigens between donors and recipients and between racial groups. Held et al. (18) noted that maximal matching for conventional HLA antigens could potentially decrease the number of HLA mismatches but this would increase 5-year graft survival by only 3.4% and reduce the percentage of kidneys allocated to blacks by 22.2% to 15%. Thus, controversy also centers around whether allocation based on matching differentially limits access to minorities, and has led some groups to advocate removing or further decreasing HLA matching criteria from the UNOS kidney sharing algorithm (19). Many of the conclusions for or against HLA matching as a major criteria for cadaveric kidney allocation, however, can be criticized because they are univariate studies, and many factors impact collectively on outcome, such as prior sensitization, race, ischemia time, transplant center effect, and gender (20).

In 1988 the United Network for Organ Sharing (UNOS) began collecting data from all transplants performed in the United States, and there is unequivocal evidence documenting excellent allograft survival of HLA-O, -A, -B, and -DR mismatched cadaveric kidneys (17). Only a small percentage of the total kidney transplant recipients during this period benefited from such mismatching, however, and this number was disproportionately lower in black (1.2%) as compared to white (5.8%) recipients. Most patients received kidneys mismatched for three or four HLA-A, -B, and DR antigens (21), which is not surprising in light of the relative infrequency of most HLA antigens and the corresponding rarity of even the most common phenotypes (7). Furthermore, most common haplotypes in white recipients differ from those of White donors because of the association of certain antigens and phenotypes with disease leading to kidney failure (e.g. diabetes) and because some HLA antigens are disproportionately expressed in the different races (22). For example, HLA-A24 was expressed on the lymphocytes of 40.1% of Asian American volunteer bone marrow transplant donors in contrast to 17.0% of white and only 6.3% of black American

donors; HLA-A30 was expressed in 22.1% of black American donors as contrasted to 4.7% of white and 3.9% of Asian American donors; and HLA-A1 was expressed on 29.6% of white donors in contrast to 11.5% of black American and only 6.4% of Asian American donors (22). Here again, strict conventional antigen matching between a predominantly white donor pool and minority recipients could substantially decrease the possibility of a phenotypic match for both black and Asian Americans.

In addition to conventional private HLA-A and -B, antigens, which are the basis of the UNOS donor-recipient matching algorithms, the existence of "public" antigens has been documented by many investigators. They were first observed after immunization with a single mismatched HLA-A or -B antigen, which resulted in antibodies that reacted with a much broader range of class I antigens. Analysis by several investigators of these and other observations following transplantation has led to the description of cross-reactive groups of these "additional reactions" called CREGs. Rodey et al. (23) documented that the serum of highly sensitized patients, that is, PRA greater than 60%, was composed of a small number of antibodies directed at public antigens rather than multiple antibodies directed at private antigens. In sharp contrast to the infrequency of most HLA class IA or B private antigens, class I public antigens (CREGs) are much more frequent for example, between 35% for the 8C CREG (composed of B8, 63, 64, 18, 38, and 39) and 88% for CREG AIC (composed of A1, 3, 11, 23, 24, 25, 26, 29, 30, 31, 32, 33, 34, 36, 66, and 74) (23). Furthermore, CREG A1C includes antigens that are more or less common in white, black, and Asian Americans (i.e., A1 in whites, A24 in Asians, and A30 in blacks); hence, racial class I HLA private antigen distinctions are blurred within CREGs. In addition to the CREGs described by Rodey et al. (24), other methods of defining them have been reported by Koneda et al. (25), Duquesnoy et al. (26), and Takemoto et al. (27). Of these researchers, Takemoto et al. (27) have based their definition on the identification of common amino acid residues rather than on empirical antibody responses, but there is close correspondence of the private antigens included within the definitions of CREGs by the other investigators.

The question arises as to whether these more frequent public antigens are significant determinants of transplant histocompatibility. Takemoto et al. (27) retrospectively compared 1-year graft survival of one conventional but zero public, antigen-mismatched transplants using their 11 amino acid residue defined CREGs (84%), the 14 public antigens of Rodey and Fuller (85%), the 41 of Duquesnoy et al. (86%), and the 108 of Koneda et al. (88%). The data showed that they all identified compatibility beyond that of conventional HLA matching. Sanfilippo, et al. (28) retrospectively analyzed 3811 cadaver transplants performed between June 1977 and July 1982 by Southeastern Organ Procurement Foundation (SEOPF) centers and reported that matching for the 14 public antigens of Rodey et al. (23) benefited a greater number of patients, with the same or better graft survival than that of conventional class I private antigen matching. Takemoto et al. (27) described the potential impact of matching for the 11 amino acid residue defined specificities versus conventional HLA class I antigens on cadaveric kidney transplant access and outcome; 1-year graft survival rate for O A, B, and DR conventionally mismatched transplants was 87% followed by an 18-year half-life, compared with 1-year graft survival rate of 84% to 88% and a half-life of 12 to 15 years for O CREG, O DR transplants. They observed less than 10% of O CREG, O DR transplants were performed but forecast that this number could have been increased to 90% with national sharing or to 66% with local sharing.

Between the initiation of the UNOS registry in October 1987 and September 1995, 12,536 first transplant cadaveric kidney transplantations were performed by centers associated with SEOPF (29). Of these, 39.5% were transplanted into nonwhite recipients, offering

the opportunity to test whether class I public antigen matching could benefit minority races to an equal or greater degree than conventional class I matching. For this analysis, a slight modification of the 14 CREGs of Rodey and Fuller (23) was again used. Access and outcome of CREG matching was compared at 1 and 3 years after transplantation to that of O B, DR; 1 B, DR and 2 B, DR mismatched kidney transplants, that is, those receiving 7, 3, and 1 points in the current UNOS sharing algorithm. Two CREG, DR groups were identified that resulted in superior 3-year graft survival for a greater number of both white and particularly nonwhite recipients. Although the total number was still small (i.e., 3.2%), 0 CREG, ODR mismatches nearly doubled the number of well-matched nonwhite transplants. Furthermore, inclusion of the second group of 1–3 CREG, O DR mismatched transplants increased the percentage to 25.6% of 4862 nonwhite recipients, with 3-year graft survival superior to the mean, as contrasted to only 13.5% of O B, DR plus 1 B, DR mismatched transplants. The percentage of 7011 white recipients with increased 3-year survival was also increased (i.e., 27.6% of 0 CREG, ODR plus 1–3 CREG, ODR versus 20.0% for O B, DR plus 1 B, D R mismatches).

Human leukocyte antigen matching has also been shown to improve cardiac transplant survival. Opelz et al. (30) reported that the 3-year graft survival of 0, A, B, DR plus 1A, B, DR mismatched allografts was 83% ± 4% as contrasted to 76% ± 2% for 2 A, B, DR and 71% ± 1% for more than 2 A, B, DR mismatched hearts. They also noted that cold ischemia time greater than 4 hours had a detrimental effect on 3-year outcome: cold ischemia < 4 hours = 73% ± 1% survival > 4 hours = 65% ± 3% survival. This short window essentially excludes national or even regional sharing of well-matched grafts. Because of the much higher frequency of public antigens, however, it could be possible to identify better matches within a local center or OPO if matching for these more frequent determinants resulted in improved survival. Therefore, a retrospective analysis of CREG versus conventional matching of cardiac transplants performed by SEOPF centers is being conducted.

III. ALLOCATION

In the 1960s, as limited but reproducible success with cadaveric kidney transplantation became possible, several southern centers, each with a small list of potential recipients, joined together to arrange to share organs that they could not use with other centers. The Southeastern Organ Procurement Foundation grew out of this early organization. A major activity was arranging to share organs, which early on began to be done with a computerized algorithm using mutually agreed-upon rules. In addition, it was decided to keep careful records for study. The mutual agreements included the stipulation that all attempted and actual transplantations would be reported to the registry, and adherence to this reporting was a major factor that enhanced the validity of the SEOPF database relative to other databases that were less intrinsically related to the actual mechanics of each transplant.

A major issue for study using the database was the nascent science of HLA typing. The attempt to improve results by use of improved matches was cause for great optimism (31). It was clear from family studies that HLA was important, and it seemed obvious that this benefit would extend to cadaveric transplants, once the typing was sufficiently accurate and sharing for good match was sufficiently extensive. Early reports from SEOPF were instrumental in delineating the extent of benefit from good matching (10,15,28).

By the early 1980s, as transplantation slowly became more widespread, the benefits of sharing by a network became even more apparent. The SEOPF organ sharing came to include a major fraction of the United States, well beyond the boundaries of the southeast,

and the United Network for Organ Sharing was formed as a spinoff of SEOPF to serve this increasingly wide participation.

The advent of cyclosporine (CYA) produced a major effect on the conceptual fabric of transplantation. Once CYA began to be used generally in the mid-1980s, it quickly produced a remarkable improvement in success, decreased morbidity, and a widened horizon for application of kidney transplantation. It eventually became clear that the improved effectiveness of immunosuppression lessened the impact of good matching in initial (1-year) graft and patient survival. Once experience was gained with CYA, centers generally found that they could produce results in poor matches nearly equivalent to those with zero antigen mismatches in the precyclosporine era. Finally, it became possible to transplant heart and liver allografts with better success than had been experienced with kidney allografts a few years previously. Success with these organs has had major impact on the science of transplantation and on the approach to allocation of all organs.

In the mid-1980s, allocation, although often arranged voluntarily through the computerized lists of the UNOS center, was a patchwork quilt of local practices. Anomalies existed. It was possible for a retrieval team to travel to a small hospital in another area of the country and be allowed by the local OPO to return with one kidney as well as the intended extrarenal organ. Strong transplant centers engendered and were nurtured by strong OPOs. However, public appeals for organs for specific patients and reports of transplants from U.S. donors into wealthy foreign citizens raised national concerns about fairness in the system and Congressional hearings were conducted.

The culmination of these hearings was the National Organ Transplant Act (NOTA) of 1984, which, among other things, established a task force. The legislation and the report of the task force together formed the basis of current transplantation policy. A network to provide a unified national system of organ sharing, the Organ Procurement and Transplant Network (OPTN), and a scientific registry to preserve and study data from this national effort, were established. In 1986, UNOS was awarded the contracts for these efforts and has overseen these processes ever since. Committees reporting to the Board oversee policy and scientific studies. A central computer system in Richmond, VA, provides real-time around-the-clock organ allocation and data processing. The bylaws and policies, although voluntary, are, in effect, the federal regulations governing practices in transplantation. It is through these rules that the science and public policy regarding HLA interface in the United States.

A simplistic response to the national concern that organs were being transplanted inequitably was to establish a single national list for allocation of organs. For example, in many areas, an organ was first offered to a list of patients at a single center. Despite problems with the single national list that were evident to most of the transplant community, this solution seemed to offer a way to remove "ownership" of the organs from the transplant centers. To overcome the drive toward this unrealistic solution, a more objective way to designate the first circle of offer and a definition of "local" were needed. The HCFA-designated OPO service areas generally include several centers, and it was determined that this would serve as a basis for allocation. Largely under the leadership of UNOS President R. Randal Bollinger, this policy was instituted and has given rise to the present general algorithm for sharing, which is "Local-Regional-National," in which local is typically the single list of patients from all centers in the OPO.

Although this policy was a major improvement and dispelled outside criticism of the system to a large degree, problems remain. A major conceptual problem with this system for persons concerned with ownership by a single center is that the first circle of allocation is still sometimes a single center. Furthermore, many centers perceived problems with this

general algorithm in the ad hoc patchwork of relationships that had grown up historically. Therefore, affected OPO members presented proposals to UNOS for "variances" to soften the problems and, although these proposals have sometimes been in the spirit of equitable allocation, they have often been controversial and have raised concerns about persisting inequities.

The set of rules within each hierarchical ring of sharing includes, for kidneys, points for waiting time, for certain degrees of HLA match as previously described, and for special considerations. Points are awarded as follows: 1 point for each year of waiting (fractional years are calculated to several decimal places to prevent ties); 5 points for a 1 B, DR mismatch, 7 points for a 0 B, DR mismatch, and 10 points for no HLA mismatch (a mandatory share across the country). In addition, pediatric patients are awarded from 1 to 3 points depending on their time waiting and age. One of the most difficult special considerations for kidneys was the long waiting times for high PRA patients. If the lists are ordered simply by number of points, a huge number of high PRA patients, unlikely to have a negative cross-match and receive the kidney, obscures the real possible candidates. This factor has been mitigated by only including high PRA patients if there is a negative preliminary cross-match with the donor. Effectively, the only way for a high PRA patient to be considered in the wide donor pool necessary to provide a chance of a negative cross-match is for the center to participate in serum sharing with other centers, using Regional Organ Procurement (ROP) trays containing serum from the high PRA patients in the sharing agreement. If there is a negative cross-match for a patient with PRA greater than 80%, then that patient receives 4 additional points. Another powerful impact on kidney allocation is the usual preferential use of pancreas plus kidney when the pancreas is transplantable, which can have the effect of directing kidneys within the local OPO from a center that does not do pancreas transplants to one that does.

It was apparent that an ongoing process of evaluation and possible further evolution in the system would be necessary. To better establish a basis for this evolution, a major effort was undertaken in the early 1990s by UNOS, in which analysis was done by committees and public comment, which then culminated in an in-depth videotaped public hearing. A wide representation from the transplant community and the public discussed the issues. John Welchel chaired this effort and authored the subsequent UNOS white paper "The Principles of Equitable Organ Allocation," which extensively details many specific issues to consider. The paper provides an overarching principle of the balance of justice and utility. Although even this concept lacks unanimous agreement within the transplant community, it serves as a solid starting place for future considerations.

All allocation is based to a large extent on waiting time. Persons who are dissatisfied with the use of HLA matching as a criterion for kidney allocation criticize the relatively large number of points awarded for the good matches. However, as described previously in this chapter, these good matches are not usual, and most transplants in fact go into patients whose points are mostly derived from time waiting. Criteria for listing a patient vary by center and within local OPO units. There is increasing conviction that a unified set of rules that define listing criteria much more strictly will be necessary. A proposal that essentially defines listing threshold at a creatinine clearance of 20 mL/min or 30 mL/min in type I diabetes, or disabling uremic symptoms, is under consideration but has generated considerable controversy.

To better define directions for future algorithms of organ allocation, UNOS has begun an extensive effort with the Pritsker Corporation to model allocation outcomes. This approach has been well developed for liver transplants. Similar investigations were planned

in 1996 for kidney allocation using this general model approach. The most sensitive area is the issue of the degree to which the definition of local may change. In one perspective, the arbitrary boundaries of a local OPO, or the state lines of regional boundaries, result in organ allocation that does not make biological sense. Divesting the process of these geopolitical boundaries for the purpose of allocation seems more appropriate.

However, there are concerns raised about the concept of removing allocation from the OPO. Some centers feel comfortable that their excellent practices direct organs to their patients and that removing that ingredient would be unfair. It is clear that the sharing of organs introduces variables and unknowns that may decrease organ function. This issue of problems with shared organs is a particular concern in areas that have a large supply of organs relative to their patients, and therefore use organs from outside their OPO more infrequently.

A major aspect of transplantation that sets it apart from all other aspects of medicine is the need for a viable organ. This places special practical and ethical concerns on the processes for dealing with recipients and organs. Moreover, there is a major concern that local organ utilization is a determinant of local enthusiasm for organ donation, and removing this aspect would decrease the success of organ donor efforts (14). Less commonly noted but clearly stated by UNOS President Bruce Lucas is the concern about centralization of transplant efforts, which threatens the maintenance of small local centers where local patients may receive a transplant. This is particularly germane to kidney transplants, less so for heart and livers, which require greater institutional resources. To the extent that centers of excellence mandate long travel on the part of the recipient, there will be a social stratification, with decreasing possibility that more disadvantaged patients will, in the end, receive a transplant. A system that protects this local advantage seems important to maintain.

The pendulum of opinion has recently begun to return, having swung far to the extreme of criticism of local, or "center-driven," as anathema, to a more balanced view of local. Centers, are, after all, where patients receive their transplants, and each patient has a stake in a particular center thriving. Furthermore, a conjunction of logistical, biological, psychological, and economic factors seem to necessitate local use of organs when there is no good reason to send them a long distance.

The arguments against sharing kidneys for good histocompatibility match tend to note the relatively modest benefit, when balanced against the logistical and other costs. It is possible that sharing on the basis of CREG or CREG+DR matching could greatly decrease the logistical costs while preserving most of the benefit of the conventional private antigen matching algorithm. Patients with a high PRA would benefit. At present, it only makes sense to ship a kidney to a patient with a high PRA if there is a negative preliminary cross-match. This test requires preparation and standardization of the ROP trays, and the size of the potential donor pool is limited by the size of the area where these trays can be served. With CREG matching, on the other hand, the pool size could potentially be the entire country. All that would be necessary would be the routine typing of the potential recipients. As soon as the donor HLA is known, a kidney could be offered for any patient for whom the analysis of CREG sensitization indicated a high likelihood of a negative cross-match. This could likewise provide benefit to patients with a high PRA awaiting an extrarenal transplant, because the negative cross-match could be predicted well before retrieval. A large sharing circle could allow more likely identification of an appropriate donor for each heart or lung recipient with a high PRA, in a way that would be logistically feasible even given the limited acceptable cold time.

Matching could likewise be done using CREGs, using a hierarchy with points for zero CREG+DR mismatch, then zero CREG mismatch, just under conventional zero antigen mismatch. There would be a high likelihood of a good CREG or even a CREG+DR match for a donor, even within regions. In centers with large lists, the kidney would be likely to be able to be placed in a recipient with a good match right in the retrieval OPO. Thus, it might be possible to convert from the present situation, in which most kidneys are transplanted into poor matches by any analysis, to a system in which most patients receive a match that should provide a real benefit over years (22). The major concern with disadvantage for blacks in the HLA rules in the present algorithm would be answered, because the use of CREGs nearly abolishes any racial difference in likelihood of a good match. Therefore, both to serve those with high PRA and to improve matching in general, the simplification provided by CREG analysis may prove to be very beneficial in ensuring optimal organ allocation.

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10

Immunological Tolerance and Its Relationship to Clinical Transplantation

ROBIN P. LOWRY, MARCUS N. SCHERER, and EDWARD K. GEISSLER

University of South Alabama, Mobile, Alabama

JENS GOEBEL

Tulane University Medical Center, New Orleans, Louisiana

I. INTRODUCTION

Protracted or permanent survival of vascularized organ allografts and xenografts has recently been achieved in small and large animals by targeting pathways presumed to contribute to the genesis of self-tolerance and immunological privilege, thus affording considerable promise that transplantation tolerance will become a clinical reality at the dawn of next millennium (1–7). Progress over the past decade in understanding the nature of self-tolerance and the genesis of specific unresponsiveness in organ transplantation must be viewed as the culmination of more than 50 years of research in cellular and molecular immunology. Essential technologies and important insights have been obtained from parallel advances in other disciplines, including biochemistry, developmental biology, genetics, oncology, and virology (8–14). Initiatives such as the Human Genome Project and accessible libraries of expressed sequence tags (ESTs) are beginning to bear fruit and accelerate the pace of research in immunology as well as other fields of biomedical endeavor (see Refs. 15–17). A series of important milestones can be identified which have contributed significantly to current understanding of transplantation tolerance.

1946. Owen, Davis, and Morgan observed red cell chimerism in dizygotic cattle twins, a consequence of blood vessel anastomosis in the fused placenta, and they concluded that self-tolerance was learned (18).

1956. Billingham, Brent, and Medawar provided experimental confirmation that tolerance could be acquired by demonstrating that a state of specific immunological unresponsiveness or "tolerance" was induced when newborn mice were inoculated with allogeneic spleen cells (19).

1961. Jacques Miller identified the thymus as the vital organ supplying lymphocytes essential for defense against infection and allograft rejection (20).

1968. Stuart et al. documented prolonged survival of experimental renal allografts in rats that had previously received an intravenous injection of donor strain mononuclear cells by the intravenous route (21).

1975. Sprent et al. documented that bone marrow–derived elements, currently identified as dendritic cells, contributed importantly to the induction of self-tolerance at the level of the thymus (22,23).

1983. Lamb and Feldmann documented that specific unresponsiveness in T cells might be due to clonal anergy (24,25).

1984. Yonagi et al. and Hendrix et al. reported the identification of genes encoding the T-cell antigen receptor (TcR) β chains of man and mouse, respectively (26,27).

1987. Kappler et al. provided the first definitive data linking self-tolerance with clonal deletion at the level of the thymus (28).

1987. Mossman et al. reported that helper T cells in mice can be divided into two functional subpopulations, Th1 and Th2, based on differences in profiles of coordinate lymphokine release upon activation (29), and they documented the primacy of the former in mediating delayed-type hypersensitivity (30). Mossman and Romagnani and coworkers, as well as others, have since identified analogous functional subpopulations in the $CD8^+$ T-cell compartment (Tc1 and Tc2) that might serve important effector and immunoregulatory roles in health and disease (31–40).

1989. Burkly et al. reported that tolerance induced by alloantigen expressed "solely" in the periphery is due to clonal anergy (41).

1990. Moore et al. reported cloning of the novel cytokine, cytokine synthesis inhibitory factor (interleukin-10 [IL-10]), which is produced by Th2 helper T cells as well as other cell types and serves, in vitro and in vivo, to suppress release of interferon (IFN)-γ, IL-2, and other cytokines by Th1 helper T cells and cells of the monocyte-macrophage lineage (42,43).

1991. Downward et al. identified activation of $p21^{ras}$ as a critical event in T-cell activation (44).

1991. Schreiber and colleagues identified calcineurin as a common target of cyclophilin-cyclosporin A and FKBP–FK506 complexes (45).

1992. Lenardo defines a role for IL-2 in T-cell activation–induced cell death (AICD) (46), an observation that has since been corroborated in IL-2 receptor gene knockout mice (47,48).

1992. Chan et al. identified ZAP-70 as a 70-kD protein tyrosine kinase (PTK) that associates with CD3-ζ and synergizes with src family PTK lck and fyn following T-cell antigen receptor cross-linking to excite downstream signaling events underlying T-cell activation (49).

1992. Turka, Linsley, Bluestone, Thompson, and Larsen, as well as other individuals and groups, demonstrated protracted allograft survival and "transplantation tolerance" following interruption of a costimulatory axis comprised of T-cell surface glycoprotein CD28 and B7 family members expressed on dendritic cells as well as other professional antigen presenting cells (APCs) (50–52).

1992. Sloan-Lancaster et al. reported that altered peptide ligands presented by live

antigen presenting cells induce T-cell anergy even in the presence of a surfeit of costimulation (53,54).

1993. Peter Morris (55) called attention to the "intriguing possibility" that allograft rejection and transplantation tolerance might be linked, at least in part, to the differential activation of functional lymphocyte subpopulations Th1 and Th2 (56–58).

1994. Su et al. demonstrated that JNK is involved in signal integration during costimulation of T cells (59).

1995. Green and coworkers as well as other groups elucidated the role of Fas/Fas ligand (FasL) interactions in induction of apoptosis (60).

1996. Li et al., Fields et al., as well as DeSilva et al. identified a deficit in Ras/JNK activation as an upstream, receptor proximal correlate of impaired AP-1 activation in T-cell clones activated by antigen receptor cross-linking without requisite costimulation (61–64).

1996. Lane et al. (65) demonstrated that in vivo induction of anergy amongst naive T cells bearing TcR that confer reactivity to staphylococcal enterotoxin B (SEB) may be linked to a positive signaling event transduced by CTLA4, an observation that has since been substantially confirmed by Abbas and colleagues (66,67).

1997. Boussiotis et al. identified Rap1 as a negative regulator of TcR-triggered Ras activation and IL-2 biosynthesis in T-cell anergy (4).

In the previous edition of this book, (68), we suggested that the modern era in the study of transplantation tolerance may truly have begun in 1987 with the landmark observation, by Kappler et al. (28), that T cells bearing serologically detectable antigen receptors, conferring specificity for self MHC antigens, undergo clonal deletion at the level of the thymus. Earlier, T-cell antigen receptors (TcRs) had been shown by Kappler and others to be derived from germ line elements that recombine during T-cell ontogeny to form the component chains of the receptor. With this new knowledge and the availability of serological/molecular probes for the TcR of mouse and humans, it became possible to design definitive experiments to probe the origins of self-tolerance as induced at the level of the thymus or in the periphery (67,69,70). Parallel studies by Ronald Schwartz and others sought to clarify the molecular basis for the induction of tolerance in vitro (71).

Determinants of conditioned unresponsiveness in organ transplantation have proven to be very complex. In this discussion, we focus on anergy, apoptosis, immune deviation/suppression, and veto as the mechanisms potentially contributing to the genesis of transplantation tolerance. The possible and probable roles of anti-idiotypic T cells and or anti-idiotypic antibodies (72–74) are excluded from further consideration here, except for brief mention of recent work that has suggested that TcR-peptide–specific regulatory cells might be induced to control expression of autoimmunity (75,76). (For discussion of idiotypic antibodies, see Chap. 4).

The primary thesis explored in our contribution to the previous edition was that transplantation tolerance, like self-tolerance (77), was both positive and assertive in nature. Specifically, we proposed that transplantation tolerance induced in adult members of the species would generally be seen to reflect both clonal anergy and specific suppression, and that clonal anergy and specific suppression would exist as a continuum. Preliminary evidence was presented to support the hypothesis that alloreactive T cells that have been rendered anergic/reprogrammed (i.e., Th0 to Th2) as a consequence of exposure to tolerogenic forms of antigen might be driven by subsequent contact with antigen in the microenvironment of a graft (and possibly elsewhere) to run their revised program and release soluble

factors in situ that blunt the generation of a local alloimmune response. The potential validity of this supposition was substantially corroborated by the in vitro demonstration by Frank Fitch and coworkers that anergic Th0 cells mature as Th2-like effectors (78) as well as recent in vivo studies (1047).

We digress briefly here to acknowledge a long-standing bias of one of the authors (RPL) that the Th1, Th2 paradigm affords an attractive "molecular" explanation for suppression, a commonly studied phenomenon in experimental transplantation and autoimmune disease models (79). The basic tenets of the Th/Th2 paradigm as might be applied to the genesis of transplantation tolerance are broadly appreciated and are briefly reviewed elsewhere in this chapter. Recent contributions to the literature (see Refs. 80 and 81) substantially corroborate and extend the view that Th2-like effectors may indeed serve as antigen-specific suppressor cells, with the potential to modulate generation of Th1 from precursors and to limit in situ activation of mature Th1-like effectors (69,82–89). Reciprocal suppression of Th2-like responses by Th1 has also been observed. These findings complement an expanding body of literature that implicates dominant suppression (infectious tolerance) as a critical component of self-tolerance and transplantation tolerance (90–92). However, we also emphasize that current literature relating to the putative roles of Th1/Th2-like effectors as antigen-specific suppressor cells is highly controversial (93–96). Briefly:

1. Neutralization of IL-12 in a solid organ mouse heart transplant model induced a Th2 shift in the profiles of cytokine mRNA expressed in the graft and marginally but significantly accelerated the tempo of graft rejection (96).
2. Genetic susceptibility to particular autoimmune and infectious diseases appears to involve more than just a predisposition to generate a Th1-like or a Th2-like response (97,98).
3. Studies in IL-4 transgenic mice and in mice rendered deficient in IL-4 by homologous recombination demonstrate no correlation between the presence or absence of IL-4, typical of Th2 responses and the tempo of acute rejection by immunocompetent recipients or the genesis of protracted allograft survival in conditioned hosts, respectively (99,100).
4. Constitutive expression of IL-4 or IL-10 has been shown to induce or exacerbate autoimmune diathesis (101,102).
5. In vitro differentiated autoantigen-specific Th2-like effectors have been shown to induce robust autoimmune disease following adoptive transfer to *immunodeficient scid* hosts and fail to modulate disease caused by Th1 effectors (103,104). Remarkably however, only in vitro differentiated Th1 cells induced autoimmune disease in normal mice in these two adoptive transfer models.
6. Orosz and colleagues determined that in vivo transfer of in vitro differentiated donor-specific Th1- and Th2-like cells into immunoincompetent *scid* recipients of vascularized cardiac allografts resulted in equivalent prompt rejection of the allografts (95).

In discussions of the potential importance of Th1 and Th2 in allograft rejection and transplantation tolerance, other authors have concluded with the comment that "it's simply not that simple" (94).

It is difficult to address these matters and other contemporary issues in transplantation tolerance without providing a brief review of selected topics in cellular/molecular immunology. Therefore, new knowledge on the T-cell receptor, T-cell activation, and T-lymphocyte subsets relevant to present understanding of the molecular basis for self-tolerance and conditioned unresponsiveness is presented. Recent reports on the pathogenesis of allograft

rejection are summarized, data on the efficacy of various experimental approaches for the induction of transplantation tolerance are reviewed, and the mechanisms responsible for maintaining such states is discussed. This review is particularly timely because recent advances in the study of tolerance have made it possible to consider strategies for the generation of specific immunological unresponsiveness in clinical transplantation. We do not attempt to provide a comprehensive review of the topic but rather to emphasize principles and recent developments. The reader is referred to a series of excellent reviews on transplantation tolerance (3,105–117) that have appeared in recent years for additional information on the evolution of thought in the field (17,69,71,83,84,86,107,118–134).

Because the serological and molecular identification of the T-cell receptor for antigen (TcR) provided the scientific basis and the reagents for recent experiments that have clarified the nature of self-tolerance, this chapter opens with a brief description of the structure and function of the T-cell receptor.

II. T-LYMPHOCYTE ANTIGEN RECEPTORS: STRUCTURE AND FUNCTION

The convergence of previously independent avenues of research surrounding (1) the consequences of T-cell antigen receptor triggering by agonist and partial agonist peptides, (2) intrathymic selection of the T-cell antigen receptor repertoire during T-cell ontogeny, and (3) the functional attributes of peripheral T-cell subpopulations has provided fresh insights concerning the coordinate contributions of diverse immunological mechanisms (anergy, apoptosis, and immune deviation) in the regulated expression of immunity (82,153). Furthermore, recent contributions to the literature defining an essential role for the thymus in the induction of peripheral tolerance in some model systems remind us that some of the earliest successful demonstrations of tolerance transfer in rodent models used thymocytes (82,135,136). Future advances in the study of transplantation tolerance will likely be obtained through a keen appreciation of the biochemistry of lymphocyte activation and a holistic view of the immune system.

A. Recombinational Events and the T-Cell Receptor Gene Product

The structure, organization, and polymorphism of murine and human T-cell receptor α and β chain gene families have been recently reviewed, and the reader is referred to these reviews for more detailed insight into the evolution of our present understanding of these receptor molecules (137–147). TcR α and β chains were first identified by Allison et al. (148) in 1982 in studies of tumor-specific antigens borne by a T-cell lymphoma and later by others (149–151) using antibodies against polymorphic determinants on T-cell clones and hybridomas. Meuer and others documented that these clonotypic antibodies could inhibit or, on some occasions, induce specific activation consistent with recognition of the antigen receptor (152). Using immunoprecipitation with these reagents, it was learned that the TcR was a disulfide-linked heterodimeric glycoprotein composed of α and β chains with molecular weights of 40–45 kilodaltons (kD). Kappler et al. performed peptide mapping studies and documented the presence of constant and variable region domains homologous to those observed in immunoglobulins (154). As is noted later, serological reagents identifying specific T-cell receptor variable region domains have proven invaluable in studies of the genesis of self-tolerance.

The study of T-cell antigen receptors was greatly advanced by the introduction of molecular approaches and the study of transgenic mice (155). In 1984, two independent

groups of investigators reported the identification of the first TcR gene to be isolated, TcR β, by screening T-cell specific cDNA libraries for clones that demonstrated rearrangement (26,27). Thereafter, three additional TcR chains were identified—γ, α, δ—and most recently, pre-Tα (155–160). Pre-Tα is now known to subserve a critical role in the development of αβT cells (161) but is dispensable for the development of γδT cells (162). Knowledge that expression of TcR β transgenes in recombinase-deficient SCID was sufficient to enable triple negative ($CD3^-$, $CD4^-$, $CD8^-$) thymocytes (TN) to advance through a developmental checkpoint ($CD44^{-/lo}$ $CD25^+$ to $CD44^{-/lo}$ $CD25^-$) that otherwise requires TcRβ rearrangement and expression (163) stimulated a search for a β-chain partner in pre–T cells. A disulfide-linked heterodimer was subsequently identified by von Boehmer's group on pre–T cells that consisted of the TcR β chain plus a nonpolymorphic 33-kD glycoprotein lacking a variable region domain that has been dubbed pre-Tα (155,160,164, 165). Their data suggest that the pre–T-cell receptor (TcRβ, pre-Tα) serves as a molecular sensor that evokes a brisk proliferative response of double negative thymocytes bearing a successfully rearranged TcR β chain destined to associate with recombined TcRα chains (155). Pre-Tα has a long cytoplasmic tail containing potential phosphorylation sites, as well as motifs that might serve as docking sites for Src homology domain 3 (SH3)-containing proteins. Although there are no data at present to indicate that preTα is directly involved in signaling (164), expression of a tailless preTα transgene restores thymocyte maturation (155), consistent with the notion that pre-Tα–associated moieties are responsible for transmembrane signaling. The pre–T-cell antigen receptor complex is functionally coupled to CD3-ζ as well as a 12-kD dimer that may serve in transmitting mitogenic signals that promote proliferation of early thymocytes (146,155,167,168).

TcR diversity was shown to be achieved by recombination of germ line elements (V, [D], J and C) to form the complete genes that encode individual chains of the TcR, a process akin to the somatic generation of antibody diversity (169). A variable number of nucleotides and codons are introduced between TcR germ line elements by the enzyme terminal transferase during recombination, and these junctional sequences may contribute significantly to potential diversity (139). T-cell receptor genes are remarkably homologous to immunoglobulin sequences (170). However, B lymphocytes continue to modify immunoglobulin genes through somatic mutation (169,171) even after isotype switching has occurred (172). In contrast, with the possible exception of γδ T cells (173), T cells do not generally augment TcR diversity through somatic recombination or mutation. This difference undoubtedly reflects requirements relating to self-tolerance in the T cell compartment (see Ref. 141).

Because most of the recent work on the molecular basis of tolerance has been performed in the mouse, a limited review of the genetic organization and expression of T-cell receptors in this species follows. The TcR β chain gene of the mouse encompasses approximately 450 Kb of DNA and is encoded on chromosome 6 (139,174). At a genomic level, the β chain gene consists of a series of variable (V) gene segments and two constant (C) region elements, which are each associated with a single diversity (D) segment and seven joining (J) gene segments (Fig. 1*a*) (175). During T-cell ontogeny V(D)J gene segments rearrange to form functional variable region genes and these are linked to constant region sequences by RNA splicing following transcription (see Fig. 1*b*). D-J rearrangement precedes V-DJ joining within the TcRβ locus (176). Nineteen Vβ gene families had been defined at the time of publication of the previous edition of this text, the majority being single member families (177). (V gene segments with greater than 75% sequence homology are considered to be members of a family.) Nested within the α chain gene are the V-D-J and C region segments, which encode the δ chain (178). The mapping of α and δ variable

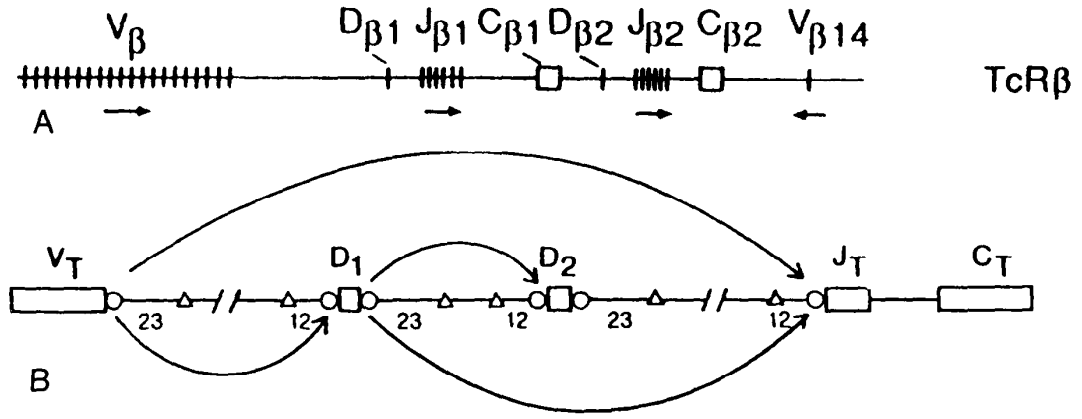

Figure 1 **A,** Genomic organization of T-cell receptor β chain locus in the mouse (not to scale). V, D, J, and C region gene segments are indicated and transcriptional orientations are marked by arrows. (From Ref. 324.) **B,** Potential rearrangements in the formation of the TcRβ chain. (From Ref. 139, used with permission.)

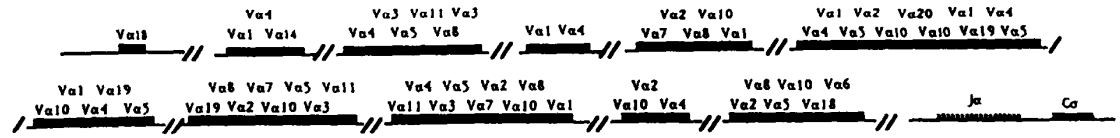

Figure 2 Genomic organization of mouse T-cell receptor α locus. The solid boxes on the horizontal line represent the V gene segments and the numbers above the boxes indicate where the V gene segment was found. Relative order of V gene segments is determined by analyzing cosmid clones and panels of T-cell lines by pulse field gel electrophoresis. (Adapted from Ref. 179.)

region segments was advanced by Kai Wang working in the laboratory of Leroy Hood (179) and the results are illustrated in Fig. 2. The TcR α chain gene situated on chromosome 14 contains only one constant region segment (180). Fourteen Vα families had been identified, each with two to 10 members (181). Interestingly, even though delta chain variable region genes (Vδ) have been shown to recombine with alpha chain constant region genes (Cα) at the level of the thymus, positive and negative selection events within the thymus or periphery appear to mitigate against peripheral expression of such aberrant products (182). Nevertheless, other lineage commitment mistakes are made within the thymus (183) that may have great biological significance (see later discussion in reference to BALB/c mice). The γ chain gene, sited on chromosome 13, is composed of a single C region segment, four J regions, and seven V region segments (Fig. 3) (184). The genomic structure and chromosomal location of pre-Tα has recently been elucidated (165). Pre-Tα is an 8.4-kb gene encoded on mouse chromosome 17 (165). The deduced configuration of the pre-Tα gene product is illustrated schematically in Fig. 4, adapted from a recent review on early αβ T-cell development (155).

The structure and organization of the human TcR is remarkably similar to that of mouse TcR (185–187). For example, only limited polymorphism has been detected when individual TcR gene segments of different inbred mice are compared but some inbred mouse strains contain broad deletions of individual Vα/Vβ gene segments (188,189). TcR Vα and Vβ chains of humans have likewise been shown to contain a similar range of deletions and polymorphisms (190–192) and these may contribute to variable reactivity to alloantigens and differences in disease susceptibility (193,194).

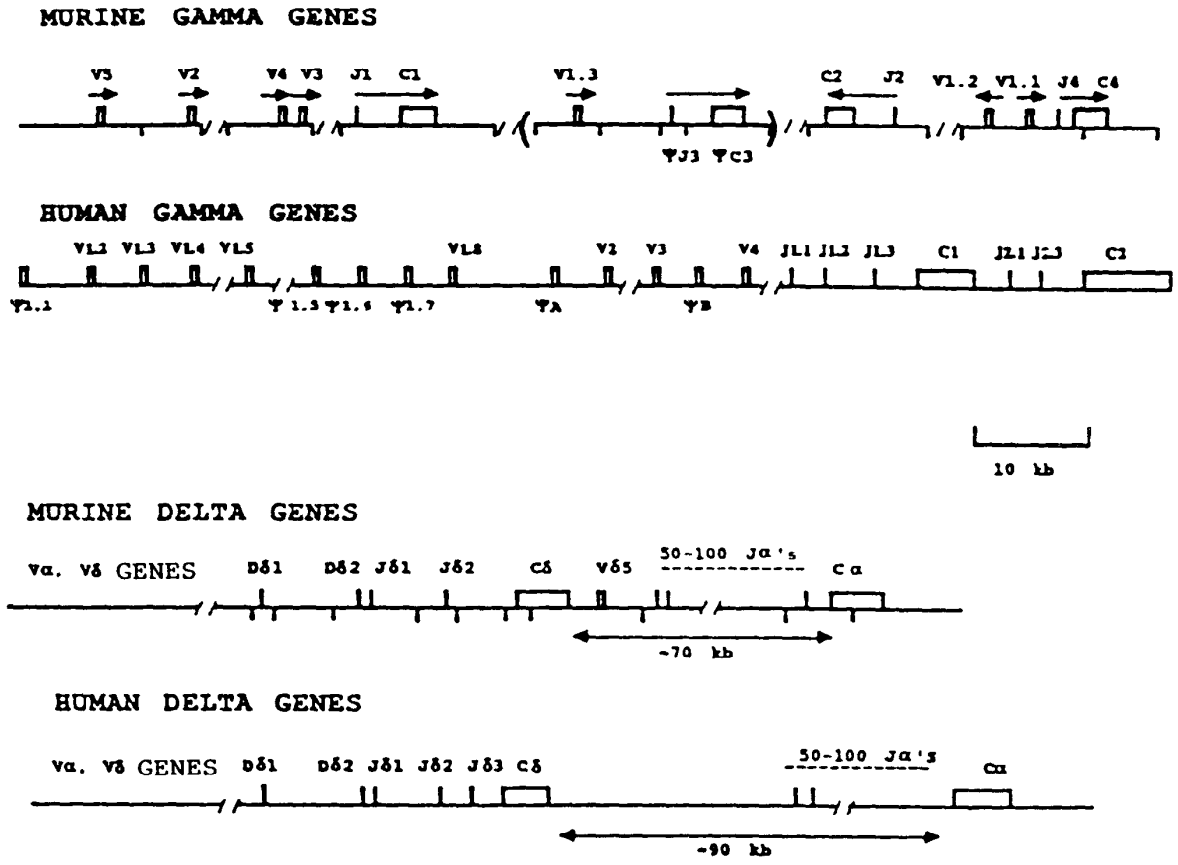

Figure 3 Genomic organization of murine and human γ and δ gene families. For simplicity, the exons of the C region genes are not depicted. Brackets ((//) indicate gaps: the murine γ genes have been linked by pulsed field electrophoresis. Functional genes are listed above the lines and pseudogenes are listed below the lines. The maps are drawn roughly to scale. The tics below the lines of the murine γ and δ genes correspond to the approximate location of Eco R1 sites of the BALB/c strain. (From Ref. 1042).

B. Mechanisms of TcR Assembly and the Regulation of TcR Expression

With the completion of the mapping and sequencing of T-cell receptor genes, attention has turned to focus on factors governing the assembly and expression of T-cell receptor genes. Recent reports have provided fresh insights into the nature of the cellular machinery responsible for the rearrangements that underlie the origins of TcR diversity. Enhancer and silencer elements that govern the ordered expression of TcR receptor molecules have been identified.

Briefly, pre–T cells entering the thymus are $CD4^-CD8^-$ TcR^- (195,196). The TcRγ chain is the first to rearrange. A substantial body of evidence has accrued to indicate that IL-7 is absolutely crucial for γ-chain V-J rearrangement (147,197). As a consequence, IL-7Rα chain gene knockout (GKO) mice are devoid of γδ T cells (147,197). This may be related, in part, to the potential of IL-7 to sustain expression of recombinase-activating genes RAG-1 and RAG-2 (147,198). However, this cannot be the whole story. TcRγ gene rearrangement is fully blocked in IL-7 receptor GKO mice, whereas other TcR (α, β, and δ) genes are rearranged and expressed in these lymphopenic mice (197). Moreover, recent literature indicates that deletion of one or other or both of the component chains of the IL-7 receptor (i.e., α and/or γ_c) by homologous recombination creates even greater deficits in thymic ontogeny than those observed in IL-7 deficient mice. This discrepancy is explica-

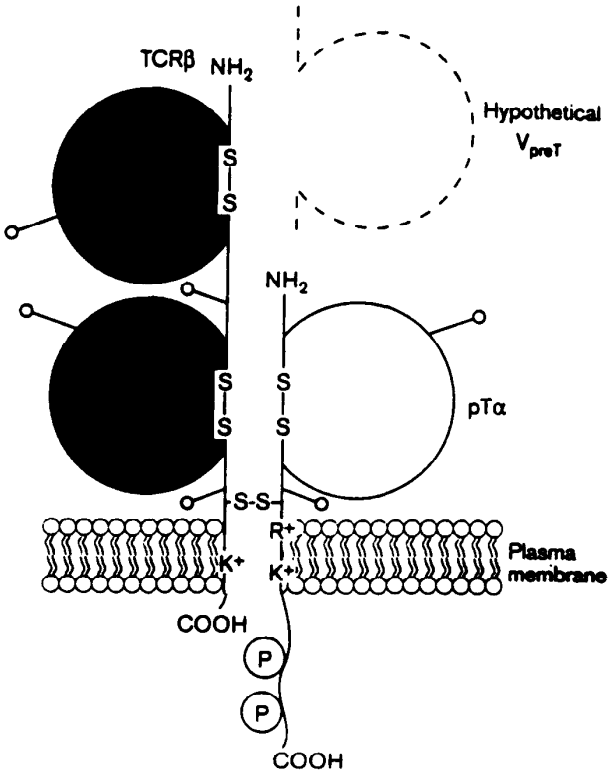

Figure 4 Schematic structure of the pre-TCR heterodimer or possible heterotrimer. Immunoglobulin-like domains are shown as circles. S-S symbolizes a disulfide bridge. Positively charged arginine (R^+) and lysine (K^+) residues within the transmembrane region of pre-Tα and TcRβ are indicated and most likely interact with negatively charged amino acids in the transmembrane region of CD3 chains (not shown). Circled Ps indicate threonine/serine residues that might serve as phosphorylation sites for protein kinase C. Bars ending in small circles indicate potential glycosylation sites. A pre-TcR–specific, variable region–like domain (V_{preT}), encoded by an independent gene, may exist, but has not yet been found and is indicated by the dashed circle. COOH, carboxy terminus; NH2, amino terminus. (From Ref. 155, © 1997 *Current Opinion Immunology*).

ble, at least in part, by the observation that there is another thymic factor (cytokine), thymic stroma derived lymphopoietin (TSLP) that targets a thymocyte receptor, which incorporates the IL-7α chain (199). It is suspected that the second chain of the TSLP receptor is something other than the γ_c chain since the thymic phenotype of γ_c chain GKO mice is mild, resembling that observed in IL-7 GKO mice (147,200). IL-7 and the IL-7R may also subserve other functions, that is, the unmasking of genes involved in survival, proliferation, and specific effector functions. Thymocytes grown in vitro in the absence of IL-7 do not acquire cytotoxic potential or competence to produce IL-4 (see Ref. 147). Moreover, IL-7R signaling activates c-*myc* mRNA expression in thymocytes as well as more mature T cells (201).

Two loci had been identified, RAG-1 and RAG-2, that acted synergistically to promote V(D)J recombination of TcR germ line elements when introduced into fibroblasts, cells in which TcR rearrangement does not normally occur (202,203). Furthermore, a DNA probe derived from one of these loci, RAG-1, had been shown to hybridize in Northern blots to a 6.6–7.0 kb mRNA species in pre-T and pre-B cells as well as fibroblasts transfected with DNA containing the RAG-1 locus. The introduction of the RAG-1 locus into fibroblasts had been found to promote V(D)J recombination only at low frequency, but this was significantly

enhanced by the subsequent introduction of the RAG-2 locus. Consistent with the above, mutant mice deficient in either recombinase gene, RAG-1 or RAG-2, have since been shown to express a SCID phenotype, with total absence of mature B and T lymphocytes (204,205). Moreover, B^- SCID patients have been identified in whom failure of V(D)J recombination has been found to correlate with lack of functional RAG gene expression (206).

Knowledge of the molecular mechanisms underlying TcR gene rearrangement have been greatly advanced since publication of the previous edition of this text (144,207). This has involved research in three areas of endeavor: (1) delineation of the V(D)J cleavage sites, (2) identification and purification of the enzymes that mediate V(D)J cleavage, and (3) identification of the ligases that reassemble coding elements of individual antigen receptor chains.

1. V(D)J gene segments are flanked by recombination signal sequences (RSSs), consisting of conserved heptamer and nonamer motifs separated by spacer regions of 12 or 23 base pairs (bp) (208,209). Homologous segments of a given TcR/Ig locus were found to be flanked by the same arrangement of RSSs (209). Recombination requires one each of the 12 bp and a 23-bp RSS, the so-called 12/23 rule of Lewis. This arrangement is enforced at the cleavage step and undoubtedly serves to enhance the efficiency of recombination by limiting unproductive rearrangements (144). Research on molecular mechanisms underlying V(D)J recombination has been advanced by the use of plasmid vectors incorporating RSS in vivo and, more recently, by the development of in vitro cell-free systems.
2. Cleavage at V(D)J recombination signals is reported to require only the RAG gene products, RAG1 and RAG2 (210–212), which recognize and bind to DNA at RSS and induce double stranded (ds) DNA breaks at the border between heptamer and coding sequence (although some controversy persists [213]). The generation of ds DNA breaks occurs in two steps: (1) the induction of single strand nicks upstream (5′-) of the signal sequence followed by (2) the generation of a hairpin loop at the cut end of the coding sequence. The presumed sequence of events is illustrated diagrammatically in Fig. 5. Herein, the formation of the hairpin loop is thought to occur through a "nucleophile attack" by the 3′-OH of the nicked strand on the phophodiester bond of the second strand.
3. Under the scenario described above, signal (heptamer) sequences may be directly and precisely ligated while the flanking coding sequences are "imprecisely" joined to create junctional diversity (210). However, the RAG gene products have no intrinsic ligase activity. Moreover, until recently, the molecular mechanisms underlying the generation of junctional diversity were not discovered. It is now appreciated that generation of maximal junctional diversity at V(D)J joints obtains through insertion of complementary (P) or random (N) nucleotides as well as through base deletion. It is rather satisfying, therefore, to be able to report that human DNA ligase IV stimulated by complex formation with XRCC4 protein is likely responsible for rejoining RAG1/RAG2-generated double strand DNA breaks to complete the process of V(D)J recombination.

Critical advances that have drawn this research saga to a successful conclusion include the following:

1. An in vitro cell-free V(D)J recombination system has been generated and incorporates recombinant RAG-1, RAG-2 ATP, dNTPs, a ligase-containing (HeLa)

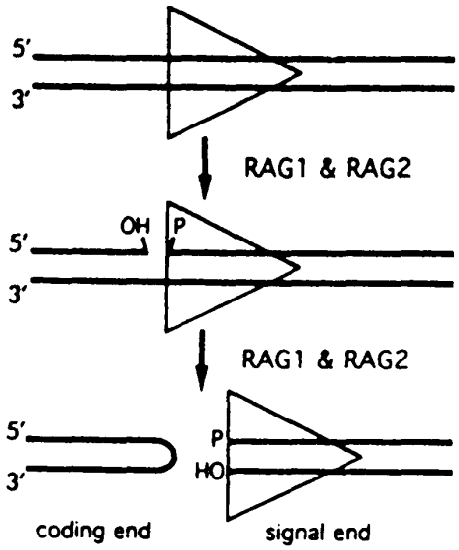

Figure 5 Cleavage at a V(D)J recombination signal required by RAG1 and RAG2 proteins occurs in two steps at recombination signal sequence (RSS) involving (1) induction of single strand nicks 5' of the signal sequence followed by (2) the generation of a hairpin loop at the cut end of the coding sequence (From Ref. 210).

cell extract, and a plasmid template containing target 12bp/23bp spacer recombination signal sequences, which "reproduces the complete set of processing events that occur in cells" (214).

2. Using this in vitro model system, it has been demonstrated that the continued presence of RAG proteins during the ligation stage significantly enhances the efficiency of ligation of coding regions (presenting as hairpin loops) relative to the efficiency of ligation of the signal sequences.
3. Ligase activity present in HeLa cell extracts was shown to variably introduce P nucleotides, corresponding to nucleotide incorporation by pairing addition, as well as deletions. Additionally, inclusion of terminal deoxynucleotide transferase (TdT) induced random (N) nucleotide additions in the junctional region. Mammalian DNA ligase 1 was shown to be active in this system though it is recognized that V(D)J recombination proceeds even in the absence of this ligase.
4. Based on evidence previously accrued to indicate that mutation of the XRCC4 gene in mammalian cells prevents formation of signal and coding joints in the V(D)J recombination reaction, Grawunder et al. (215) investigated the association of XRCC4 with DNA ligase IV. XRCC4 and DNA ligase IV were shown to associate in biological systems. Moreover, XRCC4 was shown to catalyze ligation of ds DNA breaks by DNA ligase IV.
5. Screening of the genome of the yeast *Saccharomyces cerevisiae* for potential ATP-dependent DNA ligases (15) yielded two potential homologues: Cdc9, a homologue of mammalian ligase I, and DNL4, the yeast homologue of DNA ligase IV. The former was seen to be used for repair of single strand nicks in double stranded DNA, whereas the latter was shown to serve, in conjunction with human Ku DNA binding homologues (HDF1 and Ku80), in repair of double stranded DNA breaks. These findings were seen to be entirely consistent with the proposed role of mammalian ligase IV in V(D)J recombination recounted above. The noted participation of Ku80 in this system was reaffirmed by a report of vastly reduced

recombination efficacy in embryonic stem cells deficient in the DNA bindin components (Ku70/Ku80) of DNA-dependent protein kinase (DNA-PK) (216 In contradistinction, Ku70 appears not to be required for V(D)J recombinatio (217). Of note, functional deficiency of Ku, the catalytic component of DNA PK is responsible for the *scid* phenotype mice.

6. Studies in yeast with the Ku homologue Hdf, otherwise known to be involved i "silencing," led to the identification of a new family of proteins, Sir4, Sir3, an Sir2 recruited to ds DNA breaks formed during V(D)J recombination through pri mary interaction of Sir4 with DNA-binding protein Ku, and subsequent associa tion of Sir-2 and -3 with Sir4 (218). Interestingly, mutation of Sir2, Sir3, or Sir was seen to be associated with severe defects in Ku-dependent DNA repai Moreover, rearrangements of multiple T-cell receptor loci was found to b restored transiently in γ-irradiated *scid* mice (219).

   Overall, these findings appear to delineate important common pathways i DNA repair and V(D)J rearrangement.

Regulatory mechanisms are being uncovered which provide for restricted expressio and allelic exclusion of TcR heterodimers on cells that mature under the influence of th thymus. For example, it is known that TCR γδ chains begin to be expressed on day 14 c mouse gestation, whereas TcR αβ chains do not appear until day 17 (184,220,221). Th same ordered expression of TcR chains is observed during thymic repopulation in irradi ated bone marrow chimeras (222). A T-cell–specific enhancer for the δ chain has been iden tified within the $J_{\delta 3}$-C_δ intron of the δ gene, and this activates transcription from promoter 5′ to $V_{\delta 1}$ and $V_{\delta 3}$ (223–225). An α chain enhancer was identified 3′ to Cα, which was func tional in both αβ and γδ T cells (226). More recently, the α chain enhancer has been spl into two functional domains, Tα1 and Tα2. The former contains a consensus cyclic adenc sine monophosphate (cAMP) response element whereas the latter binds the Ets-1 prc tooncogene product. Ets-1 expression is restricted to lymphocytes and appears to be criti cal for α chain expression (227). A γ chain silencing element downregulates production c TcR γ chain transcripts in TcR $V\alpha\beta^+$ T cells (228) and negative elements upstream of th α chain enhancer suppress α chain expression in γδ cells (229). Multiple regulatory ele ments have also been identified that control transcription of the TcR β chain (207,230). I is suspected that the transcriptional promoters, enhancers, and silencers that regulate Tcl transcription may also regulate accessibility of TcR genes to recombinase enzyme RAG1/RAG2 during T-cell ontogeny (147,207).

Recent contributions to the literature have also advanced our understanding of th potential mechanisms underlying allelic exclusion at the level of the T-cell antigen recep tor. It has long been appreciated that expression of a functional β chain inhibited expressio of a second β chain. Von Boehmer et al. recently reported that allelic exclusion is main tained at the level of TcR β, with consequent inhibition of additional Vβ→(D)Jβ gen rearrangement in thymocytes that have undergone successful rearrangement of the β chai locus and express TcR β, and have shown that allelic exclusion at the level of TcR β is main tained in pre-Tα and TcR α deficient mice. They suggested that the TcR β is independentl expressed on immature thymocytes, together with CD3, but without an α chain partner, an that it mediates transmembrane signaling events that advise the thymocyte that a success ful β chain recombinational event has occurred; the involvement of CD3 in allelic exclu sion was demonstrated through the observation that TcRβ transgene-driven allelic exclu sion at the TcR β chain locus is nonoperative in CD3-deficient mice (231). Nonetheless expression of a TcR β transgene was found to be less effective than an endogenous β chai

in halting further β chain gene rearrangement in pre-Tα and TcR α deficient mice. Von Boehmer and colleagues conclude, therefore, that the pre-TcR complex plays an important, albeit limited, role in maintaining allelic exclusion at the level of the TcR β locus in early thymocytes.

Although the discovery of the pre–T-cell receptor would appear to represent an important advance in understanding of T-cell ontogeny, some level of controversy persists. Thus, Lanzavecchia and coworkers reported evidence for expression of two α chains in up to one-third and dual β-chain expression in up to 1% of human peripheral T cells (232–234). Interestingly, the same group has also called attention to dual B-cell antigen receptors (235). Moreover, others have reported that allelic exclusion at the TcR β chain locus is substantially abrogated in TcR β chain transgenic mice (236). Accordingly, it has been useful to express TcR transcripts in RAG1 and/or RAG2 deficient mice to avoid the potentially confounding influence of endogenous antigen receptor chain gene products. It must also be appreciated, however, that TcR heterodimer expression and function is quite dynamic and susceptible to modulation by many influences, for example, anti-CD4 mAb (237).

TcR δ chain genes nested within the α locus are excised as circular elements at the level of the thymus, during recombinational events involving TcR α in T cells destined to express TcR α and β chains. This probably occurs through ligation of signal ends (see discussion of V[D]J recombination above). Indeed, looping out and excision is a common mechanism of gene assembly. Circular DNA products are also produced in the course of immunoglobulin heavy and light chain gene rearrangements and class switches (238–240). Although an earlier report had suggested that circular elements discovered in αβ T-cell precursors contained *unrearranged* δ chain genes (241), more recent work has documented rearrangements within δ circles (242). These findings provided the initial documentation that αβ and γδ T cells developing in the thymus belong to a common lineage, an observation that has been substantially corroborated (243). Notch, initially identified as an essential determinant of cell fate in *Drosophila,* influences whether thymocytes develop as αβ or γδ cells (8). Thus, thymocytes that have successfully rearranged γδ chain genes may be influenced to express CD4 and CD8 and evolve as αβ T cells in the presence of a constitutively active form of notch and vica versa (8,244). Notch is also reported to impact the choice of thymocyte precursors toward CD4 and CD8 cell fates (245).

Berkhout et al. as well as Carson et al. determined that individual component chains of CD3 (i.e., γ, δ, ε and ζ) are both necessary and sufficient to engender cell surface expression of TcR α and β chains (246,247). In vitro translation (using an MHC class I promoter) and assembly of a complete T-cell receptor–CD3 complex has been accomplished (248). In this system, cotranslation of CD3-γ (or CD3-δ) has been seen to engender correct folding of CD3-ε and prevent formation of disulfide-linked CD3-ε homooligomers; glycan-independent interaction between CD3-ε and molecular chaparone calnexin was shown to be dispensable for correct folding of CD3-ε. It is likely that in vitro generated TcR–CD3 complexes will be incorporated in a lipid bilayer system, together with individual receptor-proximal components of the signaling cascade excited by antigen-receptor occupancy, to further elucidate the earliest events in T-cell activation.

C. TcR $\alpha\beta^+$ and $\gamma\delta^+$ Lymphocytes

Peripheral T cells express either αβ or γδ TcR heterodimers. By far the majority of peripheral T cells express TcR αβ chains and associate recognition structures CD4 or CD8. Of these, MHC class II–restricted T cells are generally $CD4^+CD8^-$ and class I–restricted T

cells are $CD4^-CD8^+$ (249). However, alloantigen-specific TcR $\alpha\beta^+$ cytolytic T-cell clones have been identified which lack expression of either CD4 or CD8 (250). $CD3^+CD4^-CD8^-$ and $\alpha\beta TcR^+NK1.1^+$ T cells have been identified in adult murine bone marrow, which may function as regulatory elements in hematopoiesis and could be related to cells shown to protect against graft-versus-host disease (251).

TcR $\gamma\delta^+$ T cells were first reported to be $CD4^-CD8^-$ but $CD8^+\alpha/\alpha$ TcR $\gamma\delta$ T cells have now been recognized to exist in the gut and elsewhere (252–254). In such instances, CD8 expression may represent a marker of activation. The biological functions of $\gamma\delta$ T cells remain somewhat enigmatic (255,256). Experimental evidence accrued to date indicates that $\gamma\delta^+$ T cells do contribute to host defense against viral agents and bacterial and parasitic organisms, and they control inflammatory tissue necrosis (255,257). An overabundance of $\gamma\delta^+$ T cells is observed at foci of infection (258). Indeed, the role of $\gamma\delta^+$ T cells in regulating inflammation and limiting tissue necrosis may be of profound biologic significance (259). The intestinal epithelium of Tγδ-deficient mice have been shown to have reduced epithelial cell turnover, suggesting therefore, that $\gamma\delta^+$ T cells are involved in epithelial cell regeneration or differentiation (260).

In a physiological sense, γδ T cells appear to be highly analogous to αβ T cells. Autoreactive γδ T cells undergo negative selection in the thymus as do $\alpha\beta^+$ T cells (261, 262). However some γδ T cells may mature outside the thymus and even γδ T cells maturing within the thymus may be able to modify their TcR repertoire in the periphery (173). Gammaδ T cells are activated by cross-linking the TcR–CD3 complex and releasing a range of lymphokines including interleukin (IL)-2, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-3 (221). Gammaδ T cells have also been shown to release a substance, possibly interferon γ (263), which causes aggregation of monocytes in the presence of GM-CSF and which may be important in infectious loci. Published data suggest that γδ T cells may display polarized coordinates of cytokines resembling Th1 and Th2 profiles observed in typical $CD4^+$ αβ T cells (264); moreover, different γδ T cytokine profiles have been observed in particular disease states (265).

Even though γδ T cells are well recognized to function as killer cells, the target epitopes recognized by such T cells are, for the most part, as yet poorly defined (256). Importantly, however, there is substantial evidence to suggest that the antigen receptors expressed by γδ T cells "recognize" antigen directly (255). Present evidence suggests that γδ T cells interact with unprocessed antigens without the constraints of MHC restriction universally documented in the instance of αβ T cells. Cytotoxic γδ T cell clones have been isolated which demonstrate specificity for non-classical class I antigens, for example, Tla (266), Qa-1 (267). In general, however, MHC reactive γδ T cells are less common than in the Tαβ compartment and tend to be cross-reactive. Thus, Tγδ cells have been identified that appear to be loosely restricted in their cytotoxic activity to allogeneic lymphoblasts by standard MHC class I/class II alloantigens but these are uncommon. In humans, TcR $\gamma\delta^+$ T-cell lines are reported to recognize MHC-controlled elements that are not HLA-A, -B, -C, -DP, -DQ or -DR (268).

Tγδ cells have been identified which induce unrestricted lysis of tumor targets reminiscent of that obtained with natural killer (NK) cells, with good reason, because it has become recognized that most Tγδ cells express NK receptors (269). However, the pattern of target cell lysis by NK cells differs from that obtained by γδ T cells. The lytic activity of γδ T cells is inhibited by antibody to the expressed TcR chains (270), whereas lytic activity of NK cells is triggered by a 60-Kd disulfide–linked dimer identified as NKR-P1 (271).

Despite the great potential diversity that exists at the level of the genome, the

expressed γδ receptor repertoire in the fetal thymus, spleen, and amongst fetal thymus–derived dendritic epidermal lymphocytes in skin, is limited (272–274). Tγδ cells are overrepresented at the level of the intestinal epithelium (IEL) and epidermis of mouse, if not in rats or humans (254,275–277). Most IEL in the gut are CD8α/α$^+$, and a significant proportion are Thy1$^+$ (253). Tγδ cells in skin and IEL tend to express different TcR variable region elements (278). Although some have conjectured that the TcR itself might determine localization (278), studies performed in TcR transgenic mice have documented that the TcR itself does not act as a homing receptor (279).

Tγδ cells expressing Vγ9 are cytotoxic to MHC class II–bearing cells that have been pulsed with staphylococcal enterotoxin A (SEA), but do not appear to proliferate to this stimulus, at least in vitro. Some researchers have speculated that the overrepresentation of γδ Vγ9$^+$ T cells in peripheral blood, skin and GI mucosa of mice may reflect ongoing exposure to enterotoxins derived from gut flora, but this is uncertain because there is no gross difference in T-cell antigen receptor repertoire expressed by IEC of germ-free mice (280). However, there are differences in junctional diversity in γδ Vγ9$^+$ T cells obtained from different sites, and further study is required to clarify this issue (281). The overall structure of the γδ cell TcR is more similar to that of an immunoglobulin molecule than a typical αβ TcR.

Tγδ cells have also been identified with specificity for bacterial heat shock proteins (282). One report has emerged to suggest that the limited receptor repertoire expressed by γδ thymocytes responsive to purified protein derivative (PPD) might be explained if indeed such T cells recognized one of a limited number of target epitopes expressed by mycobacterial heat shock proteins (283) but this is controversial (283–285). Heat shock proteins have been highly conserved through evolution. It is possible, therefore, that sensitization to bacterial heat shock proteins might stimulate γδ T cells reactive to autologous heat shock proteins expressed, for example, by monocytes, thereby precipitating autoimmune disease (286). Some evidence to support this thesis has appeared (287). T cells reactive to heat shock proteins have been detected in arthritic joints, both in humans as well as in a number of experimental models (287–291).

Late dominance of γδ$^+$ T cells noted at sites of acute inflammation suggests that these cells may be functioning to resolve the inflammatory process (259,292). Late expansion of Tγδ cells may be driven IL-15 based on studies in salmonella infections. Of potentially greater biological significance is the fact that γδ cells have also been identified at sites of inflammation at an early stage and based on their potential to modulate the cytokine milieu likely impact the phenotype of an ensuing immune response. Thus, in mice infected with *Listeria monocytogenes* or *Nippostrongylus brasiliensis,* early expression of interferon (IFN)-γ or IL-4 by γδ cells at infectious foci determined by flourescence activated cell sorter (FACS) analysis was seen to be predictive of the phenotype of the immune response that would subsequently evolve (293,294).

From a transplantation perspective, it is interesting to note that Tγδ cells may be subject to differential immunoregulation and, in addition, may serve important immunoregulatory functions. For example, primed γδT cells have been shown to break tolerance upon adoptive transfer to mice rendered unresponsive by antigen feeding (295). A possible explanation for this effect has been proposed (296). Cyclosporine A has a profound effect on the maturation of Tαβ cells in the thymus but does not affect the development of γδ T cells (297–299).

TcR γδ$^+$ Thy1$^+$ epidermal cells have suppressive activity in a variety of model systems. For example, it is appreciated that after acute graft versus host disease (GVHD) has

been induced in the epidermis by local injection of allogeneic mononuclear cells, it is difficult if not impossible to reinduce GVHD at the same site with a second injection of allogenic mononuclear cells (300). There is an abundant accumulation of TcR $\gamma\delta^+$ Thy1$^+$ epidermal cells at sites of acute GVHD which is thymus dependent. Given the known potential of TcR $\gamma\delta^+$ Thy1$^+$ epidermal cells to mediate suppression we have speculated that these cells may be responsible for preventing a recrudescence of GVHD in this model system, and this has been confirmed based on work in T$\gamma\delta$ cell–deficient mice (301–303).

More recently, $\gamma\delta^+$ TcR hybridomas derived from mice preimmunized by the portal venous route were shown by Gorczynski to mediate anti-IL-10/anti-TGFβ-inhibitable-tolerance transfer and prolong skin graft survival in a rat model (304,305). In light of the evidence that transplantation tolerance induced by portal venous injection of allogeneic mononuclear cells is commonly associated with a Th1/Th0 → Th2 shift in donor-specific immune response phenotype (306, reviewed in 307), it is of interest that intraepithelial T$\gamma\delta$ cells within the gut mucosa, like CD4$^+$NK1.1$^+$ T cells, are readily induced to release IL-4 even in the absence of exogenous IL-4 priming (albeit in lesser abundance) (306,308,309). Finally, there is differential expression of prototypic Th2 cytokines at the maternal fetal interface (310), and extrathymically differentiated T$\gamma\delta$ cells have been found to accumulate in the decidua during early pregnancy (311).

D. MHC Restriction and the T-cell Receptor

Zinkernagel and Doherty received the Nobel Prize in Medicine in 1996 for their observation, reported in 1974, that cytotoxic T cells recognize nominal antigen in the context of self-MHC antigen, a phenomenon referred to as MHC restriction (312). These observations spawned a decade of controversy as to the nature of such dual recognition. Mechanisms of antigen uptake, MHC class I and class II structures, MHC restricted antigen presentation, and antigen recognition have recently been reviewed (313–316). It is appreciated that a single T-cell receptor will suffice for the recognition of both the MHC restricting element and antigen (142). The molecular basis for genetic restriction was further clarified by the resolution of the three-dimensional structure of MHC class I molecules and the identification of a groove in the exposed surface that appeared to contain peptide antigen (reviewed in 316,317). X-ray crystallographic studies on the human class I molecule HLA-A2 revealed two alpha helices rimming a peptide binding groove (318). Pockets were subsequently identified in the wall of the groove of a second class I molecule, HLA-Aw68, which may provide space for side groups of antigenic peptides, consistent with the evidence that MHC molecules bind nonoverlapping populations of antigenic peptides (319).

Davis and Bjorkman proposed a model for TcR - MHC class I interactions in 1988, based on the three-dimensional structure of HLA-A2 defined by X-ray crystallography and the predicted three-dimensional conformation of the TcR, predictions based on sequence data and by analogy with the three-dimensional structure of immunoglobulins. In the previous edition, we predicted that the three-dimensional structure of the TcR would shortly be elucidated because Davis and colleagues had developed a system for cell surface expression of T-cell receptors in a readily cleaved lipid linked form (320). Indeed this has been accomplished (321–323), albeit with great difficulty, because recombinant techniques have not worked as well with TcRs as they did with antibodies. Thus, Garcia et al. have reported the crystal structure of the mouse TcR $\alpha\beta$ heterodimer designated 2C that recognizes self-MHC H-2K^b in conjunction with an octameric peptide (designated dEV8) as well as the allo-MHC restriction specificity L^d in conjunction with a known "self" peptide (322). This

system promises to provide further insight into the molecular nature of allorecognition (see Ref. 145 and following discussion). In addition, Garboczi et al. have studied the crystal structure of an HLA-A2 restricted human TcR heterodimer designated A6 (321).

Davis and Bjorkman assembled data to indicate that TcR Vα and Vβ segments contact the side chains of the MHC alpha helices, whereas a more centrally located region, homologous to the third complementarity determining region of immunoglobulins (CDR3), contacts bound peptide (324). They suggested that the TcR-CDR3 region possessed the required diversity for specific recognition of the potential universe of bound peptides by virtue of the following: the potential N-region diversity noted in all four TcR polypeptides, the large number of TcR-J regions, flexibility in V region joining points, the use of more than one D region product, N region addition at three different sites, and translation of D region sequences in three different reading frames. Brown proposed a similar model to account for TcR recognition of MHC class II molecules and nominal antigen (325). Considerable data accrued to support the hypothesis that the CDR3 site of the TcR is important in TcR interactions with nominal antigen/MHC restriction element (326). Nevertheless, apparent exceptions have also been discovered. Specifically, a different site on the T-cell receptor appears to be involved in TcR interactions with the minor histocompatibility antigen Mls (327,328). These data were consistent with the evidence that superantigens were not bound to MHC class II molecules in the antigen binding groove (329) and that Mls reactivity could be transferred to transgenic mice with a single β chain, whereas the two chains of the TcR were required to confer reactivity to MHC antigens (330,331). Additional data have been reported to indicate that peptides binding to MHC class II outside the peptide-binding groove form alternate T-cell receptor ligands. For example, a peptide from the B chain of insulin, B(10–30) has been seen to compete with staphylococcal enterotoxin B (SEB) for binding to MHC class II in a murine system (332).

Such exceptions aside, present data (reviewed by Bjorkman in Ref. 145) support a new, expanded model in which the TcR interacts with membrane distal portions of MHC class I (α1 and α2 subdomains) and MHC class II (α1 and β1 subdomains), each subdomain being composed of a four-stranded β sheet and a single α helix, with associated short peptides (class I, 8–9 residues; class II 14–20 residues) embedded between paired helices. Whereas it had previously been considered that the CDR3 domain of the TcR contacted peptide within the antigen-binding groove of MHC molecules, it appears that the TcR interacts diagonally with helices of MHC restriction elements (parallel to the β strands). Thus, it has recently been discovered through the analysis of the crystal structure of TcR A6 with HLA A2 (and TcR 2C with H-2K^b) that the Vα CDR1 and CDR3 domains lie over the amino (N) terminus and middle of bound peptide, and TcR Vβ CDR1 and CDR3 domains interact with carboxy-terminus (-C) and middle of bound peptide, respectively (Fig. 6). These findings have been substantially confirmed. Thus, Garcia et al. have documented a similar orientation of the 2C TcRαβ with its target MHC-peptide complex (323,333). Importantly, however, noncomplementarity determining regions may also impact MHC class II$^+$ peptide recognition (334). Moreover, there are also data to suggest that peptide sequence and the manner in which peptides are settled into the antigen-binding groove of MHC restriction elements may also affect peptide recognition and the consequences of peptide recognition:

1. Specific T cells recognize kinetic (stable versus unstable) isomers of the binding of peptide to MHC class II (335).
2. T-cell antigen receptor interactions with peptide-MHC class II/peptide-MHC

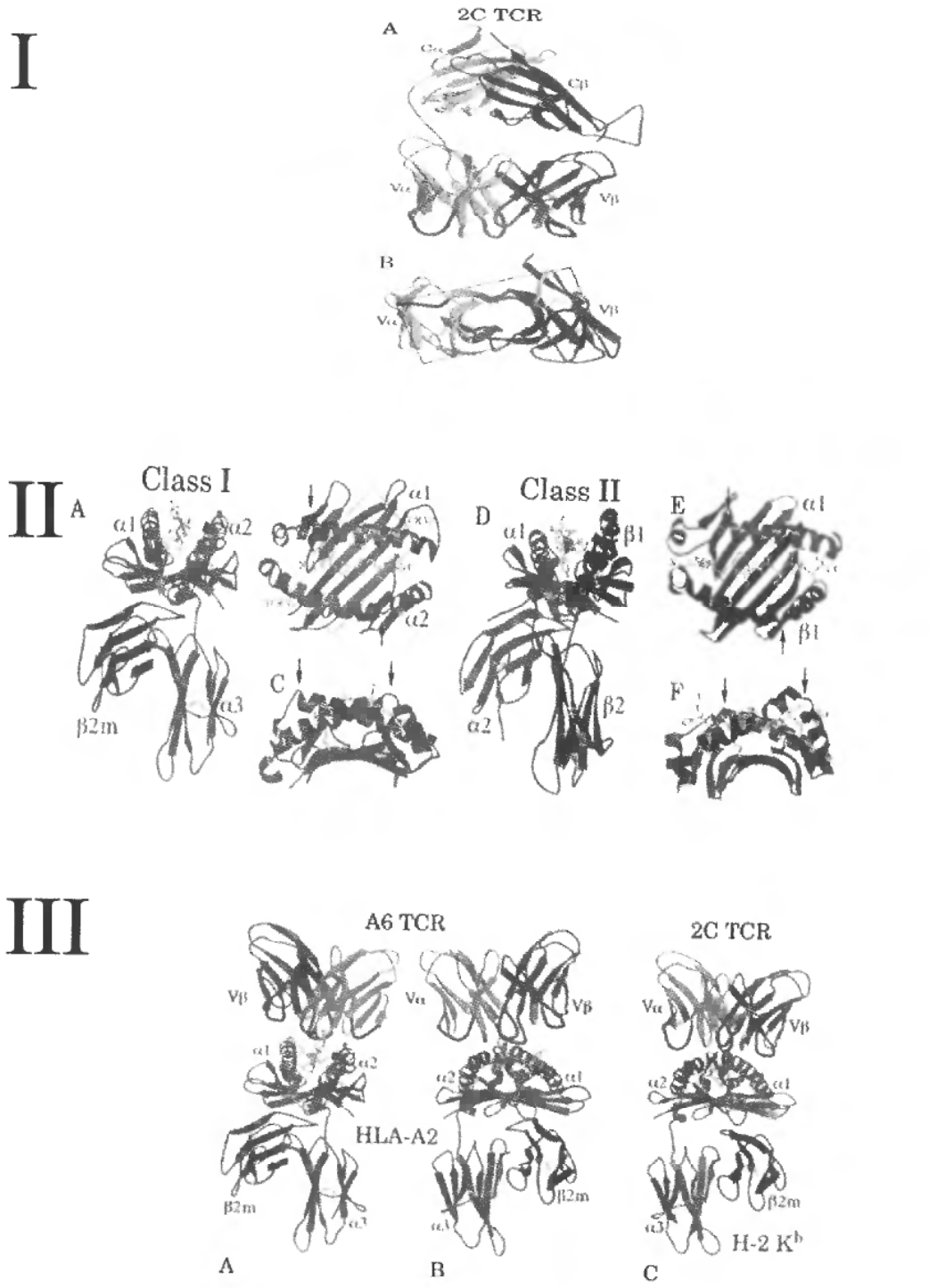

Figure 6 Structure and orientation of T-cell antigen receptors (TcR) with respect to peptide-MHC class I complexes. **I. (A)** Orientation of the complementarity determining regions (CDR): CDR1, CDR2, CDR3, of the 2C TcR. **(B)** Box corresponds to the TcR footprint on the peptide-MHC class I complex illustrated in **II (B). II.** Structures of MHC class I **(A–C)** and class II **(D–F)** molecules. Arrows in **(B). (C). (E),** and **(F)** indicate the high points of these molecules. The rectangle in **(B)** approximates the TcR footprint that misses the high points of the molecule, thus affording greater contact between the CDR of the TcR and peptide resident in the groove of the MHC class I. **III.** Structures of the A6 TcR **(A and B),** 2C TcR **(C),** and orientation with respect to HLA-A2- and H-2 K^b-peptide complexes, respectively. CDR-1, -2, -3 indicated by shades of gray as specified in (1). (From Ref. 145).

class I have been shown to be "stabilized" through associated recognition structures CD4 and CD8, respectively. HLA-A2 has recently been crystalized in association with CD8αα homodimers, and in this setting, interactions between CD8 and HLA-A2 were found to shift the position of the α3 domain of the MHC class I moiety, potentially augmenting the avidity of TcR-peptide-MHC interactions (336).

3. Th1- and Th2-like cloned T-cell lines preferentially recognize different (components of) autoantigen peptides, wherein Th2 was seen to preferentially recognize the NH2-terminal of a myelin proteolipid peptide (337).

E. Allorecognition

The process/phenomenon of antigen presentation has recently been reviewed (313,315). The presentation of soluble antigen is seen to require three steps: antigen capture, formation of stable antigen MHC conjugates, and antigen presentation/recognition (314). The requirements for effective immune surveillance necessitate that virtually all nucleated cells be capable of antigen processing and presentation to curtail (viral) infection and to provide for some measure of expression of tumor immunity, however weak and inefficient. Immune recognition of alloantigens sparks the allograft response and the generation of a variety of immune effector mechanisms competent to mediate homograft destruction. Although there are obvious exceptions, antigen presentation to $CD4^+$ T helper cells is largely restricted to MHC class II^+ professional antigen-presenting cells, to include dendritic cells, Langerhans cells in skin, macrophage, and B lymphocytes. However, other cell types, including activated endothelial cells, may also be competent to present antigen, at least to primed T cells. Particular attention has focused on the role of professional APCs in presenting antigen to MHC class I restricted $CD8^+$ T cells over the past year (338). Costimulation afforded through 4-1BB has been shown to provide a powerful costimulatory signal to $CD8^+$ T cells (339).

As evidence accrued to suggest that MHC class I and class II molecules frequently bear endogenous or exogenous peptides, uncertainty arose as to the exact nature of the target epitopes recognized by alloreactive T cells. Do T cells recognize naked MHC antigens or a complex of MHC antigen plus peptide (340)? Such uncertainties have been magnified in recent years as several groups have investigated the possibility that indirect allorecognition may contribute significantly to the pathogenesis of allograft rejection (341–346), thereby affording the possibility of peptide-based therapy for tolerance induction in organ transplantation (347). Thus, peptides corresponding to polymorphic sequences of donor MHC alloantigens may be processed and presented to host T cells by host APC. Indeed, a recent report by Vella et al. (348) indicates that renal allografts with chronic rejection demonstrate priming to representative donor MHC allopeptides. This is a particularly important report inasmuch as it provides independent confirmation of the biological significance of the phenomenon of indirect allorecognition and, as well, because it indicates that chronic rejection may obtain, in large measure, through indirect recognition and response to processed allopeptides.

Moreover, in recent years monoclonal antibodies have become available to track expression of processed self-peptides (349). A particularly attractive example is the Y-Ac antibody, which recognizes a complex formed between $I\text{-}A^b$ and an I-Eα peptide that has recently been used to delineate expression of self-peptides on dendritic cells in T-cell areas

of lymph nodes (350). The latter study calls attention to a new approach for preparing den dritic cells from lymph node and makes the important point that dendritic cells (DCs) in T dependent areas of lymph node express high levels of self-peptide. Given the high levels c expression of self-peptides by DCs, as well as their great immunogenic potential, recer documentation that dendritic cells are present in lymph node (LN) in considerable abun dance suggests that lymph node may be an important site for primary immune activation b autologous peptides.

The potential biological significance of indirect antigen presentation in transplanta tion tolerance and allograft rejection is explored in greater depth in later sections. Howeve persuasive evidence has recently been reported to indicate that peptide-independent recog nition by alloreactive cytotoxic T lymphocytes (CTL) can also occur and that such direc recognition is sufficiently robust to effect rejection of MHC class I disparate skin grafts i murine systems (351,352). Together these data corroborate the long held view that allo recognition is most likely a composite of recognition of naked MHC molecules and MH(molecules containing bound peptide, predominantly the latter (see review in 353). Fou lines of evidence were identified in the previous edition to indicate that allorecognitio involves corecognition of MHC molecule and nominal peptide:

1. Endogenously synthesized peptide ligands participate in the assembly of class proteins in living cells (354). Peptides appear to induce or stabilize the correc folding of α1, α2 domains of MHC class I heavy chains and their associatio with β2 microglobulin prior to transportation of the trimolecular complex to th cell surface (355–357). In the case of class II, the invariant chain inhibits bindin of endogenously synthesized protein to the antigen-binding cleft of the MH(class II molecules during transport from the site of synthesis in the golgi to a acidic endosomal compartment where the invariant chain is removed by protec lytic cleavage, permitting binding of peptides derived from exogenous protein (358,359). These data suggest that the majority of MHC class I and II molecule may be associated with peptide ligands.
2. Alloreactive T cells have been shown to possess remarkable tissue and/or specie specificity, data consistent with the thesis that MHC alloantigen recognition i linked to recognition of endogenous peptides. Briefly, Sherman and colleague identified alloreactive T-cell clones that recognized the mouse class I H-2K product when it was expressed on mouse cells but not when it was expressed o human cells. However, when a crude mouse cell peptide extract was added to th H-2K^b expressing human cells, the human cells became susceptible to lysis b H-2^k-specific alloreactive cytotoxic T lymphocytes (360). Similarly, alloreactiv cytotoxic T lymphocyte clones specific for H-2K^b, when expressed by the murin T-cell line EL4, did not lyse a human T-cell transfectant (Jurkat) expressing th H-2K^b molecule; the same clones were able to lyse Jurkat-K^b cells sensitized b preincubation with an EL4 cytoplasmic extract cleaved by cyanogen bromid (361).
3. Mattson et al. (362) documented that mutations in the β pleated sheet, whic forms the floor of the antigen-binding groove of the HLA-A2 molecule, tha would not appear to be accessible to the T-cell receptor, can abolish allorecogni tion in the human class II system. Similarly, in mice, mutations defining the clas I K^{bm8} allele have been identified in the floor of the antigen-binding groove (363

It is presumed that, in both of these instances, the mutations affect the binding of endogenous/exogenous peptide.

4. Finally, critical analyses of the structural basis for TcR/MHC antigen-nominal antigen interactions support the notion that T cells generally recognize a composite of MHC-restricting element plus nominal antigen. The results of these studies have led some researchers to question whether peptide-independent forms of allorecognition actually occur (364). The Davis-Bjorkmann model (324) predicts that Vα and Vβ segments contact the side chains of the MHC alpha helices, whereas the more centrally located region homologous to the CDR3 domain of immunoglobulins would interact with the bound peptide. Data consistent with this formulation has been obtained through the analysis of TcR expressed by antigen specific T-cell clones and hybridoma. In such instances, cloned TcR genes encoding receptors with specificity for a variety of peptide derived myelin basic protein and other target antigens have shown remarkable restriction of αβ TcR variable elements as well as D, J, and joining region sequences (365–367).

Conversely, a limited number of examples had been identified in which specific alloreactivity (i.e. H-2-I-E, -L^d; Mls) has been correlated solely with the use of specific Vβ chains. Analysis of the TcR αβ cDNA derived from these clones has not revealed restricted use of D or J elements or any conservation of the primary sequence at the V-D-J junctions (324). Of note, although more than 60% of H-2 L^d specific cytotoxic T cells express the same T-cell receptor variable region family (Vβ8) (340,368), there does not appear to be any conservation of the primary sequence at the V-D-J junction (unpublished observations, J. Urban, R. Lowry, J. Bluestone). These data have been cited as an example of peptide-independent allorecognition (340). These findings also formed the basis for studies documenting that anti-Vβ8 antibodies could modulate rejection of heart allografts transplanted across L^d disparities (369). Of note, L^d-restricted cytomegalovirus-specific cytotoxic lymphocytes are also reported to be $V\beta8^+$ (370). Clearly, therefore, the preferential utilization of Vβ8 by alloreactive T-cell clones with specificity for H-2 L^d may only reflect the apparent importance of $V\beta8^+$ in L^d-restricted T-cell responses. H-2 I-E reactive T cells also demonstrate marked restriction in Vβ gene segment usage (Vβ17a, Vβ11, Vβ5.1 and Vβ5.2). Deletion of T-cell clones bearing Vβ5 at the level of the thymus in $I\text{-}E^+$ mice is dependent upon the presence of a cotolerogen (presumably a peptide lying in the antigen-binding groove of the restricting molecule). Analysis of $V\beta5^+$ clones by Woodland et al. (364) revealed the probable origin of these "peptides." Intrathymic clonal deletion of T cells expressing Vβ5.1 and Vβ5.2 in recombinant inbred strains of mice of the $H\text{-}2^d$ haplotype is linked to coexpression of non-MHC–encoded gene products. One of these products maps to chromosome 12 and has been shown to be linked to the gene encoding the endogenous provirus Mtv-9. These data have been interpreted to mean that the I-E–mediated and minor lymphocyte-stimulating antigen (Mls)-determined deletions (see below) of $\alpha\beta TcR^+$ T cells from the repertoire are similar, in as much as they both require the presence of an MHC antigen and a second non-MHC gene product. Even this interpretation of the data may be an oversimplification, however, because there is evidence that the site of interaction between the TcR and particular superantigens involves a region of the β chain distinct from the CDR3 site commonly involved in TcR-antigen interactions (328,330,331).

The data reviewed above provide strong support for the thesis that "T-cell receptors interact with structures determined by both the MHC molecules, and the resident peptide"

(340). Since publication of the previous edition, it has become apparent that minor histocompatibility antigens (e.g., H-Y) are in fact represented by peptides presented in the antigen-binding groove of the standard set of MHC-encoded class I or class II (HLA) antigens (371). In at least some instances, sites of allorecognition have been mapped to sites outside the peptide-binding groove (372). However, these data raise conceptual difficulties at a clonal level. Human alloreactive T-cell clones have been identified that are reactive with particular MHC class II antigens, that is, DR1, as expressed by a panel of individuals who type serologically as DR-1 (Aftab Ansari, Emory University, personal communication). Such identifiable patterns of alloreactivity are hard to explain unless one hypothesizes that individuals sharing the same serologically defined MHC class II antigens coexpress the same (range of) peptides. Nevertheless, this formulation may not be totally unreasonable because the limiting number of MHC peptides that must be expressed to stimulate T-cell activation is quite small, on the order of several hundred molecules per cell (373). More work is required to unravel the nature of allorecognition, and this will likely be accomplished by structural analysis of the TcRs of T-cell clones with particular target specificities. However, this will not be easy because the number of clones potentially responsive to any given MHC alloantigen is likely to be great (374).

The precise nature of the antigens recognized by alloreactive cells is of more than academic interest. For example, Streinlein et al. (375) have reported that, when attempts were made to induce transplantation tolerance (assessed by skin graft survival) by injecting allogeneic mononuclear cells into neonatal mice, some mice still rejected their grafts despite marked reductions in MLR responses and CML generation. Similarly, transfusion-induced suppression of DTH did not correlate with transplantation tolerance (376). One possible interpretation of the two sets of data presented above is that the recipient mice were not tolerant of tissue-specific antigens expressed as nominal antigens in the context of allo-MHC.

III. T-CELL ACTIVATION

A. Introduction

T-cell activation is reviewed here to provide some basis for subsequent discussion of molecular mechanisms underlying positive and negative selection in the generation of the mature T-cell antigen receptor repertoire as well as the peripheral deletion, functional inactivation (i.e., clonal anergy) and/or reprogramming (Th0/Th1 → Th2) of peripheral T cells, which is known to occur during induction of states of conditioned unresponsiveness.

The T-cell antigen receptor is recognized as one of a class of multichain immune recognition structures that share similarity in structure and downstream signaling pathways (377). In the previous edition, we predicted increasing interest in the study of T-cell activation based on early reports showing that:

1. Functional T lymphocyte subpopulations Th1 and Th2 use different signal transduction pathways during activation (378–380) and vary in their susceptibility to pharmacological immunosuppression (381) and tolerance induction (382).
2. Particular (allo)antigens, that is, Mls, excite different signal transduction pathways from those activated in response to conventional transplantation (H-2) antigens (383).
3. Evidence shows that thymocytes may use different signal transduction pathways

during positive and negative selection of the peripheral T-cell receptor repertoire (384).

Most of these findings have been corroborated and extended but remain the focus of intense research endeavor. Recent findings include the following:

1. T-cell antigen receptor–triggered calcium flux profiles in Th1 and Th2 cloned T-cell lines are substantially disparate (385).
2. Antigen receptor proximal signaling events excited by altered peptide ligands differ from those stimulated by peptide agonists (54).
3. Dominant negative Ras inhibits positive but not negative selection in intrathymic T-cell ontogeny, thereby pinpointing Ras signaling as a critical event in positive selection (386,387).

B. Signal Transduction and the TcR–CD3 Complex

Knowledge of the myriad signaling events excited by T-cell antigen receptor occupancy has expanded exponentially since publication of the previous edition of this text; refer to a series of excellent reviews for more detailed expositions of this rapidly evolving field (388–390). Newcomers to the field are particularly encouraged to consult primers on signal transduction and comprehensive reviews of published work on T-cell activation. Outstanding overviews of these topics appeared in a recent text edited by Tilney, Strom, and Paul (391,392).

Recent advances in our understanding of T-cell activation has been made through the unstinting effort and perseverance of productive laboratories and the application of a broad array of highly innovative in vitro and in vivo approaches to search out the potential roles of individual signaling molecules in lymphocyte activation. In the previous edition, we called attention to a novel approach employed by Weiss and colleagues consisting of transfection of muscarinic receptors into Jurkat T-cell lines bearing mutations that interrupt normal signal transduction pathways and assessment of the consequences of activating these cells with chemical ligands to trigger the expressed muscarinic receptor (393). Even though gene transfection and transient overexpression of signaling moieties in Jurkat or other continuous cell lines are commonly applied strategies in signal transduction research, the utility of such approaches is clearly limited. Accordingly, transgenic and gene knockout (GKO) mice have been extensively used in recent years to pinpoint the roles of individual signaling molecules in T-cell activation. The use of dominant negative (DN) transgenes has aided dissection of the role of elements in redundant signaling cascades. The yeast two hybrid assay system has proven to be an invaluable alternative to coprecipitation and end terminal sequencing as the standard route to identify and clone transduction moieties that transiently associate during signaling events excited by receptor ligand binding. DNA footprinting, electrophoretic mobility gel shift assay (EMSA), and gene promoter-driven CAT/luciferase constructs are applied, in the order listed, in increasing frequency in the analysis of the specificity and functional consequences of intracellular signaling on downstream transcriptional events. Finally, immunohistochemistry and confocal microscopy have provided valuable clues as to the roles of individual gene products in different cellular compartments (e.g., PKCθ(394), Bcl-2, IκB, JIP, Jak3, STATs [394–398]).

Five years ago, it seemed reasonable to suppose that two, if not three, separate and distinct signal transduction pathways might be excited by antigen receptor occupancy in the

initial phases of T-cell activation, and that downstream signaling events transduced by TcR cross-linking intermingled with signals arising from adhesive interactions, affording critical costimulation and/or cytokine (e.g., IL-2 receptors) to drive cell cycle progression. Therefore, discussion in the previous edition focused on two TcR-triggered signal transduction pathways:

1. Receptor triggered activation of phospholipase C-γ1 with consequent activation of hydrolysis of membrane inositol phospholipids, leading to the release of diacylglycerol (DAG) and inositol trisphosphate (IP3), which serve, respectively, in the activation of protein kinase C (PKC) isoforms (a family of serine/threonine kinases) and the release of calcium (Ca^{2+}) from internal stores as well as Ca^{2+} entry from external sources (399).
2. Antigen receptor-triggered activation of protein tyrosine kinases and downstream signaling by cAMP as a second messenger, observed in Th2-like cloned T cell lines (400).

   It is understood that cAMP may serve as a second messenger in growth factor–mediated mitogenic responses, at least in neuronal tissues, based on its demonstrated potential to activate MAP kinases and Elk-1 through a B-Raf- and Rap 1-dependent pathway (401). However, cAMP is also recognized to be a potent inhibitor of proliferation of Th1-like cloned T-cell lines and has the potential to antagonize pre-TcR-CD3-$p56^{lck}$ signaling in thymocytes (402), possibly by antagonizing the Ras signaling pathway (401). Activation of the Ras, Raf, MEKK, MAPK (MAP kinase, also known as extracellular signal-regulated kinase/ERK1 and ERK2) cascade in response to TcR proximal tyrosine phosphorylation events is now identified as a preeminent feature of TcR-signaling in Th1 cells. The MAP kinase signaling pathways have been the subject of several excellent reviews (403). Moreover, activation of Th2-like helper T cells may be Ca^{2+} and phospholipase C independent. These data, together with discussion to follow, suggest that distinct molecular mechanisms may be implicated in activation and anergy of helper T cell subpopulations Th1 and Th2.

The demonstrated potential of cAMP, cited above, to activate rap1 and inhibit ras ([401], corroborated in Ref. (404) is potentially of great interest. A recent study has shown that induction of anergy in a human Th1-like helper T-cell line in the absence of B7 costimulation correlated with the fyn-dependent activation of rap1 and was reproduced by microexpression of rap1 in Th1 cells (4). A broad literature exists to show that PGE2 or its downstream effector cAMP inhibits activation of Th1 cells but has little effect on Th2 (405–408). Moreover, cAMP has been implicated, at least historically, as a second messenger in Th2 cells (400). A potential conclusion is that Th2 cells might be relatively insensitive or fully resistant to rap1-dependent anergy as initially defined in a human Th1 cell line, an observation that would be consistent with an evolving consensus that Th2 are relatively insensitive to induction of T-cell anergy triggered by TcR cross-linking in the absence of B7 costimulation (409). Conversely, however, some investigators have reported that Th2 are readily induced to become anergic through exposure to altered peptide ligands. Therefore, even though Schwartz and others have made the point that there is nothing "unique" about the receptor proximal signaling events excited by APL (410), there must indeed be something unique about downstream correlates of T-cell anergy in Th2.

The Ras superfamily of GTPases comprises more than 50 members, divided into at

least seven groups based on sequence similarity (Ras, Rho, Arf, Sar, Ran, Rab, Rap); these function as molecular switches by cycling between an active GTP-bound state and an inactive GDP-bound state (411,412). The function of Ras (and presumably most members of this superfamily) is regulated by at least three sets of modifiers: the GEFs (guanidine nucleotide exchange factors), which drive exchange of GTPase-bound GDP for cytoplasmic GTP, the GAPs (GTPase activating proteins), which enhance their GTPase activity, and the GDIs (guanidine dinucleotide exchange inhibitors), which inhibit exchange of GDP for cytoplasmic GTP. An example of the latter is Ly-GDI, a factor cloned in 1993, which inhibits GTP/GDP exchange on the Rho family of small GTPases and which is expressed preferentially in lymphocytes (413). Of note, 5 years ago Chavner et al. proposed that it might be worthwhile to look at the Rho family of GTP-binding proteins (414). Data are presented later in this chapter to suggest that members of the Rho family of small GTPases might indeed serve a critical role in activation of JNK/SAPK pathways associated with T-cell costimulation.

Furthermore, TcR occupancy and cross-linking has also been shown to activate the JNK/SAPK pathway (63). JNK/SAPK was first identified in 1993 to 1994 as a stress-activated kinase that phosphorylates and activates c-Jun (415,416). Evidence exists to show that full activation of the JNK/SAPK T cells, as obtained following exposure to TPA plus a calcium ionophore, requires simultaneous cross-linking of the T-cell antigen receptor and CD28 (59). This finding led Su et al. to suggest that JNK is involved in signal integration in CD28-dependent costimulation of TcR-triggered T-cell activation and IL-2 production. This supposition has recently been substantially corroborated by the demonstration that CD28 costimulation of TcR-triggered T-cell proliferative responses, IL-2 production, and c-Jun NH2-terminal kinase (JNK) activation are significantly, if incompletely, obliterated in transgenic mutant (gene knockout) mice deficient of SEK (otherwise called MEKK), a dual function tyrosine-threonine kinase upstream of JNK (417). However, it has been demonstrated that TcR/CD28-triggered activation of JNK is not significantly affected in Itk mutant mice even while antigen-receptor signaling is substantially abrogated (418).

Evidence that receptor proximal tyrosine phosphorylation events excited by antigen receptor occupancy may connect to downstream events ($p21^{ras}$ activation) independent of phospholipase C-γ1 (PLC) activation (128,419) supports the present construct that TcR triggering stimulates two (or more) signaling pathways that diverge, at least transiently, at the level of PLC. Additional support for this construct is obtained from the fact that TcR-triggered Ras activation is potentially one of the quintessential events in antigen receptor–triggered T-cell activation (420) and that induction of T-cell anergy interrupts Ras activation even while activation of PLC is unperturbed (61,63). However, accumulating evidence indicates that the PLC pathway and the Ras pathway may be interdependent. Substantial data indicate that G proteins and/or Ras are involved in the activation of phospholipase C (421), as well as calcium/calcineurin induced dephosphorylation (activation) of NFAT family members and IL-2 gene transcription (422,423). Such interdependency does not detract, however, from the functional integrity of the Ras signaling pathway. Although Ras has been implicated in PKC activation, downstream effects of Ras in the induction of AP-1 and NFAT transcriptional events are demonstrably PKC independent (424).

C. Receptor Proximal Events in T-Cell Activation

The T-cell receptor for antigen is expressed on the T-cell surface in association with CD3, a complex composed of six or more chains: gamma (γ), delta (δ), epsilon (ε), zeta (ζ), and

eta (η) (425–427). The γ chain of the FcεR1 receptor, cloned in 1990 (428), is also transcribed in T cells under the influence of an Alu consensus sequence in its promoter (429) and expressed as a homodimer or heterodimer with other component chains (ζ, η) of the CD3 complex (430). The component elements of the TcR–CD3 complex are deficient in tyrosine kinase activity. CD3 invariant chains γ, δ, ε, members of the immunoglobulin superfamily, express single copies of a 26 amino acid sequence known as the *immunoreceptor tyrosine-based activation motif* (ITAM) within their cytoplasmic tails (431). The gamma chain of the high-affinity IgE receptor (FcεRI), CD3-η and CD3-ζ belong to a distinct family (428) and express one, two, and three copies of the ITAM motif in their cytoplasmic tails, respectively. Employing the single letter code for amino acids (Y = tyrosine), the consensus sequence of the ITAM motif is as follows: $\underline{\mathbf{Y}}XXL(X)_{6-8}$ $\underline{\mathbf{Y}}XXL$. The tyrosines of individual ITAMs become phosphorylated following T-cell activation and are mediated by a receptor-associated tyrosine kinase of the src family (i.e., fyn) as well as a second src family member, $p56^{lck}$, which expresses and physically associates with di-cysteine domains in the amino-terminus of the CD4 and CD8 coreceptors (391). Thereafter, the tyrosine-phosphorylated ITAMs serve as docking sites for downstream signaling elements that express and associate through specific tyrosine-binding pockets known as SH2 domains (see below). The ITAMs of the CD3-ζ chain were functionally characterized by Weiss and colleagues and shown to be sufficient to couple to receptor-associated signal transduction pathways (432,433).

The potential significance of expression of the FcεRI-γ chain as a component element of the CD3 complex has drawn considerable interest. The FcεRI-γ chain is known to be an active signal transduction element when expressed as a component of Fc receptors in B lymphocytes, mast cells, and cells of the monocyte-macrophage lineage (434–437), and it seemed only reasonable to suppose that the FcεRI-γ chain might participate in transmembrane signaling when expressed as a component of the TcR-CD3 complex. We have speculated that the FcεRI-γ chain might be differentially expressed as a component of CD3 in Th2-like effectors based, in part, on evidence to show that some Th2 might express Fc receptors (438). Indeed, ongoing uncertainties surrounding qualitative and quantitative differences in the signaling properties of individual T-cell antigen receptor subunits have provided a powerful motivating force for sustained research in this arena (439):

1. Much of the original research on T-cell antigen receptor–proximal signal transduction events was performed in Jurkat cells, and there were some unexpected results when similar experiments were performed in other cell lines. For example, Cambier and colleagues recently determined that CD3-ζ signaling in non-Jurkat cells induced phosphorylation of phospholipase C but failed to induce a calcium signal (439). Conversely, CD3-ε signaling in the same cells was associated with early phosphorylation of PLC and a brisk calcium transient.
2. Expression of tailless FcεRI-γ chains as a component of CD3 in cells deficient of CD3-ζ has been found to be associated with phosphorylation of downstream substrates and IL-2 biosynthesis (440). However, the profile of substrate phosphorylation differed from that obtained with TcR containing full-length γ chains. These observations were interpreted to mean that the FcεRI-γ chain tail might serve in the recruitment of unique downstream signaling pathways.
3. Evidence has accrued to suggest that STAT6 as well as an "extrinsic" lymphocyte source of IL-4 (as well as IL-2) were required to support the differentiation of naive T cells into T cells capable of producing IL-4, IL-5, IL-6, IL-10, and IL-13

(i.e., Th2) following in vitro activation (309,441,442); this "extrinsic" source has not yet been defined. Conversely, neither IL-4 nor STAT6 are required for IL-4 production by differentiated Th2 effectors, and it is speculated that a Th2-specific transcription factor (i.e., c-maf) may supercede the need for STAT6 and IL-4 once the differentiated Th2 phenotype is acquired, that is, beyond day 7 of in vitro culture (309,443).

It became of interest, therefore, that intraepithelial CD8-α/α T$\alpha\beta$ and T$\gamma\delta$ lymphocytes in the gut, as well as NK1.1+ TcR-$\alpha\beta^+$, and NK1.1+ TcR-$\gamma\delta^+$ thymocytes were found to express high levels of FcεRI-γ chain homodimers or heterodimers with CD3-ζ as components of CD3 (444–446). Interest in this domain was greatly magnified when intraepithelial T $\gamma\delta$ cells, as well as NK1.1+ TcR-$\alpha\beta$+ and NK1.1+ TcR-$\gamma\delta^+$ thymocytes expressing high levels of the FcεRI-γ chain as a component of CD3, were shown to be readily induced to produce IL-4 in response to TcR-CD3 cross-linking (264,293,294,447–453). The subsequent demonstration that NK1.1+, $CD4^+$, TcR-$\alpha\beta^+$ T cells were capable of producing IL-4 (as well as IFN-γ) in response to antigen receptor occupancy even in the absence of IL-4 or CD28 costimulation (308) afforded the final spur that prompted several groups to make a major research commitment in this arena.

Research efforts to test the hypothesis that the NK1.1+, $CD4^+$, TcR-$\alpha\beta^+$ T-cell subset (commonly identified as natural T (NT) cells) might represent an important cellular source of IL-4 providing for the genesis of Th2 responses have taken advantage of the fact that NT cells possess a limited TcR receptor repertoire restricted by a limited set of target antigens. Thus, NT cells express predominantly Vα14-Jα281 paired with one of three Vβ domains (Vβ8.2, Vβ7, or Vβ2) and are commonly restricted by CD1, a class I–like molecule encoded outside the MHC by two sets of genes: CD1d.1 and CD1d.2 (see Ref. 454). Accordingly, β2 microglobulin GKO mice and, more recently, mice with targeted disruptions of CD1d.1 or CD1d.1 and CD1d.2 have been studied to assess the potential contribution of NT-derived IL-4 in the genesis of Th2 responses. Early studies in β2 microglobulin–deficient mice indicated that class I restricted NT cells were not required for early IL-4 production or the generation of Th2 responses (455,456). More recent work has shown that CD1d.1 mutant mice and CD1d.1/CD1d.2 double mutant mice, which are largely deficient in NT cells, are also deficient in their ability to produce IL-4 promptly in response to CD3 cross-linking (457,458). Nonetheless, CD1d.1/CD1d.2 double mutants retain the potential to generate Th2 responses (458). Overall, therefore, the literature does not support the possibility that NT-derived IL-4 is necessarily required for generation of Th2 responses (455,456,458,459), because CD1-deficient mice are still able to produce IL-4 (456,460). Accordingly, other cellular sources yet need to be defined to identify the origins of the IL-4 providing for the induction of Th2 responses in specific settings (308).

The alternative approach has been to generate CD3ζ- as well as CD3ζ/η-deficient mice and to evaluate whether residual T cells could be identified that expressed the FcεRI-γ chain as a component of CD3, and that retained the potential to produce IL-4 without exogenous IL-4. These studies have generally been confounded by the gross lymphopenia observed in such mice. Development of NK1.1+ TcR-$\alpha\beta^+$ T cells was found to be markedly restricted in CD3ζ-deficient mice; these data were interpreted to mean that there was a general requirement for CD3-ζ in development of TcR-$\alpha\beta^+$ T cells (444). This supposition has been further strengthened by recent documentation that CD3-$\zeta/\eta^{-/-}$ deficient mice accumulate only limiting numbers of seemingly conventional peripheral T cells expressing vanishingly low levels of CD3 (461).

However, documentation of a possible developmental block in T-cell ontogeny in CD3-ζ/η$^{-/-}$ deficient mice does not necessarily exclude the possibility that the FcεRI-γ chain might participate in TcR signaling in at least a subset of the peripheral αβ T cells pool. Given what is presently known about differential usage of various signal transduction moieties in thymocytes and peripheral T cells, we considered that it would be premature to exclude the possibility that expression of the FcεRI-γ chain has a qualitative or quantitative impact on immune responsiveness of peripheral (αβ) T cells. Thus, the oft demonstrated partial block in intrathymic T-cell development at the CD4$^-$, CD8$^-$ to CD4$^+$, CD8$^+$ transition (462) did not necessarily imply that TcR signaling (positive selection) is inoperative in CD3-ζ/η deficient mice; TcR-CD3ζ(η) may be critically required to subserve functions other than TcR signaling during T-cell ontogeny.

Terhorst and coworkers have since identified MHC class II/class I restricted intestinal CD4$^+$- as well as CD8αα$^+$-lymphocytes in CD3-ζ, CD3-η gene knockout mice, which express high levels of TcRαβ and the FcεRI-γ chain as a component of CD3 and produce IL-4 in response to CD3 cross-linking (462). Overall, these data suggest a potential correlation between IL-4 biosynthesis and signaling events excited by T-cell antigen receptors containing the FcεRI-γ chain as a component of CD3.

Gammaδ$^+$ TcR FcεRI-γ$^+$ intestinal lymphocytes were readily detected in γδ transgene G8$^+$-CD3-ζ deficient mice, but these were found to be incapable of responding to antigen or mitogen (Concanavlin A) by proliferation or cytokine (IL-2, IFN-γ) production. They would, however, proliferate in response to antigen receptor or anti-CD3-ε cross-linking (463). Given that TcR γδ$^+$ FcεRI-γ$^+$ intestinal lymphocytes are a recognized source of the prototypic Th2 cytokine IL-4, it is disappointing that antigen-induced IL-4 biosynthesis by γδ TcR FcεRI-γ$^+$ cells from CD3- chain GKO mice was not assessed (463). Experimental data have been reported that are consistent with the thesis that Tγδ cells excited at an early phase of an immune response might provide IL-4 needed to tolerize or otherwise deviate immune response profile of αβ T cells toward a Th2 phenotype (464). Moreover, Gorczynski has reported an association between Tγδ cells, a Th0/Th1 → Th2 switch in immune response phenotype, and oral tolerance in murine systems (304). Additionally, it has been determined that mice deficient in Tγδ cells are resistant to induction of oral tolerance (465). It seems not unreasonable, therefore, to suppose that IL-4 producing γδ intraepithelial lymphocytes might contribute to the persistence and Th2 cytokine response profile of self H-2L^d reactive TcRαβ 2C transgene$^+$ T cells (36) in target antigen–expressing mice when the majority of peripheral TcR αβ 2C Tg$^+$ T cells were found to have been deleted.

Studies were performed in the laboratory of one of the authors (RPL) to ascertain expression of the FcεRI-γ chain as a component of CD3 Th1 and Th2 cloned T cell lines. The FcεRI-γ chain was readily visualized by Western blotting of anti-CD3-ε mAb 2C11 immunoprecipitates generated from a Th2 cell line (CDC25) but not Th1 cells (D1.1) (466) (Fig. 7). Although the Th2-like mouse T-cell clone studied (CDC25) has previously been thought to be a conventional αβT cell line, it may be pertinent that NK1.1 is not a stable marker of this lineage and may be lost following in vitro stimulation of NK1.1+, CD4$^+$ T cells (467). This observation, together with evidence accrued to indicate that this cell line might be Fc receptor positive (Fig. 8), raises additional questions about the lineage of this cell line. Accordingly, although the literature does not support the possibility that an important fraction of αβ T cells or cloned Th2-like CD4$^+$ T-cell lines express the FcεRI-γ chain as a component of CD3 (446), it remains conceivable that IL-4 production by a subset of αβ T cells expressing the FcεRI-γ chain as a component of their TcR-CD3 complex contributes to immune deviation and the genesis of Th2-like immune responses (Figure 9). Recently, two independent groups have documented that the FcεRI-γ is sufficient to recon-

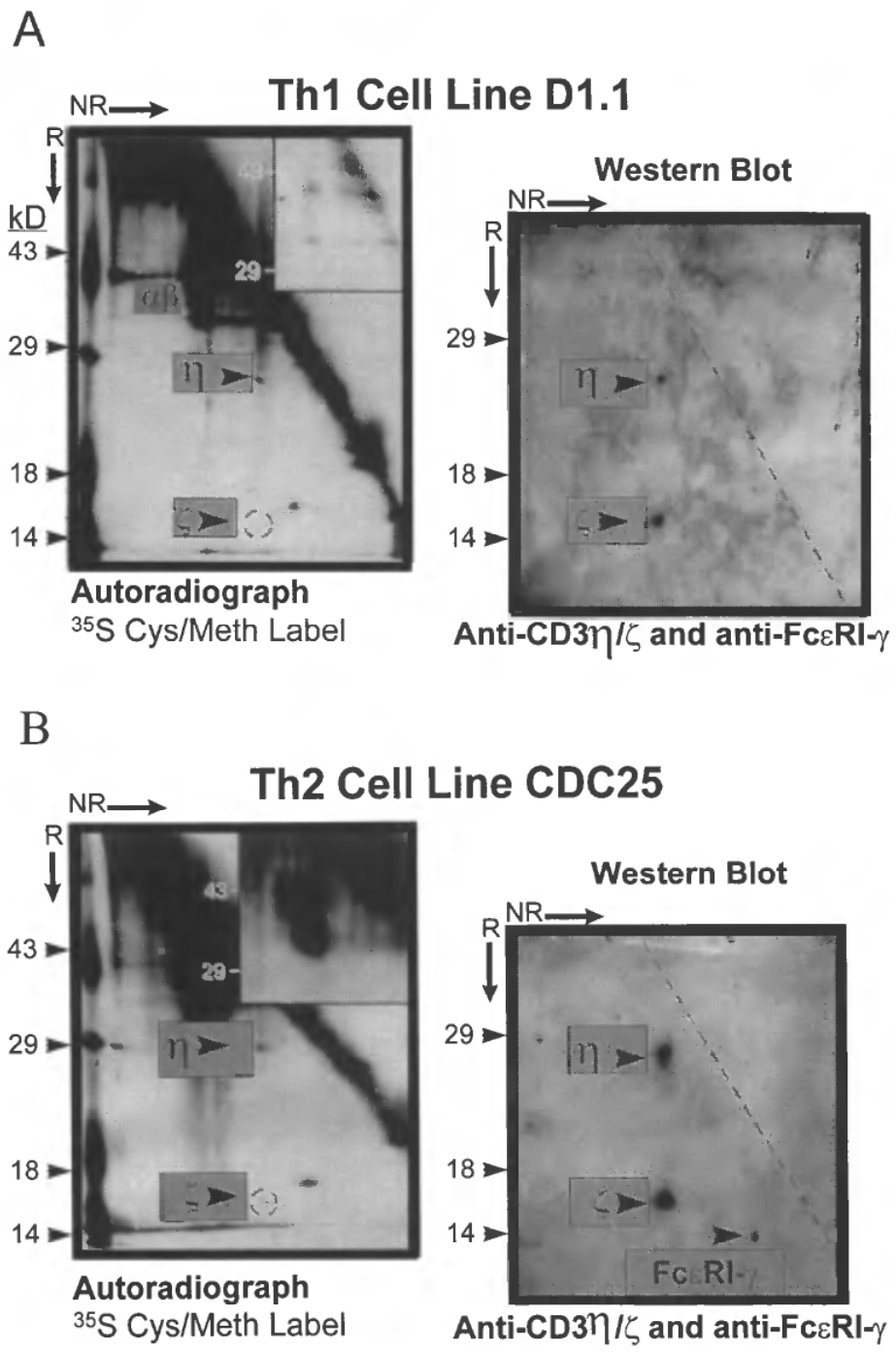

Figure 7 Differential expression of the FcεRI-γ chain as a component of the TcR–CD3 complex of a prototypic Th2-like mouse T-cell clone CDC25 **(B)** but not Th1-like clone D1. **(A),** as determined by immunoprecipitation and Western blotting. *Left panels* of **(A)** and **(B)** are autoradiographs of anti-CD3-ε mAb immunoprecipitates from digitonin lysates of metabolically labeled (^{35}S-cysteine/methionine; TransLabel) Th1 and Th2 cell lines specified, separated by two-dimensional (nonreducing/reducing) polyacrylamide gel electrophoresis (PAGE) per Current Protocols in Immunology and purposely overexposed to illustrate CD3-ζ CD3-η. Circles illustrate the position and identities of CD3 chains seen in autoradiographs but poorly visualized in photographic reproductions. Inserts represent shorter exposures of the same autoradiographs to demonstrate separation of TcR-α and TcR-β chains in the second dimension under reducing condition. *Right panels* of **(A)** and **(B)** are autoradiographs of anti-CD3-ε mAb immunoprecipitates from unlabeled Th1 and Th2 separated in parallel with the above by two-dimensional (nonreducing/reducing) PAGE, blotted to nylon membranes and analyzed by Western blotting with antisera against CD3-ζ/η (R.D. Klausner) and FcεRI-γ (# 667). (From Ref. 466 and unpublished observations, B. Konieczny, P. Selvaraj, T. Takeuchi, and R.P. Lowry.)

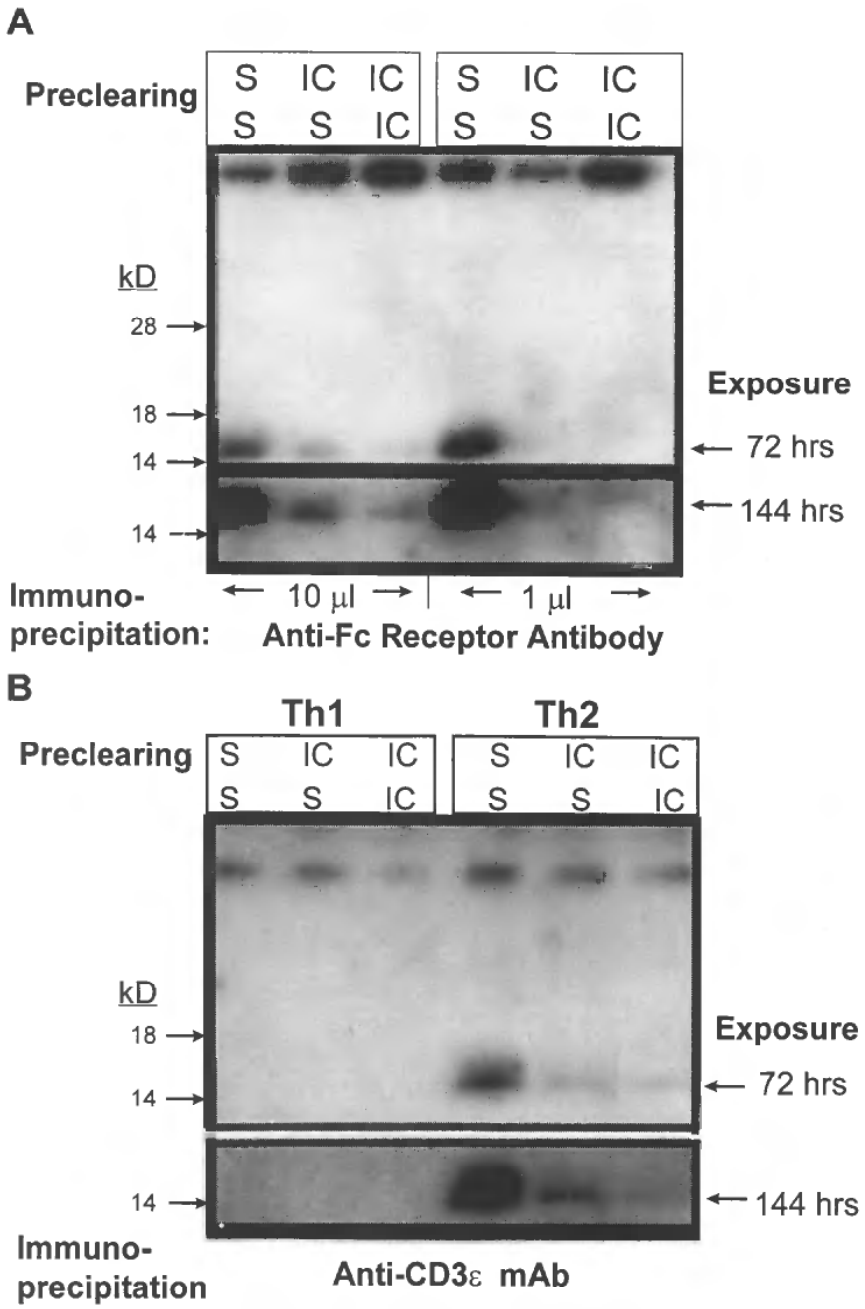

Figure 8 FcεRI-γ chains detected in (**A**) anti-Fc receptor mAb 2.4G2 immunoprecipitates of macrophage cell line P.388D.1 and (**B**) anti-CD3-ε immunoprecipitates from digitonin-lysates of Th2-like cloned T-cell line CDC25, which had previously been precleared by two rounds of immunoprecipitation with sepharose (S) and/or immune-complex (IC-) coupled-sepharose beads. (**A**) *Upper panel:* Semiquantitative assay evolved to demonstrate preclearing of Fc receptor–associated FcεRI-γ chains by one or two rounds of immunoprecipitation with (IgG–anti-IgG) IC covalently coupled to S beads. After two rounds of preclearing with S and/or IC beads, residual Fc receptor–associated FcεRI-γ chains were immunoprecipitated with 10 or 1 µL of anti-Fc receptor mAb 2.4G2 (From Ref. 1043), and detected by polyacrylamide gel electrophoresis (PAGE) and Western blotting with specific anti-FcεRI-γ chain antiserum #667. Here, extended exposure of anti-FcεRI-γ chain immunoblots has been performed to demonstrate the quantitative nature of the immunoprecipitation achieved (From (1046) and unpublished observations, R.P. Lowry, B. Konieczny, T. Takeuchi, and P. Selvaraj). (**B**) *Lower panel:* Two rounds of preclearing with IC as per (**A**) effectively removes anti-CD3ε-precipitable FcεRI-γ chains from digitonin lysates of Th2 cell line CDC25 as assessed by PAGE and Western blotting with specific antiserum #667. These data are interpreted to mean that there is an intermolecular association between Fc receptors and the TcR–CD3 complexes on the surface of Th2-like cell line CDC25. Interestingly, and consistent with the above, NK receptor and Fc receptors have recently been found to be physically associated on the surface of WK cells. Th1 cells tested, D1.1 cell line (B), do not express the FcεRI-γ chain as a component of the TcR.

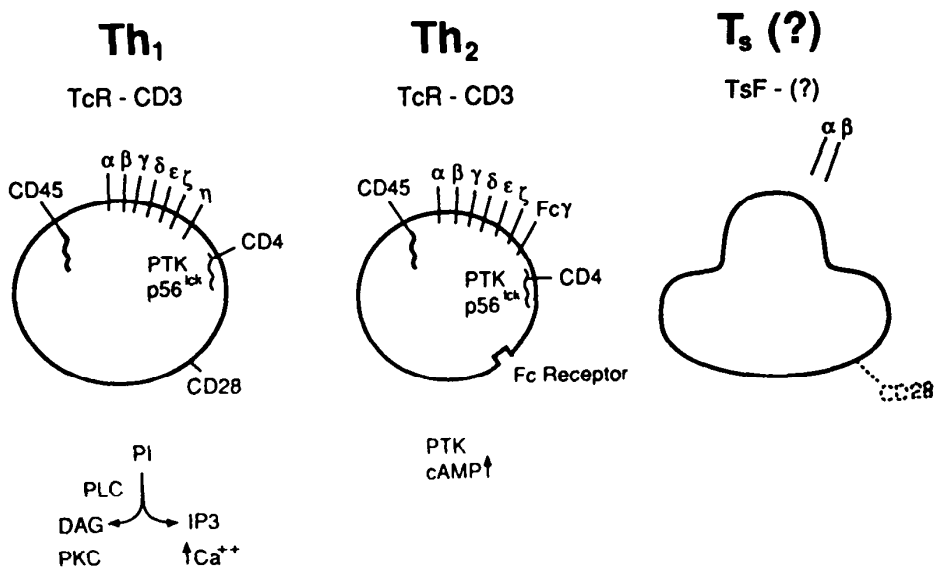

Figure 9 Hypothetical schema intended to highlight potential differences in the component chains of CD3 detected in Th1 and Th2 cell lines, wherein both Th1 and Th2 were found to express CD3-ζ and CD3-η but only the Th2 clone CDC25 is seen to express the FcεRI-γ chain (see Fig. 7) as well as the different TcR signaling pathways transduced in Th1 and Th2. It is proposed that a subset of Th2-like effectors and/or their precursors expressing the FcεRI-γ chain as a component of CD3 may be able to make IL-4 in the absence of exogenous IL-4.

Differences in the composite structure of CD3 in Th1- and Th2-like effectors may also contribute, in part, to recognized differences in the downstream signal transduction pathways excited by TcR cross-linking in these two T-cell subsets. Specifically, as illustrated in the lower part of this figure, there is substantial evidence to show that TcR-triggered activation of Th1-like effectors is associated with activation of PLC, a brisk calcium transient, as well as activation of the Ras-MAP kinase pathway. Conversely, TcR-triggered activation of Th2 has been shown to proceed in the absence of a calcium signal, at least in murine systems, and we are not aware of any data to show that the Ras-MAP kinase pathway serves any critical role in transducing TcR-triggered activation of Th2. Knowledge of the signaling pathways excited by antigen receptor occupancy in Th2 is limited. Thus, cAMP is identified as second messenger in Th2. Moreover, mutations in GATA-3 or the CLEO element abolish antigen- or cAMP-induced activation of IL-4 or IL-5 gene transcription (1044–1045). CLEO binding protein (CLEBP-1) is a second factor that may be critical for induction and expression of the Th2 T cell phenotype (1048). Thus, both IL-4 and IL-13 promoters contain binding sites for GATA-3 transcription factors (1049–1051).

Data have accrued to indicate that Th2-like effectors may function as antigen-specific suppressor cells in some systems. Nonetheless, there are abundant data to indicate that other cell types may also mediate immunological suppression (113). We have reasoned that T helper cells rendered anergic by exposure to antigen may be reprogrammed to assume a plasma cell–like phenotype (as depicted on the right), losing expression of typical surface markers of the T-cell lineage and release soluble factors that mediate antigen-specific or antigen-unspecific downregulation of the immune response. Evidence has recently accrued to support the potential validity of this paradigm (979), wherein superantigen injection induces a population of $CD4^-$ $CD8^-$ cells that mediate IFN-γ–dependent transferrable suppression.

stitute the immune system of CD3-ζ chain deficient mice (468,469). In raising issues addressed above, we are mindful that a distinct phenotype has not been observed in the thymus or peripheral T-cell compartment of FcR-γ GKO mice, even though such mice are notably deficient in phagocytosis of antibody-coated particles as well as antibody-dependent cell-mediated cytotoxicity (ADCC) responses and mast cell–mediated allergic

responses (470). It will be of great interest to evaluate T-cell function in CD3-ζ chain deficient mice whose immune systems have been reconstituted through the aid of an FcεRI-γ transgene.

Even minor differences in TcR signaling arising from differences in the affinities of TcR-peptide/MHC interactions have been shown to impact the predilection of T cells to mature as Th2-like effectors (471) or suffer "antigen-induced cell death" (AICD) (46,472–476). T cells surviving (resistant to) activation-induced cell death have been shown to express a high level of Th2 cytokines (477). It is also conceivable that Th2 cells escape antigen-induced cell death because of a deficiency of a particular cytokine; IL-2 has been shown to be absolutely required to sensitize T cells to AICD, a Fas/FasL- or TNF/TNF receptor-induced signaling event (46–48). The observation that TGFβ has the potential to inhibit AICD in Th1 cells is not inconsistent with this thesis based on evidence to show that TGFβ inhibits IL-2 receptor signaling, blocking IL-2-induced phosphorylation of Jak1 and STAT5 (478). It presently seems reasonable to suppose that TGFβ-induced interruption of IL-2R signaling might be related to the induction of a phosphatase, possibly one of the SH2-containing cytoplasmic phosphatases (e.g., SHP-1 or SHP-2), based on evidence to show that TGFβ blockade of IL-2R signaling is interrupted by the phosphatase inhibitor sodium vanadate. It may be pertinent that the differential sensitivity of Th1 to rapid FasL/Fas-mediated antigen-induced cell death (apoptosis) has previously been ascribed to the higher levels of Fas associated phosphatase (FAP-1) in Th2, a condition that might mitigate against cell death signaled by Fas-FasL interactions (479,480).

D. Early Tyrosine Phosphorylation Events in T-cell Activation

1. Clustering of T-Cell Antigen Receptors

In general, the earliest recognized events in T-cell activation include the tyrosine phosphorylation of the ITAMs within the cytoplasmic tails of CD3-ε and CD3-ζ and consequent recruitment of downstream signaling moieties bearing src homology-2 (SH2) domains that mediate their specific association with the paired phosphotyrosines of the ITAMs (481). This formulation would be consistent with evidence that aggregation or clustering of TcR or other multichain immune recognition receptors is sufficient to deliver excitatory signals. This issue has recently been reviewed (377). However, given evidence (discussed elsewhere) to indicate that one peptide/MHC complex expressed by an antigen-presenting cell may be sufficient to activate (CD8) T cells expressing an appropriate antigen receptor, it is apparent that clustering of antigen receptors must accrue by pathways other than coclustering of multiple TcR with redundant epitopes expressed at the site of cell contact. A possible explanation for this enigma has been forthcoming from work by Davis and coworkers, who have identified, through measurements of quasielastic light scatter, solution oligomerization of solubilized T-cell antigen receptors elicited by peptide–MHC complexes (482).

2. Src and Syk Kinases

Evidence to show that T- and B-cell antigen component chains are themselves deficient in tyrosine kinase activity focused attention at an early stage on the two tyrosine kinase families, srk (fyn, lck) and syk (syk, ZAP70) known to associate or to become associated with the TcR. With the identification of the lymphocyte-specific tyrosine kinase $p56^{lck}$, a member of the *src* oncogene family (483,484), there was considerable speculation that $p56^{lck}$ might be uniquely responsible for tyrosine phosphorylation of both PLC and the 16-kd CD3 ζ-chain that accompanies TcR occupancy (TcR/CD3 cross-linking) (485–487). After much

initial uncertainty (488,489), a consensus has finally been achieved surrounding the critical role served by lck in phosphorylation of CD3ζ and CD3ε, as well as recruitment and phosphorylation of ZAP70, at least in thymocytes (490). Conversely, fyn may be able to substitute, in part, in peripheral phosphorylation of TcR elements in lck deficient mice (490). Thus, fyn, and not lck, serves in the tyrosine phosphorylation of PTK Pyk2 during TcR signaling (491), an observation that might be pertinent to the induction of anergy in T cells activated in the setting of deficient costimulation (492) or exposure to APCs presenting altered peptide ligands (APL) (54); in these settings, signal transduction is associated with differential activation of fyn and hypophosphorylation of CD3-ζ.

Until recently, it has seemed premature and perhaps inappropriate to conclude that downstream signaling events excited by fyn are necessarily inhibitory. Antigen receptor cross-linking has been shown to induce an association of T-cell/myeloid cell-restricted fyn-binding protein p120/130, FYB with fyn, as well as SLP-76, and overexpression of FYB has been shown to enhance IL-2 production by a T-cell hybridoma (493,494). However, as noted elsewhere in this chapter, the reduced TcR-triggered proliferative responses characteristically observed in NOD mice were recently found to be associated with differential activation of the fyn-TcR-ζ-Cbl signaling cascade at the expense of PLC-γ1/SOS recruitment to a Grb2-pp36-38/ZAP70 complex linked to TcR-ζ (495). In view of the strong circumstantial evidence linking fyn with the induction and maintenance of T-cell anergy (496–498) and the ongoing discovery of novel proteins that associate with p59fyn in human T cells (e.g., SKAP55) (499), it is of great interest to follow the trail of discovery of downstream signaling pathways impacted by *fyn* in TcR signaling events leading to the induction of T-cell anergy. For example, SKAP55 is identified in resting T cells as a novel adaptor protein that contains a pleckstrin homology domain, a C-terminal SH3 domain as well as several potential phosphorylation sites, one of which fits the criteria to bind Src-like SH2 domains with high affinity (499).

The ITAMs of the CD3-ζ chain were functionally characterized by Weiss and colleagues and shown to be sufficient to couple to receptor-associated signal transduction pathways (432,433); the triple repeat ITAM of CD3-ζ was assumed to provide the ζ chain with unique potential to mediate downstream signaling events. Nevertheless, the recent demonstration that the CD3-FcεRI-γ chain can rescue the immune system of CD3-ζ GKO mice raises questions about the accepted primacy of the ζ chain in T-cell activation (468). Moreover, whereas peripheral αβ T-cell development is fully interrupted at the $CD4^-$, $CD8^-$ stage in fyn (–/–), lck (–/–) deficient mice (500), intraepithelial γδ T cells are present and functional, likely because these cells use Syk in lieu of ZAP70, and Syk has the potential to become activated autonomously (in the absence of fyn or Syk) upon antigen-receptor clustering (500–502).

Src kinases are the "first" (kinases) protein tyrosine kinases to be activated as a consequence of clustering of T-cell antigen receptors. The src tyrosine kinases constitute quite a large family. *Yrk,* discovered in 1993, was the ninth family member to be discovered. Five conserved sequence blocks are identified in src kinases. From the N terminus these include the extreme N terminal myristolation signal that mediates association of src kinases with the plasma membrane, the Src homology (SH) 3 and 2 domains, the kinase domain, and the C terminal noncatalytic tail containing an inhibitory tyrosine phosphorylation site conserved in all *src* family members (503). It initially seemed probable that structurally related kinases of the *src* family other than $p56^{lck}$ might be more intimately associated with antigen-receptor signaling, because a direct physical interaction of *fyn* with the TcR had been demonstrated during T-cell activation (504–506). Moreover, $p59^{fyn}$, like $p56^{lck}$, was found to be overexpressed in lymphoproliferative disorders, signifying a potential in vivo associ-

ation with a mitogenic signaling cascade (507,508). The gene encoding $p56^{lck}$ was initially recognized because it was rearranged and overexpressed in the murine T-cell lymphoma LSTRA; hypophosphorylation of lck at Tyr-505 is associated with malignant transformation in these cells (509). In humans, *lck* transcripts are detected in $CD4^+$ and $CD8^+$ T cells, as well as in partially purified B cells, Epstein-Barr virus-immortalized B-cell lines, but not in monocytes, granulocytes, or nonhematopoietic cell types (510). Moreover, it was found that CD4 cross-linking can modulate responses to TcR occupancy (511,512); in T cells, $p56^{lck}$ is bound to CD4 and CD8, and transduces signals arising as a result of cross-linking these structures (513–515). Activators of PKC cause internalization of CD4 and the dissociation of the CD4–$p56^{lck}$ complex but do not affect CD8-$p56^{lck}$ complexes (516). It is presumed, however, that the latter complexes are probably also functional because CD8a′ polypeptides, which lack the cytoplasmic tail of the alpha chain of CD8 and cannot in consequence interact with $p56^{lck}$, are inefficient in signal transduction (517). Cross-linking of CD4 in murine thymocytes activates $p56^{lck}$ (518), but in human T cells, CD4/CD8 cross-linking may not, by itself, activate the kinase (519). CD4 and CD8 associate with the αβ TcR–CD3 complex (520,521) during activation, and it is not hard to conceive how $p56^{lck}$ activation might modulate signal transduction events at the level of the TcR. The $p56^{lck}$ tyrosine kinase becomes autophosphorylated on tyrosine 505 when lymphocytes are activated (518,522), and this inactivates the kinase.

Csk is another src kinase, the sole identified function of which for many years was to phosphorylate the C-terminal negative regulatory site of fyn and lck and inhibit TcR signaling. Csk is reported to be constitutively associated with a 60-kDa tyrosine-phosphorylated protein (p60) in human T cells that appears to have GTPase activity (523,524). It presently seems conceivable that p60 mentioned above is in fact the Abl- and rasGAP associated docking protein $p62^{doc}$ (525,526). The in vivo biological significance of Csk activity was investigated in mice in which Csk was knocked into the fyn locus. This resulted in impaired $CD4^+CD8^+$ thymocyte development (527).

3. Activation and Degradation of Lck, Role of CD45

The cell surface glycoprotein CD45 possesses a catalytically active intracellular domain that functions as a specific protein tyrosine phosphatase (528,529). CD45-negative mutants fail to respond to antigenic stimuli (530). Early work demonstrated that tyrosine phosphatase CD45 was likely necessary for coupling of the T-cell antigen receptor to the phosphatidyl inositol pathway, an effect mediated at the level of $p56^{lck}$ (531). Thus, antibody cross-linking of CD45 and the TcR-inhibited T-cell activation and the generation of a Ca^{2+} signal (531), whereas coclustering of CD45 and CD4/CD8 on the cell surface altered the state of phosphorylation and kinase activity of $p56^{lck}$ and promoted T-cell activation (532,533). An autophosphorylation site within subdomain VII was identified and shown to be critical for the full activity of the kinase in the antigen receptor signaling pathway (534). CD45 has also been shown to transduce activation signals in a variety of other cell types, including thymocytes and macrophage (535).

Aebersold and colleagues (536) discovered some years ago that $p56^{lck}$ was phosphorylated on serine 59 during the course of activation. This phosphorylation decreased the activity of the kinase, induced an apparent change in molecular weight to $p60^{lck}$, and was not, apparently, mediated by protein kinase C. This observation has been substantially confirmed, and phosphorylation has been shown to modulate its substrate specificity (537). This may be due, in part, to the fact that Ser-56 phosphorylation permits phosphotyrosine-independent binding of a 62-kd serine/threonine kinase to the SH2 domain of lck (538,539).

A rasGTPase-activating protein, a negative regulator of the Ras signaling pathway, subsequently associates with this 62-kd protein. More recent data generated in the yeast two hybrid system indicates that the lck-associated 62-kd protein also has the potential to associate with ubiquitin and may thereby program lck for degradation by the proteosomal system (540). These findings are highly reminiscent of the serine phosphorylation triggered pathway of ubiquitin targeted degradation of IκB that leads to release of NF-κB and translocation of the latter to the nucleus. Together, this series of recent reports identify a molecular pathway for the downregulation of lck signaling activity. The potential validity and impact of these observations is further highlighted in a recent report by Madrenas and colleagues that there is progressive serine phosphorylation of p56lck as well as the irreversible loss of lck that appears as a counterpart of desensitization of the T-cell antigen receptor following persistent engagement with complexes of peptide:MHC molecules (541).

4. ZAP70 and SYK

ZAP70 is constitutively associated with CD3-ζ in peripheral T cells, at least in small quantities. In vivo association of ZAP70 with CD3-ζ is dependent upon the spacing of ZAP70 SH2 domains and necessary ITAM phosphorylation (542). Transphosphorylation of ZAP70 on tyrosine (Tyr-) 493 by src-PTK lck is said to be required for downstream antigen receptor–mediated activation of both the calcium and the Ras pathways (543). Milia et al. (544) identified a putative phosphotyrosine protein binding domain (PTB) in the region of Tyr-493 (Tyr-492) that mediates an association between ZAP-70 and an amino-terminal PTB of Shc, an adaptor protein with the potential to connect with downstream elements of the Ras signaling pathway. Moreover, the isolated Shc PTB, designed as a dominant negative signaling moiety, blocked TcR downstream signaling, leading to the activation of NF-AT. Conversely, dominant negative constructs containing the SH2 domain of Shc do not impact T-cell antigen receptor signaling, although they are reported to inhibit the mitogenic effect of EGF (542, 545). Shc is believed to serve in the recruitment of the Ras transduceosome to the TcR. Interestingly, the TcR and CD4 can both link to the downstream Ras signaling pathway and appear to do so using different Shc isoforms as adaptors (546). Moreover, Shc appears to associate with a small fraction of CD3ζ-bound ZAP-70, which can be found in association with pp23ζ.

Point mutations in Tyr-315 of ZAP70 lead to impaired TcR signaling and downstream phosphorylation of Vav, SLP-76, and Shc (547). Conversely, ZAP70 phosphorylation on Tyr-292 and Tyr-492 mediates negative regulation of antigen-receptor signaling (543). TcR-directed phosphorylation of SLP-76 by ZAP70 is required for T-cell receptor function (548) and leads to the association of the latter with a series of tyrosine phosphoproteins of 36, 62, and 130 kd. SLP-76 overexpression leads to dramatically enhanced TcR-mediated induction of NFAT and IL-2 biosynthesis (549). These data identify SLP-76 as one of an increasing number of recently described elements in the signaling pathways excited by TcR cross-linking, which has been found to be essential for normal T-cell activation.

Deficiency of ZAP70 itself has been found to cause a rare autosomal recessive severe combined immunodeficiency (SCID) in human subjects. Transfection of ZAP70 back into HTLV1-immortalized T cells recovered from ZAP70-deficient lymphocytes recovered from human subjects reconstitutes responsiveness to antigen receptor occupancy (550). Furthermore, dominant negative (dn) ZAP70 inhibits TcR signaling (551).

As noted, phosphorylation of SLP-76 by ZAP70 is reportedly required for T-cell antigen-receptor signaling (548), and it becomes of interest that disparate isoforms of CD45 are

reported to regulate phosphorylation and recruitment of SLP-76 to Vav (552), serving, therefore, as a marker of phenotype and function in naive and primed T cells. Conversely, the related tyrosine kinase, Syk, predominantly used as a component of the B-cell multichain antigen-receptor signaling complex and Fc receptors, can function independently of lck or CD45 (553–555). ZAP70 is substantially required for development and activation of peripheral T cells, whereas Syk is required for B-cell development (556,557). Although ZAP70 is present in all T cells (as well as NK cells) (558), expression of Syk in cells of the T-cell lineage is restricted to thymocytes, mature T-cell subpopulations such as intraepithelial γδ T cells (intraepithelial γδ T cells are substantially depleted in Syk deficient mice), and naive αβT cells (558). The noted reliance of γδ T cells on syk correlates with prior documentation of the persistence of intraepithelial γδ T cells in fyn/lck GKO mice that express the FcεRI-γ chain as a component of CD3 and raises the intriguing possibility that TcR-CD3- FcεRI-γ signaling leading to the production of IL-4 in considerable abundance preferentially involves syk. Accordingly, it would be of great interest to know whether Syk might be differentially expressed or functional in even a minority of Th2-like effectors, based, in part, on the observation that the FcεRI-γ chain is expressed in at least a minority of Th2 (see Figure 7).

Syk may substitute for ZAP70 in intrathymic T-cell ontogeny, allowing for the appearance of limited numbers of $CD4^+$ T cells in ZAP70-deficient subjects (559). Given that the p38 MAP kinase pathway has been shown to be activated in T-cell ontogeny (560), it may be of particular interest that Syk activation by recombinant molecule signaling through the FcεRI-γ chain has been shown to activate the guanidine nucleotide exchange factor (GEF) Vav and induce Rac-1-dependent activation of JNK (561). Interactions between the FcεRI-γ chain and $p72^{syk}$ have been subjected to detailed analysis. Recent reports indicate that both tyrosines of the FcεRI-γ chain ITAM must be phosphorylated and that the orientation must be such that the N-terminal phosphotyrosine associates with the c-terminal SH2-domain of Syk (562). It is widely appreciated that the paired requirement for lck and CD45 in the activation of ZAP70 and T-cell antigen-receptor signaling events relates to the singular requirement for CD45 phosphatase in lck activation (553). Lck-independent activation of Syk is likely related, at least in part, to the potential of this kinase to serve in an autoactivation-related amplification loop following initial (auto)phosphorylation induced by receptor clustering or alternate src kinases (563). The demonstrated potential for Syk to participate in an autoamplification loop may enhance the gain on B-cell antigen receptors and enhance responses to potentially suboptimal stimuli. Conversely, others have found evidence to suggest that the enhanced catalytic (kinase) activity maps to structural variations in its catalytic domain (558). Remarkably, the latter study additionally suggests that Syk, and not ZAP70, possessed the demonstrated potential to induce/mediate TcR-triggered phosphorylation of CD3-ζ in a $CD4^-$, $CD8^-$ antigen-specific T-cell hybridoma (BI-141 cells), as well as a ζ-bearing chimera, both following transient transfection in Cos-1 cells and in vitro.

5. Immunoregulatory Circuits Surrounding Cbl

Recent reports suggest that cbl may be broadly implicated in the generation of inhibitory signals that modulate T-cell activation:

1. Evidence to show that syk may participate in an autoamplification loop suggests the existence of regulatory mechanisms to downregulate the activity of Syk, and indeed such have been identified. The $p120^{cbl}$ has been identified as a direct downstream target phosphorylated by Syk in the amino-terminal proline rich domain, an event that permits

formation of an intermolecular complex that has the potential to negatively regulate the activity of Syk (564–567). Cbl was originally identified as a protooncogene that was cloned from a mouse B-cell lymphoma retrovirus (Cas NS-1) that serves as a molecular adaptor downstream of multiple receptors, including T- and B-cell antigen receptors and Fc receptors as well as EGF, GM-CSF, and erythropoietin receptors (568). Importantly, Cbl has the potential to connect the TcR with molecules that regulate guanidine nucleotide exchange factors specific for the Ras family. Vav and c-Cbl also form inducible molecular complexes in TcR-activated murine thymocytes and peripheral T cells, and it is suggested that this association regulates the function of Vav (568). Cbl associates with multiple SH2/SH3-containing adaptor proteins, including Grb2 as well as Crk and Crk-L known to interact with Ras-specific GEFs (568).

Significant interest has recently attached to the potential involvement of Crk (Crk II and Crk-L) as an adaptor molecule linking TcR signaling to downstream activation or inhibition of the Ras pathway (4). (v-Crk was the first adaptor protein identified [569]). SH3-dependent association of Crk II and Crk-L with C3G (and mSOS) guanidine nucleotide exchange factors has been documented in activated mouse T cells (570). C3G is a guanidine nucleotide exchange factor for rap1, which is 50% identical to ras. Therefore, rap1 has the potential to inhibit activation and downstream effects of ras in a variety of model systems at various levels, such as cell cycle progression (569,571–574).

A recent report by Delovitch and colleagues provides evidence that heightened signaling through the Fyn-TcR-ζ-Cbl pathway in (T cells) thymocytes of NOD mice, which are hyporesponsive to TcR-triggered proliferative responses, correlates with a reduced translocation of mSOS ras GEF from the cytoplasm to the plasma membrane and consequent exclusion of PLC-γ1 and SOS from the TcR-ζ associated Grbs/pp36-38/ZAP70 complex. This leads to the characteristic dearth of ras-MAP kinase signaling observed in these mice, which are prone to development of autoimmune diabetes (495).

2. Cbl appears to be directly downstream of CTLA4. It seems probable, at least on a population level, that B7-ligand CTLA4, mediates a tonic negative influence on the T-cell antigen-receptor signaling (reviewed in Ref. 575). Whereas CD28, the alternate B7 ligand, is constitutively expressed on the great majority of human and murine T cells, CTLA4 is an inducible membrane glycoprotein (576). Signaling through the CD28-B7 axis may mediate upregulation of CTLA4 expression in the course of T-cell activation by competent APC (576). Tonic, spontaneous activation of the Ras signaling pathway is observed in mice rendered deficient of CTLA4 by homologous recombination. Thus, CTLA4-associated phosphatase SYP might mediate tonic negative regulation of TcR signaling and thereby modulate ras activation (577). Alternatively, downstream signaling by CTLA4 transduced by Crk and Cbl might be responsible for T-cell quiescence in the resting state.

3. Abbas and coworkers have generated evidence to show that peripheral tolerance to soluble antigens in TcR transgenic mice may occur by a route independent of Fas-induced apoptosis (66). The most recent report to have emerged from this group indicates that the in vivo induction of T-cell anergy amongst naive T cells of TcR transgenic mice occurs through direct signaling mediated by CTLA4. Robust levels of T-cell unresponsiveness were obtained in response to CTLA4 cross-linking in the absence of CD28 ligand binding (67). These data substantially corroborate earlier observations of Lane et al. (65), who documented that superantigen (SEB)-induced unresponsiveness of T cells bearing TcR Vβ8 that confer SEB responsiveness was substantially abrogated in CTLA4-Ig transgenic mice; CTLA4-Ig transgenic mice inoculated with SEB manifest an initial expansion and subsequent reduction in the numbers of TcR $V\beta8^+$ T cells equivalent to that observed

in wild type mice. The key difference is the documented absence of specific unresponsiveness in the former instance compared to readily demonstrated presence of specific unresponsiveness in non-(CTLA4-Ig-) transgenic hosts.

In discussion of these observations, it is critical to note the apparent discrepancy between the recorded observations of Lane et al. (65) and Perez et al. (67) and the commonly cited observation that TcR ligation in the absence of CD28 costimulation is sufficient to induce anergy. Thus, Abbas and coworkers (67) suggest that this apparent conflict with the literature may be explicable based on the fact that many of the historical studies on the induction of T-cell anergy were performed with T cell clones.

4. Points that may explain the apparent paradox demonstrated above include:

First, T-cell anergy triggered by TcR cross-linking in the absence of requisite costimulation or on exposure to altered peptide ligand-MHC, is associated with preferential TcR signaling through fyn to cbl.

Second, activation of downstream cascades triggered by TcR cross-linking is notably costimulation dependent. Therefore, TcR-triggered activation of fyn in naive T-cells, in the absence of CD28 cross-linking, may be insufficient to trigger the crk-cbl-rap1 pathway.

Third, the demonstrated potential of CTLA4 to signal through cbl may sum with TcR-triggered activation of cbl for induction of anergy.

Alternatively, activation of CTLA4-associated phosphatase SyP (PTP1D) as a consequence of CTLA4 cross-linking may synergize with signaling through the crk-cbl-rap1 pathway to serve in the induction of T-cell anergy.

Because deficiencies of CD28 costimulation during TcR-triggered T-cell activation and CTLA4-downstream signaling events both target cbl, it is conceivable that CTLA4 draws greater efficacy in tolerance induction through its simultaneous activation of protein tyrosine phosphatase SYP or PTP-1D. Moreover, as discussed below, CTLA4 cross-linking also has the potential to induce T-cell apoptosis.

5. CTLA4 cross-linking of previously activated T cells (as required to induce CTLA4 expression) has the potential to induce apoptosis (578–580). Whereas inhibition of apoptosis by CD28 and cytokine (IL-2) receptor signaling has been linked to the induction and/or phosphorylation of the Bcl-2 family of proteins (Bcl-X_L in the instance of CD28 signaling [580–583]), an equally broad mass of literature has accrued to indicate that wartmannin-inhibitable PI-3K and downstream signaling by Akt are also important in preventing apoptosis in response to growth factor withdrawal. Activation of PI-3 kinase through growth factor receptor signaling has been shown to induce survival signals transduced by PI-3K and secondary activation of Akt (protein kinase B) (584).

It is of interest that proto-oncoprotein c-Abl has recently been shown to be capable of modulating the activity of PI-3K (585). (The role of c-Abl in cell development has recently been reviewed (586)). It is conceivable that c-Abl might be implicated in CTLA4-induced apoptosis, based in part on the observation that Cbl is reported to be a direct downstream target of CTLA4 together with evidence to show that Cbl and Abl both interact with Crk (568,587). Evidence supporting this hypothesis includes:

SH3-dependent associations of c-Abl tyrosine kinase with proline-rich sites of the SH2/SH3-containing adaptor molecules Crk-I and Crk-II have recently been demonstrated in mammalian cells (587), and such associations appear to be strength-

ened as a consequence of c-Abl-induced tyrosine phosphorylation of Crk-II on tyrosine 221 (587–589). Importantly, tyrosine phosphorylation of Crk at position 221 inhibits SH2-dependent association of Crk with other proteins (588).

Crk has previously been identified as an adaptor protein that potentially couples TcR signaling events to downstream activation of MAP kinases through its association with the guanine nucleotide exchanger C3G (570).

SH3-dependent interactions of c-Abl with other proteins (including Crk) are negatively transregulated through SH3-dependent interactions of c-Abl with Abl-associated-protein (AAP1) (590).

Constitutive SH3-dependent association of tyrosine kinase c-Abl with the p85 catalytic component of phosphoinositol 3-kinase has been described. c-Abl is activated under conditions of genotoxic stress (X-irradiation) to phosphorylate and inactivate PI-3K (585). These and other data (591), together with evidence to show that the PI-3K/Akt pathway inhibits apoptosis upon serum withdrawal, and prior documentation that c-Abl–deficient cells are resistant to irradiation-induced apoptosis, substantially implicate c-Abl as a primary cause of irradiation-induced apoptosis. DNA-dependent protein kinase (DNA-PK) and the ataxia telangiectasia gene product appear to be implicated in the activation of c-Abl under conditions of genotoxic stress (585,592–594).

Integrin-mediated adhesive interactions have likewise been shown to activate c-Abl, and it has been suggested that activation and nuclear translocation of c-Abl to the nucleus serves to integrate adhesion and cell cycle signals (595).

As adhesive interactions involving CTLA4 are variably observed to costimulate T-cell responsiveness or induce anergy or apoptosis, it is of interest that different isoforms of c-Abl are variously shown to promote cell differentiation, inhibit apoptosis, or serve in the induction of JNK as well as p38 MAP kinase pathways (595–597). Consistent with the above, peripheral mononuclear cells of c-Abl mutant mice demonstrate impaired concanavalin A–induced proliferative responses, potentially signifying that c-Abl may be directly implicated in the signaling cascade excited by antigen receptor occupancy (598). c-Abl also phosphorylates and activates the SH2-containing protein tyrosine phosphatase SHPTP1, implicating the latter in signal transduction pathways that give rise to the activation of the JNK signaling pathway (599).

Abl oncoprotein mediates Ras- and Raf-dependent downstream activation of MAP kinase pathways through protein-protein interactions that may involve RIN1 and 14-3-3 (600,601). Downstream induction of c-myc by v-Abl has been shown to be dependent on cyclin-dependent kinases CDK2 and CDK4 and accompanied by hypophosphorylation of retinoblastoma protein Rb (601). The role of c-Abl in cell cycle regulation has previously been reviewed (602).

Thus, under conditions wherein Abl is released from tonic inhibition by AAP1 and is competent to mediate SH3-dependent associations with other proteins, c-Abl may be able to mediate critical intracellular signaling events relating to ras activation and cell cycle progression. Additionally, in view of evidence that Abl mediates reciprocal consequences of TcR-triggered signaling events, it remains conceivable that c-Abl might both enhance and suppress T-cell activation. Suppression of T-cell activation by Abl may be related, at least in part, to the noted potential of Abl to activate the SH2-containing phosphatase SHPTP-1 (599).

B7 expression and potential signaling through the CD28-B7 axis is sufficient to abrogate tolerance induction by small resting B lymphocytes (603). Given that the B7 family of molecules are reported to serve equally as the counter receptors for CD28 and CTLA4, what, if any, is the counter receptor that induces differential CTLA4 signaling in the induction of T cell anergy? What reagents might be used in the clinic to induce differential blockade of the CD28-B7 axis without blunting the potentially advantageous signaling events excited by CTLA4 that serve in the induction of T-cell anergy or the maintenance of specific unresponsiveness? This is not a moot point because inadvertent blockade of CTLA4 has the potential to exacerbate (autoimmune) disease (604). Moreover, the potential efficacy of tolerance induction protocols targeting the CD28-B7 axis might clearly be significantly enhanced if naturally occurring negative signaling events via CTLA4 were not incidentally foiled. As discussed elsewhere, the tolerogenic potential of CTLA4-Ig by itself is relatively limited and investigators, of necessity, have been obliged to look at combination approaches to obtain desired efficacy (605–614). There are potentially other important costimulatory pathways for T-cell activation (615) and other tolerogenic pathways (616,617).

Although the answers to these questions are not readily available, it becomes of great interest that alternate CTLA4 counter receptors have been identified on nonclassical antigen presenting cells (i.e., B lymphocytes). Murakami et al. (618) have reported the preliminary identification of an alternative CTLA4 binding protein (ACBP), a disulfide-linked moiety of approximately 130 kd found on small resting B cells, using a search strategy that relied on use of recombinant CTLA4-Ig as well as a modified form of CTLA4-Ig that does not bind B7 because of the presence of two amino acid substitutions in the conserved motif MYPPPY identified in B7 ligands. Of note, there is a substantial body of data to suggest that small resting B lymphocytes have unique tolerogenic potential; this has been ascribed to the fact that small resting B cells express low levels of costimulatory molecules of the B7 family (619–621). Moreover, antigen-unspecific B cells and lymphoid dendritic cells have been found to show extensive surface expression of processed antigen–MHC class II complexes after soluble protein exposure in vivo and in vitro (349). This observation stands in striking contrast to the long-held supposition that surface Ig represents the main, if not the only, mechanism of antigen capture by B lymphocytes (314,622). It has been hypothesized that the numerical advantage enjoyed by TcR-ligand (antigen) bearing small resting B cells (following in vivo antigen administration or release) relative to the numbers of professional antigen presenting cells might permit them to interact first or more often with naive antigen-specific T cells, contributing to the induction of high-dose T-cell tolerance or immune deviation. Indeed, effort has been made to determine whether small resting B cells might actually be required for induction or maintenance of peripheral tolerance, but this seems not to be the case (623). It is also of interest that alternatively spliced forms of the B7 family of molecules have been identified (624). An alternately spliced form of CD80 (B7-1) named B7-1a, lacking the second Ig-like domain encoded by exon 3, has the potential to costimulate proliferation of primed but not naive T cells (625–627). Because CTLA4 has also been shown to be able to process some costimulatory signals, it would be of interest to know the relative potential of B7-1a to excite CD28 versus CTLA4 signal transduction pathways.

Negative signaling by CTLA4 has been linked to constitutive or inducible association between SHPTP-2 (PTP1D, SYP) and the cytoplasmic domain of CTLA4 (628). Conversely, CTLA4 GKO mice demonstrate sustained tyrosine phosphorylation of Cbl and its intermolecular association through tyrosine 699 with the SH2 domain of Vav. TcR-stimulated association of Vav and Cbl is likely related to direct effects of TcR-associated tyro-

sine kinases Syk or src family tyrosine kinases, because the nonspecific phosphatase inhibitor vanadate also induces association of Vav and Cbl. Syk is not unique in its interactions with Cbl. ZAP70 also phosphorylates Cbl, and ZAP70 as well as fyn (fynT) have been shown to associate with Cbl following T-lymphocyte antigen-receptor cross-linking in a response that is dependent upon fynT or lck (629). Also of note is the fact that Cbl undergoes serine phosphorylation in response to T-cell antigen-receptor occupancy or stimulation with phorbol esters; this permits Cbl to associate with other downstream signaling moieties, including the tau and tau + zeta isoforms of 14-3-3 protein.

6. Protein Kinase C and 14-3-3 Proteins

The role of protein kinase C (PKC), a serine threonine kinase, in T-cell activation presently appears somewhat enigmatic. Many recent studies cite the fact that the particular signaling pathway under study is apparently PKC independent. Accordingly, it is of interest to note that PKC has recently been identified as a primary determinant of quiescence and survival of single-positive T cells. Preliminary data have accrued to indicate that PKC is responsible for phosphorylation and ultimate ubiquitination/degradation of lymphocyte Kruppel-like factor (LKLF) (630). Kuo et al. (630) have identified LKLF as a transcriptional regulator of quiescence of single positive thymocytes and peripheral T cells; peripheral T-cell lymphopenia of $LKLF^{-/-}$, $RAG^{-/-}$ chimeric mice was shown to correlate with apparent expression of activation markers (e.g., CD69 and Fas) leading to enhanced spontaneous apoptosis of peripheral T cells.

The 14-3-3 proteins are abundantly expressed acidic moieties of 30 kd reported to serve as adaptor proteins (as well as a wide array of other functions), creating opportunities for numerous downstream intermolecular associations (631–634). The human 14-3-3 isoform (tau) dimer has been characterized at the 2.6Å level (633). The dimer 14-3-3 forms a negatively charged channel, the base of which forms a binding site for protein kinase C. Consistent with the above, other researchers have reported that 14-3-3 protein coimmunoprecipitates with protein kinase C-theta from Jurkat T cells and that the two moieties are readily shown to associate in vitro (635). The association of 14-3-3 with PKC isoforms appears to be biologically significant. Thus, 14-3-3 has been seen to inhibit T-cell activation–induced translocation of T-cell–specific theta isoform of PKC from the cytosol to the plasma membrane, where it has been shown to cap at the site of contact between T cells and antigen presenting cells mediating T-cell activation. Moreover, overexpression of 14-3-3 has been shown to suppress induction of the IL-2 promoter by PMA plus inomycin, whereas induction of the IL-4 promoter is unaffected. These observations might seemingly place 14-3-3 at a crossroads in T-cell activation as a potential determinant of precursor T-cell differentiation into Th1 or Th2 effectors. It would be of great interest, therefore, to know how 14-3-3 might interact with STAT4/STAT6 signaling pathways as well as nuclear regulatory factors (e.g., c-maf) with restricted expression in Th1 or Th2 (443,636).

However, efforts to sort out the role of 14-3-3 in T-cell activation will likely be frustrated by the multitudinous signaling pathways impacted by this protein. Thus, the 14-3-3 protein, as well as KSR, have been identified as positive regulators of the Ras pathway (637,638). The 14-3-3 protein has been shown to form a complex with Raf-1 as well as KSR, which was initially identified in *C. elegans* as the kinase suppressor of Ras (639). In Xenopus oocytes, overexpression of KSR as well 14-3-3 induced mitotic maturation and cdc2, an effect blocked by dominant negative Raf-1. KSR has binding sites for 14-3-3 and also binds to Raf-1. Conversely, the unphosphorylated (beta and zeta) isoforms of 14-3-3 bind to both the (regulatory) amino- and (catalytic) carboxy-terminus of Raf-1 in resting

cells, but activated Ras displaces 14-3-3 from the amino-terminus following cell activation (640). Studies in yeast have indicated that zeta and beta isoforms of the 14-3-3 protein may be as effective as Ras as an upstream activator of Raf (641).

Knowledge of factors impacting 14-3-3 phosphorylation is accumulating. Tyrosine phosphorylation of the B-cell antigen receptor (BCR) by receptor-associated PTK c-FES is essential for SH2-dependent recruitment of Grb-2 to tyr-177 of the BCR and BCR-induced activation of the Ras pathway. It may be pertinent therefore, that tyrosine phosphorylation of the BCR by FES prevents BCR-induced (serine/threonine) phosphorylation of 14-3-3 (642). Various isoforms of 14-3-3 are expressed in human lymphoid cells; total cellular lysates of human spleen contain epsilon, gamma, beta, and zeta isoforms (of which epsilon and gamma are uniquely associated with centrosomes) as well as eta, which maps to human chromosome 22q12.1-q13.1 (637,643). Evidence has accrued to show that 14-3-3 also interacts with cdc25, a phosphatase implicated in the activation of cyclin-dependent kinases (644) as well as glucocorticiod receptors (645).

E. TcR-CD3 Downstream Signaling Events

Downstream signaling events excited by TcR-CD3 cross-linking include the activation of PTK as well as an increase in intracellular calcium (648,649).

1. Phospholipase C-γ1

Amongst the earliest recorded consequences of TcR occupancy by antigen (or TcR/CD3 cross-linking) is tyrosine phosphorylation and activation of phospholipase C (PLC) (650,651). Complexities surrounding the activation of PLC have recently been the subject of intense research endeavor, and yet there is still no clear understanding of how PLC is activated following TcR cross-linking. Thus, even though there were recent strong indications to suggest that Lnk, a 38-kd phosphoprotein (containing a single SH2 domain as well as tyrosine phosphorylation sites) distinguished from the more abundant 36-kd phosphoproteins that appear to be activated in T cells, might serve a critical role in the T-cell antigen-receptor activation of PLC and linking Grb2, phospholipase C-gammal, and phophatidylinositol 3-kinase to the TcR (652), overexpression of Lnk in thymocytes of transgenic mice provides no evidence that Lnk plays any limiting role in antigen-receptor–triggered activation of PLC in T cells (653). Nonetheless, evidence to suggest that a moiety of approximately 36 kd is importantly involved in activation of PLC is substantial. Parham and coworkers have determined PLC activation in NK cells correlates with the association of a 36-kd adaptor phosphoprotein (pp36) with PLC and, further, that killer cell inhibitory receptor (KIR) receptor recognition of MHC class I activates a phosphatase SHP-1, which, in turn, inhibits PLC activation and the association of pp36 (654). Moreover, transfection of Jurkat with cDNA encoding a hybrid linking Grb2 with a phosphatase domain (655) inhibited TcR-triggered phosphorylation of p36, PLC activation, and an increase in intracellular calcium, but it did not impact the phosphorylation of PLC or downstream activation of Ras-MAP kinase pathway. Moreover, p36 adaptor homologues have been implicated in activation of PLC downstream of other receptors. APS is a B lymphocyte specific adaptor molecule phosphorylated following BCR stimulation (656). Other groups have described the assembly of a series of multimolecular complexes incorporating PLC as well as the other common (e.g., lck, hSos, ZAP70, Grb2, CD3-ζ, SLP-76, p36/38) or uncommon (e.g., Sam68, Grap, [the latter a lymphoid-restricted protein containing SH3-SH2-SH3 domains]) molecules (548,655,657–660).

Upon activation, PLC causes hydrolysis of phosphatidylinositol 4,5 bisphosphate (PI) to yield diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (661). Diacylglycerol and a second signal provided by IL-1 or phorbol ester (PMA) activates protein kinase C (PKC) and causes its relocation to the cell membrane (662,663). Protein kinase C is responsible for the phosphorylation of the γ and ε chains of the CD3 complex on serine residues that accompanies T-cell activation (664). As part of the regulation of the activity of individual elements of the signal transduction pathways involved in T-cell activation, (665), the activity of PKC may be controlled, at least in part, by diacylglyerol kinase (DAK), which rephosphorylates DAG as the first step in the resynthesis of phosphatidylinositols (666). Structural data suggest that lymphocyte DAK may be a calcium binding protein, because its sequence contains two E-F hand motifs typical of Ca^{2+} binding proteins (667). It seems plausible that increases in intracellular calcium brought about by the second messenger IP3 may activate DAK. This, in turn, would cause a decline in PKC activity as part of a feedback loop.

2. Calcium

T-cell receptor cross-linking has previously been reported to induce a sustained (>15 minutes), oscillating increase in intracellular calcium through release of IP3 from a small, labile precursor pool (661,668,669). IP3 was reported to provoke an initial transient increase in intracellular Ca^{2+}, which accompanies T-cell activation by binding to specific receptors (670–673) and releasing Ca^{2+} from intracellular stores in the endoplasmic reticulum. It is understood that the demonstrated expression of oscillating calcium tracings correlates with the degree of transformation of the target cell under study (674). IP3 also opens small conductance (7 pS) calcium permeable channels to cause a sustained increase in flux of Ca^{2+} across the cell membrane and a sustained increase in intracellular Ca^{2+}. A second low conductance calcium permeable channel has been described which is linked to the TCR/CD3 complex and functions independently of IP3. In the previous edition of this text, we noted that a prolonged elevation of intracellular calcium (greater than 2 hours) had been thought to be a necessary correlate of T-cell activation; the substance of this observation has recently been confirmed and extended (675). Moreover, observations relating to the nature of the calcium signal evoked by agonist peptides, altered peptide ligands, or peptide antagonists presented by MHC restriction elements provide important insight regarding the nature of the signaling events associated with TcR/BCR cross-linking, and downstream events associated with tolerance induction (675–677). Additionally, recent discoveries relating to calcium signals generated in the course of T/B cell activation also provide new insights relating to the potential inputs that derive from costimulation, including the inhibition of T-cell anergy.

T cells express approximately 30,000 antigen receptors whereas APCs may express as few as 200 complexes of MHC with specific peptide. Accumulating evidence indicates that the process of T-cell activation is a dynamic process wherein multiple individual TcRs can interact sequentially with a limited number of peptide/MHC complexes expressed by APC for varying intervals (678,679). According to this paradigm, the number of TcRs productively engaged by peptide-MHC complexes presented by stimulator cells correlates with the probability that individual T cell–APC engagements will result in T-cell activation. Thus, it is supposed that TcR recognizing and binding to peptide/MHC complexes with sufficient affinity, or with a sufficiently slow dissociation rate (680), transduce excitatory signals to the intracellular milieu and undergo downregulation. Additionally, data show that a sufficient number of TcRs must be productively engaged by peptide/MHC within a defined period to provide a stimulus of sufficient intensity to provoke T-cell activation. It has been

hypothesized that T-cell antigen-receptor triggering leads to the production of a short-livec molecule or complex, possibly a tyrosine phosphorylated receptor element, that serves a: an essential trigger for T-cell activation (674,675).

The presumption is that the concentration of the short-lived signaling moiety will b a function of the numbers of antigen-receptors engaged and qualitative features of engage ments between individual TcRs and MHC/peptide to generate bites of the short lived sig nal, and, finally, the rate of degradation/disappearance/dephosphorylation of the short-livec product in T cells excited by antigen receptor cross-linking. This hypothesis is based, ir part, on prior documentation to suggest that approximately 8000 TcRs must be triggered tc induce full T-cell activation (681) and that lesser numbers (approximately 1500) of antiger receptors may need to be engaged to trigger the cytotoxic programs of CTL (682), as wel as on the following three sets of observations:

1. Wulfing et al. (674) determined that MHC I-E^k–restricted presentation of ago nist, partial agonist, and antagonist peptides elicit disparate calcium signals by single cell of a $CD4^+$ moth cytochrome C (MMC)-specific cell line derived from an MCC-reactive TcR transgenic mouse (5C.C7). Peptide agonists were shown to elicit a brisk sustained high intensity calcium signal in 100% of the cells studied. Partial agonists were seen to evoke a signal that was sometimes reduced, partial or transient (i.e., delayed, fluctuating to baseline or terminated prematurely) in at least a portion of the cells studied. Conversely, antagonis peptides failed to induce a calcium signal. Further, dilution of partial agonist (MMC) inte neutral peptide, or agonist into partial agonist or antagonist substantially reduced the cal cium signal detected as well as the ability of the peptide mixture to elicit an in vitro prolif erative response.

2. Preckel et al. (683) demonstrated that hapten antagonists inhibited TcR down regulation by hapten agonists, suggesting that antagonism is directly related to "competi tive engagement of the TcR in unproductive interactions."

3. Sprent and coworkers (684) studied conditions surrounding the downregulation of the clonotype $1B2^+$ TcR transgene on $CD8^+$ T cells of 2C mice that are positively selected on H2-K^b but demonstrate alloreactivity to the MHC encoded class I alloantigen H2-L^d in conjunction with an 8-mer peptide p2Ca derived from the housekeeping gene 2 oxoglutarate dehydrogenase, as well as a series of related peptides that bind to H2-L^d with greater or lesser affinity. This group found that TcR downregulation was "(a) peptide dose dependent, (b) most prominent with high affinity peptides, (c) independent of CD8 expres sion, and (d) apparently unrelated to the subsequent functional responses of the cells." Additional studies involving expression of L^d on *Drosophila* cells indicated that parallel expression of costimulatory molecules ICAM-1 and/or B7-1 enhanced responsiveness (e.g., activation-induced expression of CD69 and CD25) but did not impact TcR downreg ulation. Thus, "TcR signaling is generally abortive unless amplified by corecognition of MHC molecules by CD4 or CD8 and accompanied by delivery of "second signals" through recognition of costimulatory molecules, e.g., B7." The authors concluded that TcR down regulation is most closely linked to the affinity of TcR interaction with peptide/MHC restriction element.

The conclusion from the work reviewed above is that antagonist peptides may medi ate their ability to block responses to peptide agonists by serial "spoiling" of TcR through low affinity/transient nonproductive interactions (685–687). Conversely, altered peptide ligands may engage MHC-peptide complexes with sufficient intensity to evoke downstream signaling events but of insufficient magnitude or duration to propel T cells to commit to full-fledged activation. Parallel contributions to the literature appear to suggest that CD4 or CD8 coreceptor engagement has the propensity to convert signaling events elicited by par-

tial agonists into agonistic type responses (688,689). Several authors have suggested that TcR downregulation may serve as a route to prevent excessive T-cell stimulation.

3. Costimulation, T-cell Activation and Priming

Previous discussion in this section has focused on antigen-receptor proximal signaling events leading up to the generation of a calcium signal, the activation of protein kinase C, and potential linkages to activation of MAP kinase signaling modules. Integrated downstream signaling events occasioned by T-cell antigen-receptor triggering, costimulation, and cytokine receptor occupancy drive T cell maturation, expression of effector phenotype, and cell division. Available evidence indicates that circumstances surrounding antigen receptor triggering, delivery of particular costimulatory stimuli, and the cytokine milieu govern the consequences of (auto/allo) antigen challenge.

Potential outcomes following T-cell antigen receptor triggering include retention of the naive phenotype, priming for second set responses, anergy, apoptosis or immune deviation. Given that immunological memory is one of the hallmarks of the immune system, the relative contributions of proliferation and maturation in the genesis of immunological memory continue to be debated. Thus, Zinkernagel and coworkers have suggested that, "the phenotype of immunologic memory is the best correlated with antigen-induced activation of low frequency effector T cells and plasma cells," a phenomenon that may require continued presence of antigen (690).

Additional evidence linking prepriming as the hallmark of memory in the $CD8^+$ T cell compartment has recently been reported by Lalvani et al. (691) who demonstrated the persistence of CD8 T cells capable of rapidly producing IFN-γ in primed mice in the absence of detectable CTL. This report and references therein raise the interesting speculation that the demonstrated potential of primed CD8 T cells to produce interferon gamma rapidly following antigen receptor triggering by the priming antigen might be of greater importance than perforin-dependent cytolytic activity vis-a-vis the control of viral infections. Thus, interferons induce apoptosis via the 2-5A system in conjunction with double stranded RNA in virally infected cells (692).

Conversely, others report that T-cell priming can occur in the absence of proliferation and in the relative absence of costimulatory signals (693). Thus, whereas paraformaldehyde fixation is reported to reduce the capabilities of antigen presenting cells to deliver costimulatory signals (signal 2), Gupta et al. discovered that MHC mismatched APC fixed with low concentrations of paraformaldehyde are equivalent or superior in their potential to prime human T cells to make an enhanced proliferative response. Moreover, inhibition of T-cell proliferation in primary cultures with aphidicolin, pentoxifylline, or low dose cyclosporine A likewise did not inhibit priming.

The above notwithstanding, recent work including experiments using a model system evolved by Mark Jenkins and coworkers (70) has provided important new insights into the cellular and molecular basis for priming. In the Jenkins model, CD4 T cells from TcR-transgenic mice are adoptively transferred in limiting numbers to naive syngeneic hosts that are then challenged with antigen subcutaneously or intraperitoneally or i.p. in incomplete Freund's adjuvant (IFA) to induce priming, or tolerance, respectively. Thereafter, the phenotype and functional responsiveness of transferred T cells expressing the TcR transgene can be visualized directly using using an anti-clonotypic antibody. The data indicate that CD28 costimulation and/or other costimulatory signals (e.g., heat stable antigen-HSA) are generally necessary for T cells to acquire a primed phenotype, i.e., early peak of antigen stimulated IL-2 biosynthesis, capacity to generate IFN-γ (67) and the generation of functional effectors (e.g., CTL). These findings have been substantially corroborated by others

(694–699). Moreover, CD28 appears to be particularly important for the delivery of B-cell help, because CD28-deficient mice are deficient of germinal centers (700).

Interestingly, some specific type 2 responses (e.g., to *Heligmosomoides polygyrus*) are well preserved in CD28-deficient hosts (701,702). However, in the instance cited (*H. polygyrus*), it might appear that another T-cell B7 counterreceptor (possibly CTLA4) provides a critical second pathway, because B7 blockade with CTLA4-Ig or anti-B7-1/anti-B7-2 antibodies substantially inhibit the response (703).

The induction of a primary (adaptive) immune response and the generation of a broad range of antigen-specific effector mechanisms (CTL generation, B-cell help for antibody production, delayed-type hypersensitivity) result from MHC class I-, MHC class II-restricted presentation of processed antigen (agonist peptides) by professional antigen presenting cells (e.g., dendritic cells) expressing or induced to express a series of costimulatory molecules, including B71-, B7-2, CD40, within lymph node germinal centers. Costimulatory signals and signaling events excited jointly by antigen-receptor occupancy and CD4/CD8 coreceptor cross-linking are integrated at various levels to trigger a heightened/sustained calcium transient, induce antiapoptotic factors (Bcl-2, Bcl-X_L), enhance transcription ± stabilize cytokine message (IL-2, IL-4), induce expression of late activation genes (granzymes, perforin, IL-10, CTLA4), and trigger cell cycle progression. Antigen-induced cell death, in its various manifestations, is a characteristic feature of a "normal" immune response. Therefore, factors potentially impacting the incidence of apoptosis, induction of anergy, or immune deviation affect qualitative and quantitative features of the immune response. Outcome variables include the following: (1) the duration or affinity of antigen-receptor interactions with nominal antigen/MHC restriction element and engagement of coreceptors CD4/CD8, (2) magnitude and sustained duration of calcium transients, (3) nuclear translocation of NFAT (calcineurin, Ras), (4) generation of and response to antigen-induced growth factors IL-2, IL-4 within the local milieu, and (5) integration of delivery of antigen receptor– or cytokine receptor–induced mitogenic signals required for cell cycle progression. It is interesting to suppose that costimulation (e.g. CD28-B7 axis) modulates TcR downregulation and enhances the efficiency of TcR signaling through effects targeting Rab 5 GTPase (1052).

4. Calcineurin

The discovery of cyclosporine A, FK506, and rapamycin as potent new immunosuppressive agents and subsequent identification of their mechanisms of action provided fresh insight into critical signaling events in T-cell activation (45,704–708). Cyclosporine (CsA) and FK506 binding to specific immunophilins, cyclophilin and FK-BP, respectively, forming complexes with the potential to bind to a tripartite complex of calcium, calcineurin, and calmodulin leads to the inhibition of calcineurin phosphatase activity. Calcineurin activation in the course of T-cell activation is linked to the antigen receptor–triggered calcium transient. Calcium-dependent regulation of intracellular processes is mediated by proteins that, on binding calcium, assume a new conformation, which enables them to bind to their target proteins and modulate their function (709). Calmodulin contains a helix-loop-helix (EF-hand) motif that has been linked to calcium sensitivity in other proteins. Moreover, data have been accrued to indicate that calcium-induced opening of the interfaces between the helical segments of calmodulin serves, at least in part, to mediate calcium/calmodulin-dependent activation of calcineurin phosphatase activity (709). Thus, interactions between residues Val115/Leu116 of the calcium-binding calcineurin B subunit with hydrophobic residues 328–390 of the A subunit mediate the calcium-dependent activation of the phosphatase activity of the A subunit (710,711).

Calcineurin is believed to play an essential role in antigen-receptor–triggered proliferation of T cells as well as other cell types, including fibroblasts (712,713). The critical finding that pinpointed TcR-directed activation of calcium/calmodulin-dependent calcineurin phosphatase activity as a critical early event in T-cell activation was the discovery that CsA and FK506 inhibit calcineurin phosphatase activity. Calcineurin phosphatase mediates dephosphorylation of NFAT family members preexisting in the cytosol, thereby permitting their transit to the nucleus where they bind to enhancer elements within the IL-2 promoter and promoters of other (cytokine) genes (e.g., IL-4 [714–719]). Phosphorylation sites in the N-terminus domain of NFATx1 mediate retention of NFAT in the cytoplasm, evidenced by the fact that NFATx1N-terminal deletion mutants spontaneously migrate to the nucleus (720). NFATx1 (also called NFATp) is preferentially expressed in the thymus and peripheral leukocytes. NFATx1 binds to calcineurin at multiple sites through a domain in its N-terminus, causing a size reduction in NFAT consistent with dephosphorylation of the molecule (720). Association of CsA or FK506, together with their respective immunophilins, inhibits binding of NFAT to calcineurin (721).

Activated calcineurin, p21ras, and serine/threonine kinase Raf have all been implicated in some of the earliest phenotypic correlates of T-cell activation, such as for example, upregulation of CD69, evidenced by the fact that dominant negative ras, dominant negative raf, and CsA, an inhibitor of calcineurin phosphatase have all been shown to block upregulation of CD69 induced by TcR cross-linking or increased intracellular calcium (722,723).

Calcineurin has been implicated in positive selection of double positive (DP) cortical thymocytes, which may be linked, in part, to its potential to inhibit glucocorticoid-induced apoptosis (724–726). It has previously been suggested that positive selection is accomplished through antigen receptor–triggered signal transduction events, including calcineurin activation that counters glucocorticoid-induced death of double positive thymocytes. This paradigm would be consistent with studies in yeast, indicating that calcineurin plays a critical role in maintaining viability in the face of a calcium signal triggered in response to mating pheromone (727). However, a recent publication indicates that glucocorticoids antagonize signaling precipitated by TcR cross-linking within the thymus to achieve positive selection of T cells bearing TcR of low to medium avidity and that introduction of antagonists of glucocorticoid biosynthesis in vitro (thymic organ cultures) or in vivo (TcR transgenic mice) leads to deletion of thymocytes that would otherwise be positively selected (728).

Calcineurin has also been implicated in cell death precipitated by calcium transients in the absence of requisite growth factors (729). This study demonstrated that a constitutively activated calcium-independent mutant of calcineurin could precipitate calcium-independent cell death, suggesting that calcineurin signaling plays a role in antigen receptor–triggered T-cell death, discussed elsewhere in this chapter. Negative selection of thymocytes also occurs at the double positive stage; depending on TcR "affinity" for peptide and MHC, there is a requirement for a calcium signal and calcineurin in the negative selection process. However, the role of calcineurin in negative selection is controversial (730–732). For example, FK506 was not seen to impact deletion of $\alpha\beta$ thymocytes of a TcR transgenic mouse in a negatively selecting environment (733).

As discussed in greater depth later, the TcR-induced calcium transient also mediates other important downstream events. TcR signaling excites Ca^{2+}/calmodulin-dependent protein kinase (CaMK) type IV/Gr, a kinase that is selectively expressed in T lymphocytes and has the potential to promote IL-2 transcription by inducing the formation of AP-1 (734). Calmodulin-dependent activation of CaMK type IV/Gr kinase is negatively regulated by serine threonine phosphorylation of calmodulin by casein kinase II, but serine threonine

phosphorylation of calmodulin has no effect on its ability to serve in the activation of calcineurin. Calcium also mediates calcineurin-independent polarization of T cells and stabilizes their interactions with antigen presenting cells (735).

A great deal has been learned about the physical and biochemical basis for the inhibition of the calcium/calmodulin-dependent phosphatase calcineurin by CsA and FK506 coupled to their respective immunophilins. The crystal structure of calcineurin and the calcineurin FKBP(12)-FK506 was uncovered several years ago (736,737). Calcineurin is composed of two subunits (A and B), of which, the A subunit is known to possess serine-threonine phosphatase activity and the B subunit binds calcium. Calcineurin phosphatase 2B activity appears to be regulated, at least in part, by intramolecular and intermolecular interactions involving its C-terminal autoinhibitory domain and its association with calmodulin (738). Mutations in calcineurin that inhibit binding by CsA and FK506 coupled to their respective immunophilins have previously been identified. Evidence to show that calcineurin is the only "CsA sensitive component limiting T cell activation" was obtained by expressing mutations known to inhibit binding of CsA and FK506 coupled to their respective immunophilins in mice (739,740). FKBP12-FK506 displaces the autoinhibitory element from the active site of calcineurin (736). P21 Ras and calcineurin synergize in the activation of the nuclear factor of activated T cells (74). Thus, neither mutationally activated calcineurin or activated p21ras alone is sufficient to mediate NFAT transactivation in T lymphocytes (741).

5. MAP Kinase Cascades in T-Cell Activation

The unfolding story of the role of MAP kinase modules in lymphocyte activation has been considered one of the most important discoveries in cell biology and molecular immunology in recent years (742). Downward et al. determined in 1990 that p21ras was stimulated upon T-cell activation (420), and subsequent work has demonstrated, that ras activation is an essential correlate of TcR signal transduction (743). Ras is a member of a family of small GTP-binding proteins. The phosphorylation state of the guanidine nucleotide bound by these small GTP-binding proteins determines their ability to bind to and stimulate downstream enzymatic activities. Thus, p21ras-GTP is active while p21Ras-GDP is inactive. Ras and other small GTP-binding proteins possess low basal GTPase activity, hydrolyzing GTP with a rate constant of only 0.02 min^{-1} (744), but this is enhanced by a factor of 10^5 by GTPase-activating enzymes (e.g., p120GAP) (745,746). Overexpression of rasGAP was seen to inhibit T-cell activation (747). Conversely, exchange factors modulate ras activity by increasing the rate of exchange of the guanidine nucleotide bound to ras with cytoplasmic guanidine nucleotides. Such exchange factors tend to be activating factors because the predominant guanidine nucleotide in the cytoplasm is GTP. The major thrust of research in the field of signal transduction has been the identification of factors mediating or modulating activation of p21ras and other small GTP-binding proteins and the elucidation of the their downstream signaling cascades or modules. The three modules that have been most carefully studied to date include ras-activated MAP kinase, Rho/CDC42-activated jun-c-terminal kinase (JNK), and the p38 pathway.

The possible role of ras as a participant in cell differentiation and mitogenesis was obtained from the early demonstration of the transforming potential of mutated ras (e.g., H [Harvey] Ras) (748). It has come to be appreciated that ras and myc synergize to induce mitogenesis (749). The molecular correlates of ras activation have been extensively studied (750). CD2 mitogenic activity has been linked in part to its demonstrated potential to mediate ras activation (751). The critical role of p21ras in TcR-triggered IL-2 biosynthesis was discovered by Downward and coworkers (752). It was subsequently discovered that ras was

a critical determinant of NFAT activation, and this effect was shown to be independent of downstream activity of protein kinase C (422,753). CD28 cross-linking by antibodies, but not through interaction with its natural ligand, B7, was also found to activate ras (754). Nonetheless, recent work indicates that it may be the JNK/SAP kinase pathway that is most uniquely required in CD28 signaling, evidenced by studies in SEK gene knockout mice.

The pathogenesis of p21ras activation following T-cell activation is yet to be fully elucidated. Early work in other systems indicated that linker molecules with the potential to bind hSOS exchange factor were part of the signaling cascade that mediated ras activation in other systems, and these findings focused attention on the potential recruitment or activation of such signaling molecules following TcR triggering. Sos was found early on to bind to CD3 (755), and an association of Sos, Grb-2, and a 36–38 kd phosphoprotein, was readily demonstrated in T lymphocytes (424,756). Evidence was accrued that such interactions might be essential for T-cell activation. Nonetheless, it has been difficult to define a definite role for Grb-2 and hSOS in TcR-triggered ras activation in human T cells. Interest has focused on the possibility that Vav, a GTP exchange factor (GEF), might be an important activator of ras following T-cell activation, although this is thought to be unlikely by some parties (757). Vav bears sequence homology with exchange factors for the Rho family of small GTP-binding molecules (758), and definitive data have accrued that Vav is an exchange factor for Rho and Cdc42, small GTP-binding proteins implicated in activation of the JNK/SAP kinase pathway. Crk and downstream exchange factor C3G are suspected as having a primary role in ras activation following TcR triggering (570). Pathways that serve in downmodulating ras in vivo have become the subject of intensive investigative effort (see page 297). Farnesyl transferase inhibitors are also being explored as potential treatments for neoplasia associated with ras transformation because of their potential to block ras activation by inhibiting its translocation to the cell membrane (759–762).

Data have accrued to indicate that TcR signaling has the potential to activate a second MAP kinase pathway leading to the activation of JNK/SAPK (see below). At the time of preparation of this review, upstream signaling events that mediate TcR-triggered JNK/SAP kinase activation had not been clearly delineated, although circumstantial evidence suggests that the signal is transduced through TcR-triggered activation of Vav (763–765). Vav has been identified as a guanidine nucleotide exchange factor (GEF) for at least one (Rac-1) of the several small GTP-binding proteins of the Rho family (e.g., Rho, Rac, and Cdc42Hs) that lie upstream of the JNK/SAPK pathway, based on transient transfection studies employing oncogenic Vav as well as wild type and mutated forms of Rac-1 in Cos 7 cells (63,561,758,763,766–768). These studies have recently been extended to document that activated (tyrosine phosphorylated) Vav catalyzes GDP/GTP exchange on Rac-1 and stimulates c-Jun kinase (JNK) in vivo (769), a finding consistent with recent reports that place Vav upstream of ZAP70 as an essential mediator of antigen receptor–triggered T-cell activation (770,771). Rac1 (as well as Cdc42) has previously been shown to associate with the Pak1 kinase, suggesting that the latter may be the most upstream member of the c-Jun N-terminal kinase (JNK) pathway. Evidence has recently accrued that would place the dual leucine zipper-bearing kinase (DLK) downstream of Rac1 and Cdc42Hs but proximal to MEKK1 (772). Downstream signaling below PAK (and possibly DLK) involves MEKK (773), and its target SEK (774), also known as MKK4 or JNKK (775), which is the direct activator of JNK (416,774,776). Finally, new data have accrued to indicate that Cdc41 and all Rho proteins (RhoA, RhoB and RhoC), but not Rac, can also activate JNK through a Pak-independent pathway, at least in human epithelial cells (411).

A second mammalian stress-activated protein kinase cascade has also been well characterized (MKK3/MAPKK6 (MKK6)/p38^{Hogg}) in which p38 is activated by tyrosine-182,

threonine-180 phosphorylation within the dual phosphorylation motif (Thr-Xaa-Tyr is a Glu, Pro, or Gly group for the ERK, JNK, and p38 group of kinases, respectively [777]) in response to cytokine stimulation and other stresses (778). There is a family of p38 stress-activated protein kinases (α, β, γ, and δ) that demonstrate significant differences in tissue distribution, stimulus activation, and substrate specificity as well as inhibitor sensitivity (779).

Although promiscuous in vitro (775), recent evidence documents the individual functional integrity of the three mammalian MAP kinase signaling modules (780). Nonetheless, upstream kinases and pathways have been identified that might serve in the activation of two or more downstream MAP kinase signaling modules. Thus, TNF-induced apoptosis has been linked to TNF-induced activation of ASK1, a MAPKKK, and downstream activation of both SAPK and P38 in Jurkat as well as other cell types (781). ASK1-induced apoptosis was inhibited by a catalytically inactive form of ASK1. Conversely, Tpl-2 protooncoprotein, a novel MAP kinase kinase kinase, has been shown to activate MAP kinase signaling modules, leading to activation of ERK and SAPK (782).

Interestingly, tandem activation of pairs of MAP kinase signaling modules may be required to signal downstream mitogenic responses, as noted in the instance of healing of gastrointestinal epithelium (ERK, p38) (783), and this might be related to the differential effects of individual MAP kinase modules on downstream signaling events. Evidence has accrued to show that MAP kinase pathways converge on the ternary complex factor Sap-1a. Phosphorylation of Elk-1 and Sap-1a (components of the ternary complex factor [TCF] constitutively associated with the c-fos promoter element SRE), by particular MAP kinases, initiates c-fos transcription. Janknecht and Hunter as well as other groups (784–786) have recently reported that ERK and JNK but not $p38^{Hog}$ phosphorylate Elk-1, although there is some controversy relating to p38 in these regards (786). Conversely, TCR Sap-1a is efficiently phosphorylated by ERK and $p38^{Hog}$, but not by JNK; in the latter instance, there is again some controversy that may be related to cell type and promoter specificity (786). This redundancy allows for targeted downstream events even by stresses or signals that activate only pairs of MAP kinases (e.g., CD40 and IL-1 target both JNK and p38 pathways) (787). 3pK is a novel mitogen-activated protein kinase-activated protein kinase known to be activated by all three MAP kinase pathways (788). Other downstream targets of MAP kinases include kinases that activate the cell cycle. Thus, in yeast, SMK1, a developmentally regulated MAP kinase, phosphorylates and activates cyclin-dependent kinase–activating kinase (CAK) (789). Phosphatases PAC1, MAP kinase phosphatase-2 (MKP-2), and MKP-1 have been implicated in the in vivo inactivation of ERK and p38, ERK and JNK, and ERK, JNK, and p38, respectively (790–792).

The above notwithstanding, evidence exists to show that full activation of the JNK/SAPK T cells, as obtained following costimulation of T cells, requires simultaneous cross-linking of the T-cell antigen receptor and CD28 (59), leading Su et al. to suggest that JNK is involved in signal integration in CD28-dependent costimulation of TcR-triggered T-cell activation and IL-2 production. This supposition has been formally tested by a recent study in transgenic mutant (gene knockout) mice deficient of SEK (otherwise called MEKK), a dual function tyrosine-threonine kinase upstream of JNK (776), that demonstrate impaired responses to costimulatory signals (417). Thus, $SEK^{-/-}$ peripheral T cells demonstrate reduced proliferative responses and IL-2 production after CD3/CD28 cross-linking. These findings are particularly attractive because tandem activation of at least two MAP kinase signaling cascades are apparently required to activate transcription of c-fos, whereas JNK (cJun N-terminal kinase) downstream of SEK activates and stabilizes c-Jun

(415). Therefore, simultaneous activation of MAP and JNK, leading to transcription of c-fos as well as the activation and stabilization of c-Jun, are sufficient to constitute the transcription factor AP-1. MAP kinase cascades have singular importance in biology of T-cell activation and prevention of anergy as implied by work of Li et al. and Fields et al. (61,63); a recent publication characterizing a novel p38 mitogen-activated protein kinase (779) identifies four groups of MAP kinases (ERK1/2, JNK, p38, and ERK5).

The recent discovery of additional redundancies among MAP kinase signaling modules may be particularly warmly received by persons engaged in research on Itk (also called Emt and Tsk), a member of the protein tyrosine kinase (PTK) family (Tec, Btk, Itk, and Bmx [793]), and transduction of costimulatory signals arising from CD28 cross-linking. Whereas there are data to indicate that the "integration of the CD28 costimulatory signal and the TcR signaling pathway occurs at the level of c-Jun NH2-terminal kinase (JNK)," Liao et al. (418) recently reported that they observed no difference in JNK activation between $itk^{+/+}$ and $itk^{-/-}$ mice, even though their own data as presented indicate that the nonreceptor protein tyrosine kinase *itk* markedly impacts "the interplay between the TcR and CD28 signaling pathways" (418). Thus, Liao et al. have confirmed their earlier observation that T cells of *itk*-deficient mice demonstrate reduced or absent proliferative responses to anti-CD3ε antibody stimulation or allogeneic MHC (794), and they report that T cells of $itk^{-/-}$ mice mount supranormal (3×) proliferative responses upon concurrent exposure to anti-CD3 and anti-CD28 antibodies, even while IL-2 production is somewhat reduced (418). These findings are particularly welcome because advances in understanding of the signals transduced in response to CD28 cross-linking have not kept pace with research on receptor proximal events triggered by T-cell antigen receptor occupancy or CD3 cross-linking.

Indeed, the role of the Tec family of PTK in T-cell activation is presently the subject of intense investigative effort by several large laboratories and, accordingly, future progress in this arena is likely to be swift. It seems that the human variant of mouse Itk was initially cloned in a polymerase chain reaction (PCR)-based search of T-cell–specific tyrosine kinases and was shown to contain, in order, a Pleckstrin homology (PH), Tec homology (TH), SH3, SH2, and tyrosine kinase domains, and to lack the N-terminal regulatory phosphorylation site of src kinases (795). Subsequent studies revealed that Itk is tyrosine phosphorylated and activated by CD28 or CD2 cross-linking (796,797). Recent physical studies have shown that an intramolecular association occurs between the SH3 domain of Itk and the adjacent N-terminal proline-rich TH domain that likely serves to block intermolecular interactions through these sites in the resting state (798). Accordingly, a multistep model has evolved wherein interactions between the SH3 domain of Itk and the N-terminal diproline motif within the cytoplasmic tail of CD28 serve in the activation of the kinase (799). Recent documentation that Itk forms an inducible complex with CD28 in vivo provides fresh insight as to mechanisms whereby CD28 cross-linking transduces downstream PTK-dependent signaling events. Itk may also interact with other proteins engaged in transducing signals associated with lymphocyte activation. Thus, the SH3 domain of Itk has been shown to interact with the proline-rich sequences of Sam68 and the Wiskott-Aldrich syndrome protein (WASP), although weaker interactions with Cbl have also been documented (793,800,801).

Even though it is presently unclear what signals promote Itk interactions with CD28, Sam68, or WASP during lymphocyte activation and the potential downstream consequences of same, it is potentially of interest that protein kinase C is reported to mediate Itk activation in response to Fc receptor cross-linking (802). Itk/EMT/Tsk has been found to

bind to several PKC isoforms in intact cells. The PKC may also participate in Itk activation following TcR signaling as has been documented in Jurkat cells (803). Members of the Rho family of small GTPases (e.g., Cdc42) are also reported to interact with WASP (804). Accordingly, one could speculate that activation of Itk as a consequence of CD28 cross-linking might be intimately linked to downstream activation of MAP kinase pathways and prevention of T-cell anergy, but this is likely simplistic. As discussed below, there are data to show that Gβγ subunits of heterotrimeric G proteins, together with unidentified membrane factors, can increase the activity of immunoprecipitated Itk (805).

Other members of the Tec family (including, perhaps, more distant relatives) also appear uniquely positioned to serve important roles in T-cell activation or the expression of particular helper T-cell phenotypes. Rlk was identified as a novel member of the Tec family in the mouse that is preferentially expressed in Th1 cells relative to Th2 (806). A human homologue of Rlk has been discovered, i.e., Txk, whose expression is restricted to T cells and several myeloid cell types (807). Of note, Txk shares 57% amino acid homology with Bruton's tyrosine kinase (Btk), and mutations or deficiency of Btk have been associated with X-linked hypogammaglobulinemia, a primary immunodeficiency disease. It becomes of interest, therefore, that the G_q-protein of the α subunit of heterotrimeric G proteins has been shown to activate (stimulate) Btk activity (808). These data are consistent with genetic evidence from the same group for the existence of a tyrosine kinase bridge (involving Csk, Btk, or Syk) that mediates signal transduction from G proteins to the MAP kinase pathway in avian lymphoma cells. In these cells, targeted deletion of Csk blocks stimulation of MAP kinase by C_q (809). G-protein–coupled receptors and the potential relationships between Ras-related and heterotrimeric GTP-binding proteins have recently been reviewed (810–814). Although there are no data to suggest that heterodimeric G proteins are coupled directly to the TcR signaling, it may be pertinent that heterodimeric G proteins are involved in signaling by C-C chemokines (MCP-1, RANTES) and thromboxane receptors in T and NK cells (815–817), that thromboxane A2 receptors are highly expressed in immature thymocytes (818), and that thromboxane receptor antagonists have been shown to inhibit tolerance induction following intrathymic injection of MHC allopeptides (115,819).

Recent contributions to the literature provide evidence for higher order regulation of the JNK signaling pathway. Thus, a cytoplasmic inhibitor of the JNK signaling pathway has recently been identified by Dickens et al. (398), which appears to function, at least in part, by cytoplasmic retention (inhibition of nuclear translocation) of JNK and by interrupting the association of JNK with c-jun, thereby inhibiting JNK-dependent phosphorylation and activation of the transcriptional activity of the latter.

To summarize, it has become appreciated that there are two or more distinct pathways of T-cell activation, one that is PLC and calcium dependent and one that is PLC and calcium independent and that involve cAMP and tyrosine kinases. T cells may use different signal transduction pathways at different stages of T-cell ontogeny within the thymus. There is evidence to suggest that clonal deletion at the level of the thymus is accompanied by activation-induced increases in intracellular calcium. It seems reasonable to suppose, therefore, that positive selection will be shown to involve a calcium-independent signaling pathway (820). Peripheral $CD4^+$ helper T cells of the Th1 subset may be activated by signal transduction mechanisms involving PLC and Ca^{2+} signals (which are tyrosine kinase independent), whereas the activation of the Th2 helper T-cell subset may be Ca^{2+} and PLC independent. Both pathways appear to be able to stimulate IL-2 release, presumably in $CD4^+$ helper T cells of the Th0 subset, as evidenced by a report of a PLC, Ca^{2+}-independent, pertussis toxin inhibitable pathway of T-lymphocyte activation leading to IL-2 secretion (821). Finally, the regulated activity of MAP kinase signaling modules by the ras fam-

ily of small GTP-binding proteins has been identified as a primary determinant of cell fate in T (and B) lymphoid compartments.

F. Interleukin-2/Interleukin-2 receptors in T-Cell Activation

The regulation of IL-2 gene transcription and IL-2 biosynthesis has continued to draw a great deal of attention in recent years as a critical marker of productive T-cell activation (822,823). Such interest is obtained, in large measure, from a vast body of evidence to show that T-cell costimulation through the CD28/B7 axis enhances IL-2 transcription, stabilizes IL-2 message, enhances IL-2 biosynthesis, and prevents anergy (409,824–828). Factors binding to NFAT-, AP1-, octamer-, and κB-binding sites have all variously been considered to promote IL-2 gene transcription. CD28 costimulation enhances TcR-triggered IL-2 biosynthesis 30- to 100-fold. In molecular terms, CD28 cross-linking in IL-2 biosynthesis is recognized to correlate most particularly with binding of NF-ATp as well as NF-κB/Rel species to the CD28RE (829). Thus, ligation of CD28 by B7 counter receptors superinduces IL-2 biosynthesis and generates long-lasting T-cell proliferation through enhanced expression of AP-1, activation of NF-κB, and stabilization of IL-2 message (825,830).

The NFAT family of transcription factors has recently been reviewed (718). As discussed in previous sections, activation and nuclear translocation of NF-AT is a Ras-dependent event that has been linked to activation of the calcium/calmodulin-dependent phosphatase calcineurin. The mechanism of NF-κB activation has been extensively elucidated in recent years (831). As is generally understood, the NF-κB family of nuclear transcription factors are generally maintained in an inactive state by an anchor protein, I-κB, serving to maintain κB transcription factors within the cytoplasm. In the past 2 years, it has been discovered that NF-κB is activated through targeted destruction of I-κB. This has been found to involve the phosphorylation of serines (Ser32, Ser36) in the N-terminus of I-κB-alpha by the cytokine-responsive I-κB kinase, which, in turn, promotes subsequent ubiquitinization of I-κB on lysines 21 and 22, thereby targeting the molecule for degradation by the proteosomal system (832–836). The corresponding serines of I-κB-beta are constitutively phosphorylated and, accordingly, inducible phosphorylation of these sites in the beta-isoform does not necessarily constitute the signal-induced event that targets the molecule for proteolysis (837). Finally, serine phophorylation (Ser 894, Ser908) of the inhibitory C-terminal portion of the NF-κB precursor p105 is known to release the active moiety by targeted degradation of the C-terminal end of the protein (838). It is of interest that cyclosporine interferes with the inducible degradation of NF-κB inhibitors I-κB alpha and I-κB beta (839).

Defective IL-2 transcription in anergic T cells has been linked to reduced AP1, obtained from impaired expression of c-Fos, FosB, and JunB (840,841). These data are consistent with evidence to show that anergic T cells are deficient in c-Jun-NH2-terminal kinase and ras-MAP kinase signaling pathways (64). Defective IL-2 transcription in c-Rel GKO mice has been linked to the potential of c-Rel to enhance transcriptional activation by AP1 (842). Additionally, there are data to show that c-Rel associates with JNK and may serve as a docking factor for a second ligand, which is a target of JNK kinase activity (843).

The structure of the IL-2 receptor (IL-2R) has recently been reviewed (844). Briefly, the IL-2 receptor consists of three chains: the activation-dependent IL-2Rα-chain, and two chains constitutively expressed on resting T cells, that is, the IL-2Rβ and IL-2Rγ, which together constitute the high-affinity IL-2R. IL-2R signaling is intimately linked to the association of the Jak family kinases, Jak1 and Jak3, with IL-2Rβ and IL-2Rγ chains, respectively (845). There are data to suggest that Jak1 is dispensable both for IL-2–induced cell

growth as well as Jak/STAT5 activation in a human T-cell line (846). Importantly, however, the IL-2Rβ chain contains four distal tyrosine phosphorylation sites, of which, the most proximal appears to be essential for IL-2–induced activation of c-fos, whereas the c-terminus tyrosine residues (Tyr-338 and Tyr-510) appear to be critical for growth and (STAT5) signaling (847). Greene and coworkers discovered that STAT5 associates with sequences spanning Y392 and Y510 of the IL-2Rβ-chain, and that such association is a critical initial event in IL-2–induced STAT5 (tyrosine phophorylation/dimerization) activation (848). However, it is currently appreciated that STAT5 can undergo direct JH2-dependent association with JAK1, JAK2, and JAK3 and be activated in this context (849).

Two other IL-2Rβ chain domains, the serine-rich region and the acidic region, have been found to serve essential roles in IL-2–induced cell growth and Lck src kinase activation, respectively (850). Syk protein tyrosine kinase is associated and activated through interactions with the serine-rich region of the IL-2 receptor and affords a possible link with IL-2–induced activation of c-*myc* (851). Tyrosine 338 of the IL-2Rβ chain has been found to be essential for binding and induced tyrosine phosphorylation of the 52-kd adaptor molecule Shc, as well as IL-2R–transduced Ras activation and downstream signaling through the MAP kinase pathway (852). A recent report indicates that the phosphotyrosine binding domain (PTB) of Shc mediates the association of Shc with a phosphotyrosine residue y338 of the IL-2Rβ chain (853). IL-2R signaling induces tyrosine phosphorylation of Shc.

A STAT5-binding site has been discovered in the IL-2R-α chain promoter, consistent with previously accrued evidence to suggest that IL-2 served, in part, to upregulate expression of its own (high affinity) receptor (854). New data are beginning to accrue on factors that impact STAT5 signaling. First, the glucocorticiod receptor has been shown to interact with STAT5 and enhance STAT5-induced transcriptional events (855). More recently, it has been determined that STAT5 has to undergo both serine and tyrosine phosphorylation for activation (856). Different cytokines induce different patterns of STAT activation. Thus, IL-4 induces STAT6 and IL-12 induces STAT4. Some data have accrued to indicate that expression of STAT4 or STAT6 may be linked to the evolution of particular phenotypic properties of differentiating T helper cells (857).

It may also be of profound biological significance that IL-2R signaling induces IL-2Rβ chain–dependent (acidic domain) phosphorylation and activation of the SH2-containing tyrosine phosphatase SHP-2. Involvement of the acidic domain of the IL-2Rβ chain may implicate src kinase p56lck in SHP-2 phosphorylation, based on evidence to show that interactions of lck with the IL-2Rβ chain have previously mapped to the acidic domain. Receptor-associated constitutive protein tyrosine phosphatase activity maps to JAK1 and controls its activity (858). These insights may be particularly important because data accrued in our own laboratory in collaboration with individuals at the NIH (J. Goebel, A. Franks, F. Robey, H. Young, R. P. Lowry, unpublished observations) and reports by several other groups are beginning to indicate that the regulation of IL-2R signaling may contribute to the genesis of self-tolerance and conditioned unresponsiveness in the setting of experimental organ transplantation. Indeed, phosphatases are seen to have a broad role in mediating and regulating lymphocyte activation as documented elsewhere in this chapter (859–865).

Two signals are required, as a minimum, to elicit T-cell activation. Physiologically, these are likely supplied through TcR interactions with antigenic peptide/MHC restriction element as well as interactions between CD28 and inducible B7.1/B7.2 counter receptors expressed on antigen presenting cells. Antigen-receptor occupancy/cross-linking coupled with relevant costimulation through the CD28-B7 axis is sufficient to drive IL-2 biosynthesis and cell cycle progression. The five primary stages of the cell cycle (G0, G1, S, G2,

and M) as well as critical events accompanying the two primary cell cycle check points have been extensively reviewed (866). Transition of G1 and G2 cell cycle check points in human systems are most intimately associated with the function of complexes containing cyclin E/cyclin-dependent kinase 2 (CDK2) and cyclin B/Cdc2 (also called CDK1), respectively (867,868). Function of the cyclin E/CDK2 complex prior to G1 is regulated in part by $p27^{kip1}$, a cyclin-dependent kinase inhibitor cloned in 1994 by Polyak et al. (869). Importantly, as illustrated in Figure 10, dual signals encompassing antigen-receptor cross-linking (or low doses of PHA as demonstrated in this figure), together with CD28 cross-linking, or exogenous IL-2, serve in the induction of CDK2 and Cdc2, as well as the down-regulation of the CDK2-inhibitory protein $p27^{kip}$ (870,871). Remarkably, downregulation of $p27^{kip1}$ in response to IL-2, as observed in the data shown, is driven at least in part, by the ubiquitin-triggered degradation of the inhibitor (872). The mechanisms wherein protein

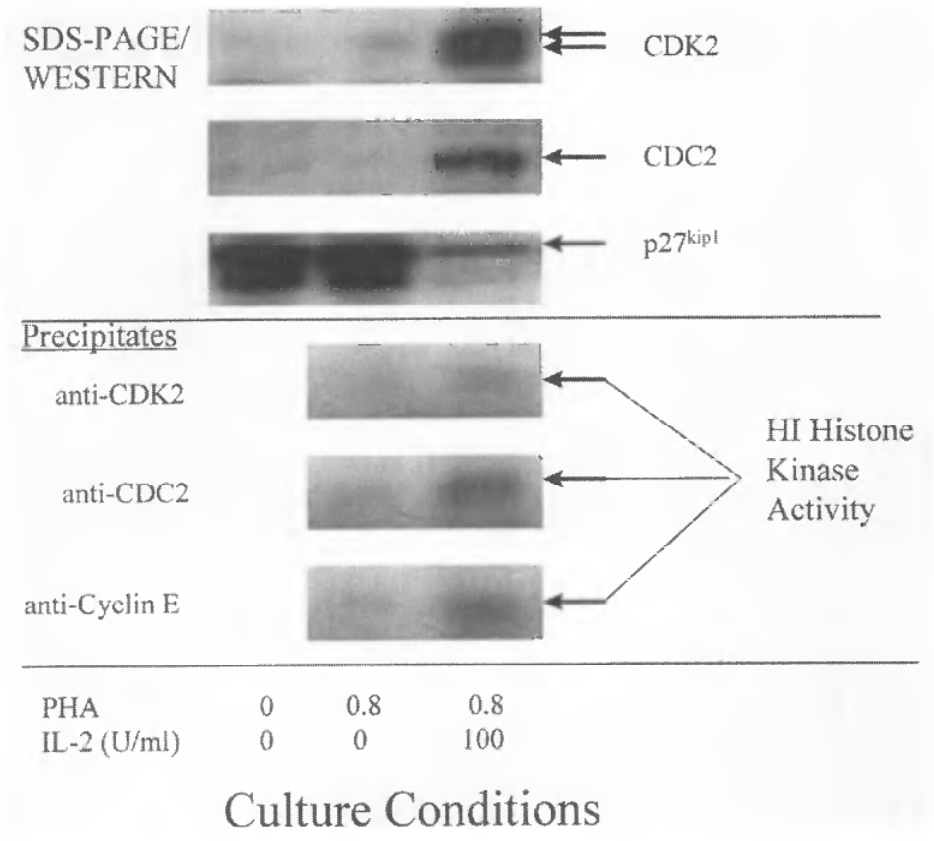

Figure 10 Two signals (e.g., phytohemagglutinin and IL-2) are required to promote cell cycle progression in human T cells, evidenced by the appearance and induced in vitro kinase activity of cyclin-dependent kinases CDK2 and $p34^{cdc2}$ (CDK1) as well as cyclin E–associated kinases. Briefly, human peripheral blood mononuclear cells were recovered from healthy volunteers by leukophoresis. Small resting T cells were then isolated by sequential ficoll hypaque gradient density centrifugation, Percoll step gradients, and nylon wool filtration and cultured for 3 days in complete medium, in complete medium with T-cell mitogen phytohemagglutinin (PHA-P, Sigma, 0.8 μg/mL), or PHA (0.8 μg/mL) plus IL-2 (100U/mL) prior to lysis, essentially as reported by Firpo et al. (871). *Upper panel:* Immunoprecipitates were separated by electrophoresis on 5% to 15% polyacrylamide gels, transferred to nylon, immunoblotted with antisera against cyclin-dependent kinases CDK2 and $p34^{CDC2}$ or cyclin-dependent kinase inhibitor $p27^{Kip\,1}$ (all from Upstate Biologicals). Alternatively, *lower panel,* lysates were immunoprecipitated with anti-CDK2, anti-CDC2, or anti-cyclin E antisera (Upstate Biotechnology, Lake Placid, NY) coupled to protein A-sepharose (Santa Cruz) and in vitro kinase assays were performed using ^{32}P-γ-ATP and H1-histone substrate; subsequent analysis of the latter was by electrophoresis on 15% polyacrylamide gels and autoradiography.

phosphatase Cdc25C mediates G2 check point control has just been elucidated (873). Briefly, the dephosphorylation of Cdc2 by Cdc25C is one of the key steps in regulating entry into mitosis. It is appreciated that the 14-3-3 protein discussed earlier, binds to serine-216 (ser-216) phosphorylated Cdc25C and maintains the latter in an inactive state through sequestration. Studies with synchronized cells have shown that Cdc25C is maintained in vivo in a ser-216 phosphorylated state by human kinase Chk1 and thus is sequestered by 14-3-3, except during the G2-M phase of the cell cycle.

Recent contributions to the literature indicate that IL-2 and IL-2 receptor signaling play a central role in the prevention of T-cell anergy as well as the induction and prevention of antigen-induced cell death in the T-cell compartment. Work by Boussiotis et al. (874) established that IL-2Rγ_c chain cross-linking was sufficient to prevent anergy of human T cells triggered by T-cell antigen receptor occupancy in the absence of B7 costimulation (blockade by CTLA4-Ig). Subsequent work by Boussiotis and coworkers determined that T cells stimulated by exposure to alloantigen in the absence of CD28 costimulation activates CD3ζ and promotes association of the latter with fyn (496). Although the exact pathogenesis of this is unclear, it appears that Csk may be involved. Thus, the activity of Csk is increased some sixfold in proliferating cells (875). Alternatively, it should also be noted that the increased fyn kinase activity observed in anergic cells requires calcium and protein synthesis and is inhibitable by cyclosporine A (497). The mechanisms whereby IL-2 blocks induction of anergy following TcR cross-linking in the absence of B7 costimulation have not yet been elucidated. Not unreasonably, therefore, Boussiotis et al. suggest that costimulation provided by B7 signaling through CD28 must facilitate association of CD3ζ with lck, thereby inducing productive downstream interactions with ZAP70. It seems not unreasonable to suppose, however, that at least one of the numerous downstream signaling cascades (e.g., the Ras-MAP kinase pathway) excited by IL-2R occupancy interrupts active processes that characterize states of T-cell anergy (497). As an example, a recent contribution to the literature by Chen et al. (876) indicates that calcium-dependent immediate early gene induction in lymphocytes is negatively regulated by p21Ha-ras; this negative effect of p21Ha-ras was seen to be prevented by cyclosporine A. Given that maintenance of T cell anergy has recently been linked to activation of rap1, it would be of great interest to determine whether IL-2 induces or activates rap1-specific GAPs (GTPase activating proteins) GAP 3m or Spa1, previously identified as mitogen-inducible GAPs (4,412). T cells activated by CD3 cross-linking are highly susceptible to apoptosis, and this response is inhibited by exogenous IL-2 and to some extent by several other cytokines (877). Interestingly, p21ras has been shown to inhibit TcR-triggered apoptosis of resting cells (878), as evidenced by studies incorporating a dominant negative or inducible ras mutant. Additional studies with dominant negative Ras mutants has revealed that Ras regulates activation of the JNK in response to simultaneous TcR cross-linking and CD28 cross-linking (879), and this is probably due, at least in part, to transcription (or activation) of the PAC1 phosphatase gene (880). Thus, to the extent that combined triggering of the JNK and p38 MAP kinase pathways has been associated with apoptosis, ras may be able to inhibit apoptotic cell death. JNK activation has also been linked to Fas-mediated apoptosis in human T cells (881), but this is quite controversial. Alternatively, it may be of note that IL-2 also activates PI-3K as well as PKC-ζ (882); PI-3K (as well as Akt) has been directly implicated in the inhibition of cell death as occurs from growth factor withdrawal (584).

Sizable literature has also accued to indicate that IL-2 promotes TcR-triggered cell death (apoptosis) of previously activated T cells in a response that is both Fas (CD4 T cells) and/or TNF p70 (CD8 T cells) dependent (46,472,473,475,476,883–889). The mechanism

wherein IL-2 predisposes T cells to AICD is not clearly defined. Evidence indicates that variables relating to the TcR signal are important determinants but that downstream signaling events have not as yet been identified. Although studies in p53 GKO mice by Boehme et al. (886) would appear to exclude potential involvement of the p53 tumor suppressor gene as an important participant in the genesis of AICD, it may be pertinent that a p53 homologue has recently been identified which appears to subserve most of the functions of p53 (890). Evidence has recently appeared to show that IL-2 in fact has the potential to program activated T cells to undergo activation induced cell death (Figure 11). Studies in IL-2Rα chain gene knockout mice have shown that IL-2 receptor signaling is indeed required for activation-induced apoptosis of activated cells (47,48). It becomes of great interest, therefore, that IL-2– and IL-2R–deficient mice develop autoimmune diatheses. Moreover, work by Konieczny and Lakkis and coworkers has revealed that IL-2/IL-2R–deficient mice are resistant to tolerance induction as generally is obtained following parenteral administration of CTLA4-Ig. These data potentially pinpoint a requirement for IL-2–induced cell death as an important pathway in the genesis of transplantation tolerance. Nonetheless, the work by Abbas and coworkers as well as Strober and coworkers cited above also provide evidence to indicate that biosynthesis of prototypic cytokines by Th2-like effectors is IL-2 dependent. It remains conceivable, therefore, that autoimmune disease in IL-2/IL-2R–deficient mice, and resistance of same to tolerance induction, may also be related, at least in part, to a possible role for Th2-like effectors in the maintenance of self/transplantation tolerance.

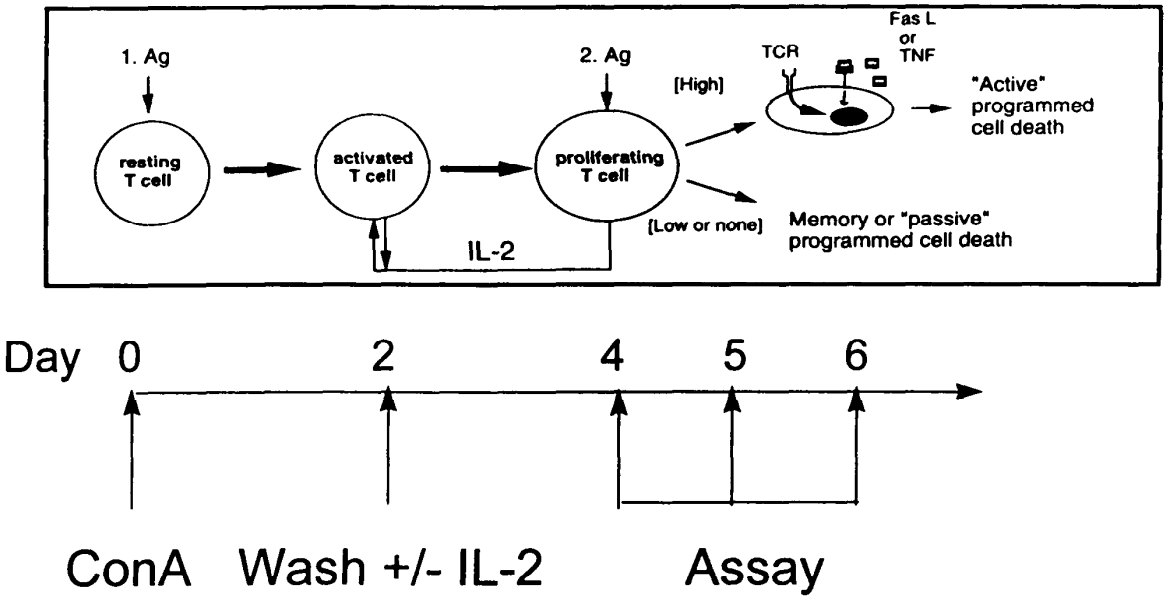

Figure 11 Interleukin-2 subserves an essential function in the induction of antigen-induced cell death (AICD) of activated T cells upon repeated exposure to antigen (46) (Adapted from Ref. 884, with permission.) In the Lenardo model, lymph node cells are activated by exposure to Con A on day 0, washed on day 2, and recultured in the presence or absence of IL-2 through day 4. Thereafter, as illustrated in the lower panel, exposure to plate-fixed anti-CD3 on day 4 precipitates Fas/TNF-dependent apoptosis of T cells that have been maintained in IL-2. Data recently reported by Lakkis (Annual Meeting, American Society of Nephrology, 1997) indicate that IL-2 as well as IL-2 receptor gene knockout mice resist antigen-receptor–triggered apoptosis in vivo and are not susceptible to tolerance induction by parenteral administration of CTLA4-Ig.

III. HOMOGRAFT REJECTION

Immunological mechanisms underlying acute and chronic organ allograft rejection are addressed in depth elsewhere in this book. Nonetheless, it is difficult to address transplantation tolerance without at least brief discussion of current thought about the pathogenesis of allograft rejection.

A. CTL in Allograft Rejection

Recent contributions to the literature indicate that expression of mRNA for granzyme B, perforin, Fas, FasL, IL-10, and possibly IL-2 is linked to acute rejection and mRNA for TGFβ to chronic rejection (891–897). These data have been interpreted to indicate that allograft rejection is perhaps most closely correlated with the activity of cytotoxic T cells. However, transcription of many of these messages examined in these studies occur in the later stages of lymphocyte activation. Moreover, Strom and coworkers have also identified CTLA4, another late lymphocyte activation marker, believed to deliver negative signals that might serve to limit or arrest lymphocyte activation, in human renal transplant biopsy specimens in the setting of acute rejection (898). Accordingly, it is of interest that the tempo of allograft rejection by recipient mice deficient of perforin and FasL is only mildly retarded or unchanged (899).

B. DTH as an Effector Mechanism of Allograft Rejection

One of the authors (RPL) has previously been a proponent of the thesis that allograft delayed-type hypersensitivity might be an immediate proximate cause of allograft rejection. As reviewed elsewhere (900), there is a significant body of data that might be consistent with the hypothesis that allograft rejection should be viewed as the product of a complex, immune/inflammatory process (901). There is support for the hypothesis that allograft rejection might be most closely linked to the activity of Th1- and/or Tc1-like effectors (see Fig. 12). However, this paradigm is also controversial. Thus, evidence has accrued to indicate that the tempo of rejection of experimental cardiac allografts in IFN-γ gene knockout mice may even be accelerated when compared with the tempo of rejection in wild-type littermate controls (902). Lakkis and coworkers have proceeded to show that IFN-γ GKO mice also reject heart grafts bearing an isolated MHC class I disparity, even while class II disparate grafts are not rejected by IFN-γ–deficient hosts (903). Their interpretation of these latter observations is that IFN-γ may be required to upregulate MHC class II expression on target tissues; this is consistent with work by other investigators in the field (904). These observations are seemingly inconsistent with the previously documented role of IFN-γ in mediating protection against certain infectious organisms or in provoking prototypic Th1-induced tissue injury in autoimmune disease models (905–907). Nonetheless, parallel contributions to the literature have documented high susceptibility to autoimmune diatheses in IFN-γ and IFN-γ receptor gene knockout mice (908,909).

Although the potential implications of these data are far from clear, it is perhaps of interest that there may be both IFN-γ–dependent as well as IFN-γ–independent but IL-12–dependent pathways of expression of Th1-type responses, as evidenced by work in a *Chlamydia* model (910). Additionally, some insight has been obtained with regard to the accelerated tempo of tissue injury with IFN-γ/IFN-γ receptor deficient mice. Evidence has accrued to indicate that IFN-γ may be implicated in retarding cell cycle progression and/or induction of apopotosis (911,912). Furthermore, there are data to indicate that IFN regulatory factor-1 (IRF-1) plays a central role in the regulation of the expression of class I and

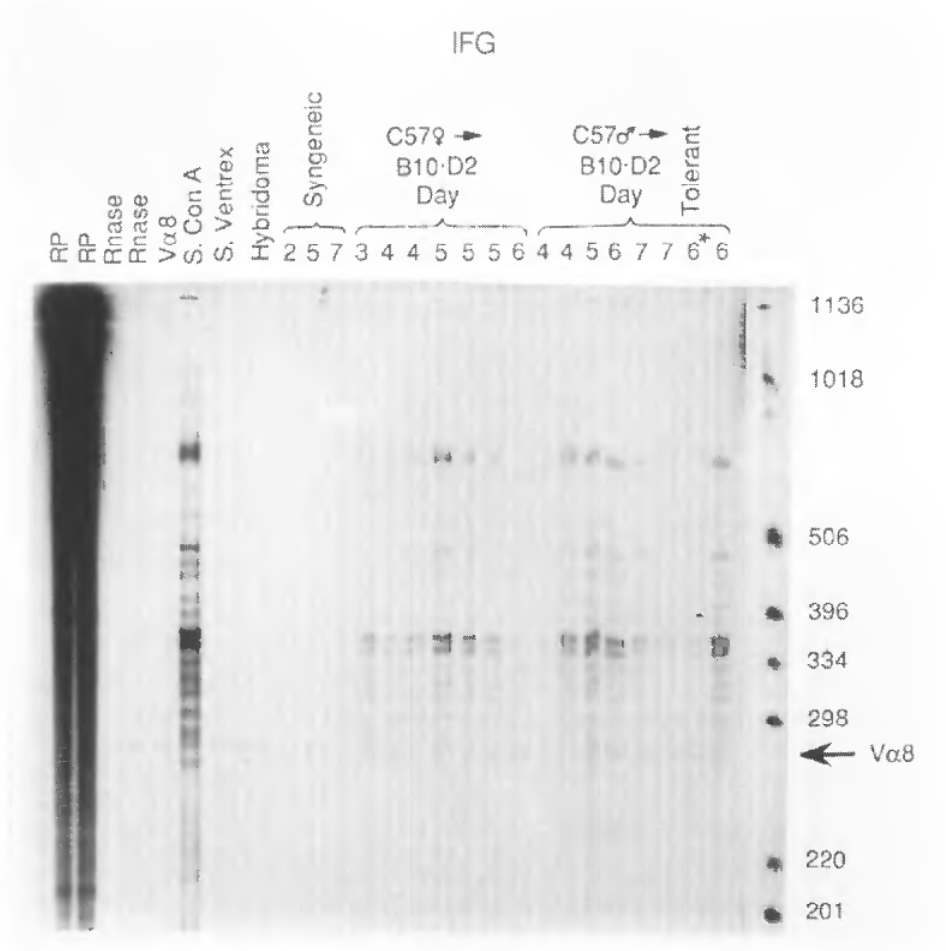

Figure 12 Initial documentation of the time course of appearance of interferon-γ mRNA transcripts in mouse heart allografts during the phase of acute rejection. Total RNA from the graft was extracted from heart grafts transplanted in the strain combinations indicated. IFN-γ transcripts were assessed by solution hybridization to specific antisense RNA radioactively labeled during transcription from a plasmid template (RNase protection assay). Following hybridization, RNase is added to the mix and unprotected probe is degraded. The digested/protected products are then identified by polyacrylamide gel electrophoresis and autoradiography to allow a precise determination of the size of the protected product, if any. Multiple bands are seen in positive lanes resulting from variations in the size of the antisense transcripts employed. Both male and female C57BL/10 donors were used in these studies and results are shown separately. The data shown indicate the time course of appearance of IFN-γ transcripts in mouse heart allografts during the phase of acute rejection (From Ref. 68.)

class II MHC genes, based on work of Halloran and colleagues (913). Additionally, IRF-1 has also been shown to lie upstream of ICE in radiation-induced cell death and may be directly implicated in activation-induced cell death (914). In studies in one of our laboratories (Franks A, Goebel J, Lowry RP, unpublished observations), we have observed a close correlation between the amount of apoptosis induced following ConA activation of lymphocytes from wild-type and IFN-γ GKO mice and mitogen-stimulated induction of IRF-1. Importantly, IL-2 inhibits apoptosis in this model system. Moreover, others report STAT5 represses transcription of IRF-1 (1053). Accordingly, it seems not unreasonable to suppose that the heightened T-cell proliferative responses and exaggerated autoimmune diathesis of IFN-γ/IFN-γ receptor GKO mice may be related to the demonstrated potential of IFN-γ to down-modulate immune responsiveness and/or induce apoptosis. It also seems conceivable that the documented potential of anti-IL-12 antibody to exacerbate cardiac allograft rejec-

tion may relate, at least in part, to some of the issues addressed in this section but see also (1054).

To note, in closing, Tilney and coworkers have developed attractive data to suggest that cells of the monocyte-macrophage lineage may be recruited to the graft bed by a variety of factors (chemokines), some of which may be produced by parenchymal elements of the graft, contributing to tissue histopathology in states of chronic rejection (915–917).

IV. TRANSPLANTATION TOLERANCE

Protracted or permanent survival of vascularized organ allografts and xenografts has recently been achieved in small and large animals by targeting pathways presumed to contribute to the genesis of self-tolerance and immunological privilege, thus affording considerable promise that transplantation tolerance will become a clinical reality at the dawn of the next millennium (1–7). This chapter has attempted to cover, in a comprehensive manner, recent advances relating to the physiology of lymphocyte activation potentially bearing on the induction of conditioned unresponsiveness.

Our perspective is that transplantation tolerance is obtained through simultaneous or sequential induction of T-cell anergy, apoptosis, and immune deviation. The following discussion addresses the sources of greatest current controversy in this arena. Thereafter, molecular mechanisms known or presumed to mediate specific unresponsiveness in preclinical solid organ transplant models are discussed in the context of present knowledge of the biology of lymphocyte activation.

A. Apoptosis and the Induction of Peripheral Tolerance

Prior documentation by Kappler, Marrak, and others demonstrating elimination of single and double positive thymocytes bearing T-cell antigen receptors conferring reactivity to "self-antigens" provides a powerful impetus to consider clonal deletion as a key mechanism underlying the induction and maintenance of peripheral tolerance (28,918–922). Indeed, evidence has accumulated to indicate that Fas/FasL- and TNF-dependent mechanisms mediate apoptosis of perhaps the majority of cells activated in response to antigen- or superantigen-challenge (60,923). Evidence was reported to show that FasL-dependent mechanisms might be an important cause of immunological privilege in the eye and testis, and potentially in other sites (924). However, as reviewed recently by Green and Ware (925), substantial controversy has recently appeared in this field. A considerable number of recent contributions to the literature indicate that local expression of Fas ligand is in fact a cause of localized autoimmune injury, such as thyroiditis and multiple sclerosis (926–928).

Data have accrued to indicate that apoptosis may be an important mechanism underlying the genesis of transplantation tolerance (929). A particularly attractive demonstration of apoptosis in action is found in spontaneous acceptance of liver allografts in the mouse (930,931). Particular antigen presenting cells may have special properties that enable them to kill antigen-reactive T cells (932), consistent with evidence collected by Thomson and coworkers to show that some populations of cultured dendritic cells are deficient or express only low levels of the B7 family of molecules (933–936) and the parallel demonstration by the same group that interruption of the B7-CD28 axis can exaggerate TcR-triggered apoptosis induced by exposure to competent APCs (937). Notwithstanding existing controversies in the field, clonal deletion will likely be seen as an important contributing factor in the genesis of peripheral tolerance. Given the exaggerated sensitivity of anergic cells to antigen-induced cell death, interruption of apoptosis may be required to fully gauge the contributions of other pathways potentially implicated in the induction of peripheral tolerance (938).

B. T-Cell Anergy and the Induction of Peripheral Tolerance

An enormous body of literature has now accrued on the subject of T-cell anergy as a mechanism of peripheral tolerance (939). Moreover, there are also very good data to indicate that induction of T-cell anergy is a pivotal mechanism underlying induction of transplantation tolerance. However, much of the evidence is indirect. It has been difficult to construct appropriate model systems to definitively identify anergy as a cause of protracted allograft survival in tolerant hosts. Accordingly, some of the data implicating anergy as a cause of transplantation tolerance are obtained from knowledge of the strategy employed in inducing tolerance. Antecedent in vitro studies have substantially implicated anergy as a cause of sustained, specific unresponsiveness when T cells encounter antigen in the absence of B7 costimulation (63,940).

Anergy has been shown or been presumed to contribute to the genesis of peripheral tolerance in bone marrow transplanted mice prepared with a nonmyeloablative regimen (941). However, there is some evidence to suggest that cytokines (i.e., IL-10) may contribute to the induction of T-cell anergy in this setting (942). Anergy has also been implicated as one of the mechanisms underlying tolerance induction following anti-CD4 (and/or anti-CD8) monoclonal antibody administration (943,944), interruption of single or multiple costimulatory receptors (62,116,609), oral feeding (945–947), or donor-specific transfusion (948).

In some of the models cited above, however, it may be difficult to exclude the potential involvement of Th2-mediated suppression in the genesis or maintenance of specific unresponsiveness. Nonetheless, recent work by Lakkis demonstrating protracted if not indefinite survival of mouse heart allografts in CTLA4-Ig treated treated IL-4 deficient (GKO) hosts substantially confirms that isolated T-cell anergy may be sufficient (99). The IL-4–deficient mice used by Lakkis and coworkers were also notably deficient in their ability to make other Th2 cytokines (e.g., IL-5 and IL-13).

C. Immune Deviation or Suppression in Transplantation Tolerance

Specific suppression has commonly been demonstrated in experimental transplant models and affords an attractive explanation for the pathogenesis of transplantation tolerance, based in part on the presumed need for mechanisms to hold in check potential donor reactive cells among recent thymic migrants (949–952). With the discovery that functional helper cell subsets Th1 and Th2 released cytokines with the potential to inhibit activation or clonal expansion of the other cell type, it became attractive to suppose that Th1 and Th2 might serve as antigen-specific suppressor cells in vivo to regulate expression of the reciprocal immune response phenotype (953). In the ensuing years, the Th1/Th2 paradigm has withstood an avalanche of focused, rigorous scientific scrutiny. Work in a number of experimental models has identified evidence for a Th1 (Th0) shift to Th2 in immune response phenotype in association with the induction of transplantation tolerance at the level of the graft bed or the draining lymph nodes. Such models include the following: classic neonatal tolerance (954–956), tolerance induction by total lymphoid irradiation (957,958), anti-CD4 mAb pretreatment (959,960), CD28-B7 blockade (961), donor-specific transfusion (DST) (58), and others. In the instance of transplantation tolerance induced by donor specific transfusion (DST) in a mouse heart transplant model, work performed by Takeuchi and Konieczny in the laboratory of one of the authors (RPL) reveals early downregulation of prototypic Th1 cytokines in the graft bed as well as sustained in situ expression of IL-4 and IL-10 (Fig. 13).

In reviewing these data, however, it should be noted that this field of inquiry is

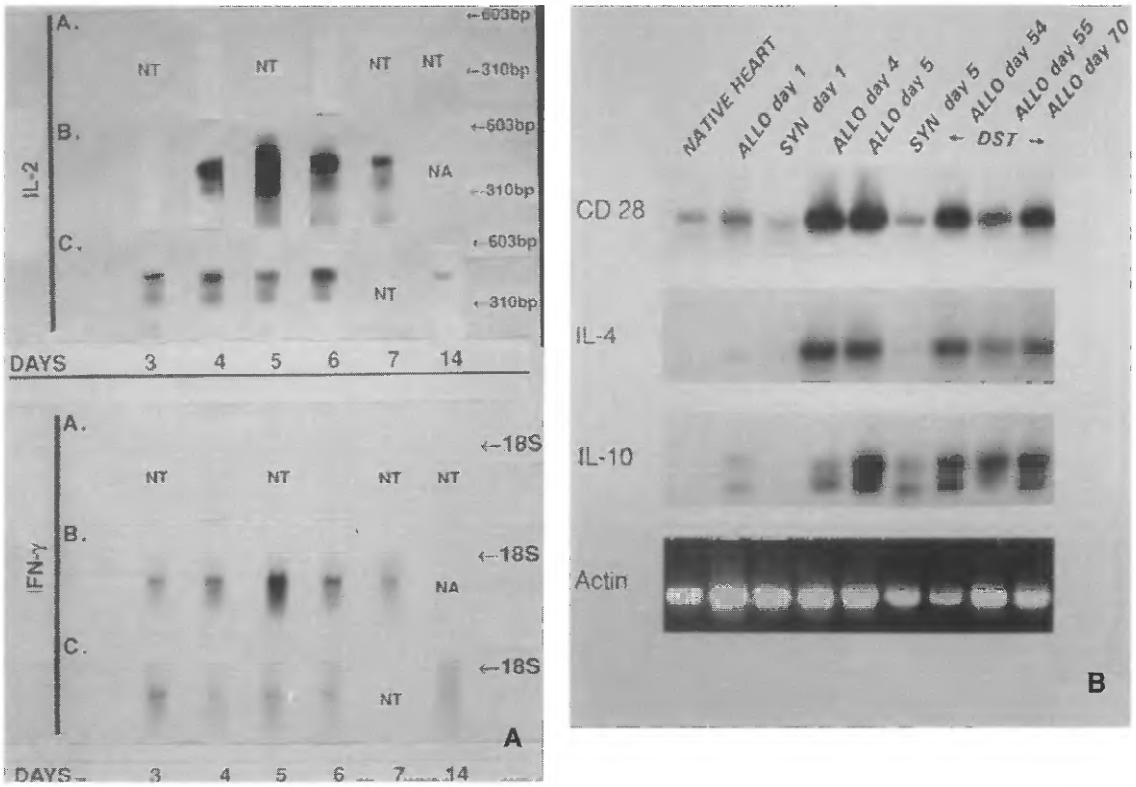

Figure 13 **A,** The time course of expression of message for prototypic Th1 cytokines IL-2 and IFN-γ, as assessed by RT-PCR and Northern analysis, respectively, in syngeneic mouse heart isografts (C3H-C3H) as well as allografts (C57BL/10-C3H), in unmodified hosts as well as hosts rendered tolerant by antecedent donor-specific transfusion (Adapted from Ref. 58.) **B,** Induced expression and persistence of message for prototypic Th2 cytokines IL-4 and IL-10, determined by RT-PCR, in syngeneic mouse heart isografts (C3H-C3H) as well as allografts (C57BL/10 - C3H), in unmodified hosts as well as hosts rendered tolerant by antecedent donor-specific transfusion at the posttransplant intervals specified. Expression of CD28 and actin transcripts were assessed in parallel by RT-PCR as a measure of T-cell infiltration and equivalency of template RNA (Konieczny and Lowry, unpublished data evolving from work pioneered by Takeuchi [58]).

extremely controversial. Nonetheless, we have been encouraged to proceed by recent contributions to the literature that further define the phenotype and function of Th1- and Th2-like effectors (962) and seek to address contemporary controversies in the field.

V. CURRENT CONTROVERSIES SURROUNDING THE GENESIS OF TRANSPLANT TOLERANCE

A series of recent contributions to the literature have raised important questions relating to the genesis of transplantation tolerance that have provoked a great deal of thought and inspired further research.

A. Interleukin-12

Neutralization of IL-12 in a solid organ mouse heart transplant model induced a Th2 shift in the profiles of cytokine mRNA expressed in the graft and marginally but significantly accelerated the tempo of graft rejection (96). In contrast, IL-12 has been identified as a critical mediator of GVHD in mice (963), and, in this setting, neutralization of IL-12 has been shown to modulate disease while simultaneously inducing a Th1 to Th2 shift in immune response phenotype (964). The authors of the latter study were, however, keenly aware that GVHD in other strain combinations may be a Th2-mediated disease. Indeed, in their own work, in just such a combination, anti–IL-12 therapy was associated with induction of autoantibodies that appeared to be without harmful effect. However, even though there is some conflict between different model systems, it is important to ask how graft rejection might be accelerated by neutralization of IL-12 without reference to any model system or paradigm. New data have accrued to indicate that IL-12 may serve to downregulate the late phases of an immune response (965). Additionally, the proinflammatory effects documented in the studies cited above that were obtained with neutralization of IL-12 or INF-γ might relate to a resultant increase in TGF-β production, as has previously been observed in the instance of antigen feeding (966). Finally, new data show IL-12 p40 reduces Th1 responses (1054).

Thus, there is substantial data to suggest that resultant TGF-β should be antiinflammatory or immunosuppressive, based on work in TGF-β GKO mice (967) and evidence to show that TGF-β has potential to inhibit macrophage activation (968), modulate nitric oxide production (969), downregulate the activity of Th1-like effectors (970,971), suppress IFN-γ–mediated induction of MHC class II (972,973), and reduce expression of adhesion molecules (ICAM-1) (974), and induce apoptosis or cell cycle arrest of T cells (975,976).

TGF-β is also reported to inhibit IL-2R signaling (478), and recently accrued data indicate that IL-2R signaling is the sine qua non for AICD of restimulated cells (47), a phenomenon initially described by Lenardo et al. (46) (see Fig. 11). Indeed, there are experimental data to indicate that Fas- and antigen-induced apoptosis are reduced in T cells preactivated in the presence of TGF-β (977). Nonetheless, there are also data to show that TGF-β has the potential to exacerbate apoptosis under particular conditions (975). Indeed, overall, it must be stated that the effector functions of TGF-β are ambiguous, and that TGF-β has been identified as both a cause and a cure of inflammation (978).

B. Interleukin-4

Data have accrued to indicate that genetic susceptibility to particular autoimmune diseases appears to involve more than just a predisposition to generate a Th1-like or a Th2-like

response (97,98). Moreover, studies in IL-4 transgenic mice, and mice rendered deficient in IL-4 by homologous recombination, demonstrate no correlation between the presence or absence of IL-4 and the tempo of acute rejection by immunocompetent recipients or the genesis of protracted allograft survival in conditioned hosts, respectively (99,100,922). Evidence to document that transplantation tolerance may be obtained in the absence of IL-4 or expression of other prototypic Th2 cytokines is not inconsistent with the thesis that transplantation tolerance in such systems results exclusively from anergy and/or clonal deletion. However, data have accrued to indicate that even states of profound unresponsiveness may be the result of cell–cell interactions masquerading as anergy. Thus, a recent report indicates that $CD4^+$ $TcR^{Transgene+}$ T cells from pigeon cytochrome C-specific $V\alpha11^+V\beta3^+$TcR transgenic "AND" mice inoculated with superantigen, SEB become profoundly anergic, failing to proliferate or secrete cytokines in response to antigen-receptor cross-linking (979). Surprisingly, splenocytes from SEB-injected mice were also shown to inhibit mixed lymphocyte cultures in coculture experiments. Finally, manifestations of T-cell anergy obtained following in vivo injection of SEB in this model substantially resolved when $CD4^+$ splenic T cells were highly purified. Together, these data implicate a suppressor cell as a cause of functional anergy in the T-cell compartment of SEB-injected mice. Data suggest that suppression in this model is IFN-γ dependent and effected by an as yet poorly characterized $CD4^-CD8^-$ cell potentially analogous to a DN $TcR\alpha\beta^+$ $CD3^+$ T-cell precursor of putative thymic origin. This line of research is particularly intriguing because it affords the potential for the identification of an alternate previously undiscovered pathway of antigen-specific dominant immunosuppression that might be amenable to experimental/clinical manipulation.

C. Tissue Injury by Th2 Cells

Neither induced nor constitutive expression of prototypic Th2 cytokines is necessarily benign (perphaps particularly in *scid* mice [95,103]). For example, constitutive expression of IL-4 induces autoimmune disorders in mice (101). Moreover, in vitro differentiated autoantigen-specific Th2-like effectors have been shown to induce robust autoimmune disease following adoptive transfer to *immunodeficient* hosts and failed to modulate disease caused by Th1 effectors (103,104). The results of these studies are remarkably similar to those obtained by Orosz and colleagues, who recently reported that in vivo transfer of in vitro differentiated donor-specific Th1- or Th2-like cells into immunoincompetent *scid* recipients of vascularized cardiac allografts resulted in equivalent prompt rejection of the allografts (95). It is perhaps remarkable, therefore, that, in the above mentioned autoimmune disease models, only in vitro differentiated Th1 cells induced disease upon adoptive transfer to normal mice. Conversely, the induction of disease by autoantigen-reactive Th2 in *immunocompromised* hosts was ascribed simply to the heightened activity of the innate immune system in the setting of immunodeficiency. One particularly appealing hypothesis is that normal resting lymphocytes in immunocompetent mice serve as a decoy for IL-4 since they express BCL-6 which serves to downregulate IL-4 receptor (STAT6) signaling (104). Finally, it should be noted that the failure of autoantigen-specific Th2-like effectors to modulate the disease, inducing potential of autoantigen-specific Th1-like effectors in the two autoimmune disease models mentioned above, contrasts with the demonstrated immunosuppressive potential of autoantigen-reactive Th2 in other model systems (80). It might also be argued that Th2-like effectors would be expected to be more effective in sup-

pressing the induction rather than the effector phase of an immune response based on evidence that IL-4 inhibits generation of Th1 from naive precursors (980).

D. Potential Avenues for Resolution of Existing Controversies

It is unclear how existing controversies surrounding the disparate roles of functional lymphocyte subpopulations Th1 and Th2 will ultimately be resolved. Other researchers have concluded discussions of the potential importance of Th1 and Th2 in allograft rejection and transplantation tolerance with the comment that "it's simply not that simple" (981). One eventuality, therefore, is that a more complex paradigm will evolve, one that would provide, an explanation for the parallel demonstrations that IL-4 deficient mice resist leishmania infection while innate and acquired immunity to the same organism is increased in IL-10–deficient hosts (982–984). However, speculation on the exact nature of that paradigm is likely premature, for the following reasons:

1. First, we are reminded that the Th1/Th2 paradigm evolved directly from the first comprehensive application of recombinant technologies and new knowledge of lymphokine biology to the study of the phenotypes and functions of a random assortment of cloned T-cell lines, thereby reaping the benefits of decades of achievement in immunology research. It might be said, therefore, that "Th1" and "Th2" first appeared in the literature as a rational initial attempt at sorting out a new type of data, and subsequent popularity of the concept evolved from the broad success of the paradigm.

2. Second, the recent identification of novel pathways impacting cytokine receptor signaling events afford fresh vistas for sorting out the biology of functional lymphocyte subpopulations. Thus, recent contributions to the literature (985–987), coupled with an isolated historical report (988), define a new family of proteins, variously identified as CIS, SOCS 1–4, JAB, and SSI, that serve to mediate feedback (STAT inducible) regulation of cytokine receptor signaling. Generally, it appears that this occurs by direct SH2-dependent binding to Jak proteins 1–3 and consequent inhibitory interactions between the SOCS domain with the JAK kinase domain. CIS (Cytokine [STAT5] inducible SH2-containing protein) may be an important exception because it has been shown to bind directly to the tyrosine phosphorylated β-chain of the IL-3 receptor as well as the erythropoietin receptor (988,989).

Furthermore, protein tyrosine phosphates are recognized currently as critical regulators of signal transduction in the immune system, and several important findings in this domain are presented in later sections (628,654,859,990–996). Recent publications have implicated other SH2-containing proteins, the SH2-containing tyrosine phosphatases (e.g., SHP-1 [otherwise called HCP, PTP-1C] and SHP-2 [PTP-1D]) as important regulators of the signaling properties of several individual cytokine receptors. Ihle, a coauthor of the report of the initial discovery that mutations in the murine motheaten locus were within the gene encoding the hemaotopoietic cell protein-tyrosine phosphatase (HCP, PTPC1) that contains two SH2 domains (997), subsequently generated data to show that HCP was also associated with the IL-3β chain. Interestingly, increasing levels of HCP obtained in target cells by overexpression of the transfected gene were found to cause quantitative reduction in IL-3R signaling (998). Since that time, SH2-containing phosphatases (SHPs) have been discovered in association with other cytokine receptors or shown to modulate their function (999) (e.g., the erythropoietin receptor [1000]). In the latter instance, PTP1-C has been shown to inactivate Jak2 and terminate erythropoietin-induced proliferative responses

(1001,1002). Conversely, SHP-2 (PTP2) has been shown to mediate positive downstream signaling events below Jak2 (1003). Recently accrued indirect evidence suggests that SHPs may also be important downstream targets of the IL-2R (1004), may associate directly or indirectly with the IL-2 receptor to mediate vanadate-inhibitable TGF-β induced abrogation of Jak1 phosphorylation and STAT5 signaling in response to IL-2R occupancy (478), or modulate IL-2R signaling directly through association with STAT5. These data corroborate and extend earlier work demonstrating that cytokine receptor signaling is substantially gated at multiple levels, to include early or late activation-dependent induction of component chains of the receptor (i.e., IL-2 versus IL-12) as well as receptor downregulation (e.g., IL-12) associated with assumption of the mature Th2-cell phenotype (1005). Clearly, there is a great deal more work to be done, and the unexpected results of individual experiments must be sorted out one by one.

A great deal has been learned in recent years about other basic immunological mechanisms, including anergy, apoptosis, and veto, to name a few, that are now known or presumed to contribute to the genesis of self-tolerance and that undoubtedly contribute to the genesis of transplantation tolerance. Accordingly, the central perspective explored in this chapter is that anergy, apoptosis, and immune deviation function in concert or in sequence to mediate the regulated expression of alloimmunity during the induction and maintenance phases of transplantation tolerance. Several factors are identified that might potentially have clouded the in vivo demonstration of the coexpression or sequential involvement of particular processes in many well-studied tolerance models (114). First, it is apparent that highly sophisticated models are required to identify in vivo induction of T-cell anergy and/or clonal deletion in experimental organ transplantation. Experimental studies designed to follow clonotypic T-cell expansion and/or the simultaneous or sequential induction of T-cell anergy or clonal reduction through apoptosis have generally necessitated the use of mice bearing TcR transgenes for highly defined antigenic specificities, as well as clonotype-specific monoclonals to follow T-cell fate. Given that relatively few such studies have been performed in realistic transplant models, it has generally been necessary to draw inferences about the mechanisms underlying the genesis of tolerance induction in particular model systems based on an examination of demonstrable residual immune reactivities in tolerant hosts (e.g., MLR proliferative responses, CTL generation, percursor frequencies, or expression of cytokine message/cytokine biosynthesis). Alternate strategies that are beginning to provide useful data include the use of mice bearing particular deficiencies that preclude their ability to undergo AICD or generate particular cytokines (e.g., IL-4, IL-10 GKO mice). However, the conclusions reached about mechanisms of tolerance induction in individual model systems are often inferential and based on one's best guess as to the origins of data observed. Several factors might potentially cloud the in vivo demonstration of the coexistence or sequential involvement of particular tolerogenic mechanisms in certain well-studied models sytems (577):

1. First, expression of the Th2 phenotype may be limited in states of profound T-cell anergy and accompanying clonal reduction through antigen-induced cell death, based on evidence to show that IL-2 is required for IL-4 biosynthesis. This apparent but as yet poorly documented requirement for IL-2 for production of IL-4 was first documented by Seder et al. (441) and was recently confirmed in two independent reports involving work with IL-2Rα chain gene knockout (GKO) mice (47,48). A recent report by Lanoue et al. (1006) identified clonal deletion and anergy as the cause of specific unresponsiveness among influenza hemmagglutinin-(HA) $CD4^+$ T cells from TcR transgenic mice exposed to antigen through adoptive transfer to syngeneic mice bearing an HA transgene. At early

time periods after adoptive transfer, $TcR^{transgene+}$ $CD4^+$ T cells produced IL-4 as well as IFN-γ. Conversely, residual TcR transgene-bearing CD4 T cells recovered at late intervals demonstrated proliferative unresponsive and failed to produce IL-4 upon TcR cross-linking. Formal proof that donor-specific Th2-like effectors might accrue in the graft bed or elsewhere in tolerant allograft recipients, without inducing in situ expression of prototypic Th2 cytokines, resulting from a local deficiency in IL-2 biosynthesis, is lacking. Nonetheless, the paradigm recounted above offers a potential explanation for the recent demonstration that splenic T cells from tolerant allograft recipients lack in situ expression of prototypic Th2 cytokines in their allografts yet serve to mediate tolerance transfer and induce expression of prototypic Th2 cytokines in grafts placed in naive test recipients (1007). This assumption is substantially corroborated by published work of Waldmann and colleagues who found that the administration of neutralizing anti–IL-4 antibodies inhibit transfer of infectious tolerance in their model system (92).

2. Second, expression of prototypic Th2 cytokines in situ or in local lymph node might commonly be missed if the process of immune deviation and associated differential expansion of donor alloantigen-specific Th2-like effectors was a late event following tolerance induction in many of the experimental transplant models presently being studied. Thus, Bruce Hall and others were routinely able to demonstrate transferrable suppression with $CD4^+$ spleen cells recovered day 50 or beyond (and less reliably at earlier time points) from heart allograft recipients rendered tolerant by passive enhancement or a brief course of cyclosporine A by adoptive transfer to test graft recipients (949,950,1008). Recent contributions to the literature provide broad hints as to the potential sources of delay in the appearance of antigen-specific suppressor cells. Thus, whereas it has been popular to consider that AICD might be an important independent cause of peripheral tolerance (as potentially obtains in the instance of central [thymic] tolerance) (66,475,887,922,938,1009–1014), recent literature indicates that peripheral Th2-like effectors may be uniquely resistant to antigen-induced apoptosis (479,480,1015). To the extent that donor-reactive T cells expressing a Th2-like phenotype represent but a small fraction of effectors maturing at early time periods following organ transplantation under tolerogenic conditions, some time lapse might be required until the Th2 phenotype could achieve ascendency if this necessitated antigen-induced apoptosis of donor-reactive T cells with alternate phenotypes.

VI. IMMUNOMODULATION BY SOLUBLE ALLOANTIGEN

Because much of this chapter has focused on lymphocyte activation and signal transduction pathways potentially implicated in the genesis of T-cell anergy, we have chosen to conclude with a brief description of an integrated piece of work in the laboratory of one of the authors (EG) relating to tolerance induction by soluble alloantigen. This work provides a useful perspective on the range of immunological mechanisms that may contribute to the genesis of transplantation tolerance.

Donor-specific immunosuppression is an exciting avenue to achieve immunosuppression in transplant recipients, in that currently used immunosuppressive drugs lack specificity for donor tissue, and therefore must be designed to downregulate the immune system against all foreign antigen. In addition, drug therapy has not proved to be effective at preventing chronic rejection. One approach to achieve donor-specific immunosuppression could involve the pretreatment of organ recipients with sources of soluble donor MHC antigen. In the following discussion, data concerning the potential for soluble donor MHC

to induce immunological tolerance are reviewed, along with possible mechanisms of action.

Solubilized antigenic molecules have long been recognized as having potential immunosuppressive properties. In 1961, Shapiro et al. (1016) induced permanent skin graft acceptance in mice by administering large doses of solubilized donor antigen before transplantation. Gowland (1017) later reproduced this effect in other animal strain combinations. Further studies suggest that various MHC antigen preparations might be important for inducing antigen-specific immunosuppression (1018,1019). An important study by Sumimoto and Kamada (1020) suggested that MHC class I antigen from serum complexed with allospecific antibody could substantially extend heart allograft survival in a rat model. In contrast, other researchers have not been able to demonstrate an immunosuppressive effect with injection of purified soluble MHC class I antigen (1021,1022). The dose and ability to continuously infuse the antigen appear to be critical factors in these studies to induce donor-specific unresponsiveness. However, because cells and semipurified cellular extracts have been the principal source of MHC antigen in these studies, it has been difficult to determine exactly which MHC antigen forms are best able to induce donor-specific unresponsiveness. An additional problem is that injections of crude preparations (blood cells, tissue extracts) containing primarily membrane-associated donor MHC class I antigen carry a risk of sensitization, precluding the use of this type of therapy for clinical transplantation—with the exception of pretransplant blood transfusions (1019).

New experimental molecular biology approaches are beginning to more clearly demonstrate a potential for donor MHC alloantigen as immunological downregulators. For instance, Ferber et al. (1023) have shown that antigen-specific immunosuppression can be achieved in transgenic mice by induction of allo-MHC class I antigen expression on hepatocytes. In these animals, the TCR on self-reactive $CD8^+$ cells was downregulated in a dose-dependent manner. In a different model, in which transgenic mice express a foreign MHC class I antigen in a secreted form, animals have demonstrated normal allospecific CTL and antibody responses (1024). However, a small population of T cells were rendered tolerant in this model when the alloantigen was indirectly presented in the context of self-MHC antigen. Other researchers have made recent contributions in this respect using similar technological advances. Madsen and coworkers have used DNA-mediated gene transfer to recipient blood cells to determine the relative contribution of donor-derived MHC class I and class II antigen in the induction of donor-specific unresponsiveness (1025). Furthermore, a genetically engineered secreted H-$2K^b$/IgG fusion molecule, consisting of the extracellular domains of a murine class I polypeptide H-$2K^b$, has been shown to have potent immunosuppressive properties (1026). This chimeric protein specifically inhibits CTL in vitro at nanomolar concentrations.

We have used two gene transfer strategies to study the immunological effect of different forms of MHC class I antigen. Studies in which we expressed allo-MHC class I antigen in skeletal muscle, by direct DNA injection into muscle tissue, suggested that soluble forms of the antigen are less likely to cause immunological sensitization, compared to membrane-bound forms of the same antigen (1027).

More recently, we have developed a gene transfer protocol in the rat to study the immunological effects of soluble versus membrane-bound allo-MHC antigen in an organ transplantation model. In this model, primary cultured Lewis ($RT1.A^1$) hepatocytes are transfected by lipofection with plasmids encoding allogeneic membrane-bound or secreted $RT1A^a$. Rats injected via the portal vein with the genetically altered hepatocytes secreting alloantigen showed extended ACI ($RT1^a$) liver allograft survival and decreased CTL activ-

ity (1028). Consistent with these observations, allo-specific CTL were also inhibited in vitro when exposed to transfected hepatocytes secreting allo-MHC class I antigen (unpublished data). In contrast, the same antigen in a membrane-bound form primes CTL in vivo and in vitro, and accelerates ACI allograft rejection. Further studies in our laboratory using portal vein injection of transfected hepatocytes, suggest that soluble donor MHC antigen is capable of reducing the sensitizing effect of membrane-bound alloantigen, as demonstrated by the ability of soluble alloantigen to abrogate acceleration of liver allograft rejection induced by the membrane-bound form of the same antigen (unpublished data). Therefore, we have been able to use a well-defined system to provide evidence that soluble MHC class I molecules may have immunosuppressive properties that can be used to promote allograft survival in an organ transplantation situation.

Although the exact mechanism by which soluble alloantigen downregulates the immune response is unknown, there is evidence for several potential modes of action. For instance, Zavazava and coworkers have recently shown soluble allo-MHC antigen can inhibit allospecific CTL cell lines in vitro (1029), and more recently they have provided data indicating that the CTL inhibition is mediated by CD95/CD95-ligand-mediated apoptosis (1030). It is also possible that secreted MHC class I molecules directly interact with the allospecific CTL receptor, thereby blocking CTL killing of target cells. Another mechanism of inhibition, especially with regard to CTL, could relate to the binding of soluble allo-MHC molecules to nonspecific (but relevant) cell surface molecules, such as CD8 and the IL-2 receptor. Reports of these types of interactions were recently summarized by Buelow et al. (1031). There is also evidence that HTL responses that promote sensitization can be inhibited by soluble molecules. In this regard, soluble non-MHC molecules can downregulate IL-2 production by Th1 cells and have also been shown to be capable of expanding potentially downregulatory HTL that secrete cytokines including IL-4 and IL-10 (Th2 cells) (1032,1033). This type of immune deviation may be related to the processing and presentation of soluble antigen by APC not expressing costimulatory activity (1036), such as resting B cells. A recent study by Zhong et al. (1035) support the theory that antigen-unspecific B cells are fully capable of processing intravenously injected soluble alloantigen.

There is also evidence suggesting that suppressor-type cells may be generated following exposure to soluble alloantigen. More specifically, suppressive-type T cells have been described following exposure to non-MHC soluble alloantigens (1036), and development of suppressor cells has been demonstrated following transplantation of the liver, which is an organ that produces large amounts of soluble MHC class I antigen (1037). The mechanism of suppressor cell development is unknown, but it is possible that soluble MHC class I antigen could aggregate to form multivalent complexes that lead to antigen-specific unresponsiveness by cross-linking allospecific T-cell receptors, without delivery of costimulation (1034).

Finally, there is evidence to suggest that soluble alloantigen is capable of inhibiting the humoral immune response. Soluble non-MHC antigen has been shown to dramatically reduce the number of alloreactive memory B cells (1038) and to impede antibody affinity maturation (1039). Grumet et al. (1040) have shown that soluble MHC class I molecules can have a similar effect, specifically suppressing the humoral alloimmune response. The mechanisms responsible for the immunosuppressive effects of soluble antigen on B cells is not completely understood. However, there is evidence that the B-cell inhibition is related to the formation of antigen–antibody complexes, particularly where there is an excess of antibody. It has been hypothesized that the situation of antibody excess in the presence of antigen may result in cross-linking of a critical number of F_c and antigen-specific receptors

on the B cell, resulting in transduction of a negative intracellular signal. Alternatively, Nossal et al. (1038) have suggested that soluble antigen may negate T-cell activation, thereby depriving B-cell development of critical cytokines.

In conclusion, the mechanisms by which soluble alloantigen can potentially induce antigen-specific unresponsiveness are likely very complex and require further investigation.

VII. CONCLUSIONS

Recent documentation that combined blockade of the CD40L-CD40, CD28-B7 costimulatory axes serves to induce protracted survival of skin and/or solid organ grafts in large and small animals has raised hopes and expectations that similar strategies may be applied in clinics soon. We have addressed a limited subset of present knowledge that might support a future quest for an effective strategy for inducing specific unresponsiveness in organ transplantation. Although we are somewhat doubtful that any strategy will suffice to induce indefinite organ allograft survival without the benefit of limited concurrent immunosuppression, we are particularly hopeful that the application of useful tolerance induction strategies will allow the doses of immunosuppressive drugs to be greatly curtailed. Such an approach, we suggest, holds the promise of improved allograft survival and a lesser risk of life-threatening complications. Great care will be required, however, to prevent coninduction of tolerance of infectious organisms [see (1055), reviewed (79)].

ACKNOWLEDGMENTS

This work was supported in part by research grants, including an NIH award to RPL (AI30732-06) and an NIH award to EG (AI139741-01). The authors are also obliged to the the editors for their patience and encouragement, and to their colleagues and their families for their forebearance as the work proceeded.

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11

The Pathology of Kidney Transplantation

LORRAINE C. RACUSEN
The Johns Hopkins University School of Medicine, Baltimore, Maryland

KIM SOLEZ
The University of Alberta, Edmonton, Alberta, Canada

STEEN OLSEN
University Institute of Pathology, Municipal Hospital, and Kommune Hospital, Aarhus, Denmark

I. INTRODUCTION

Evaluation of the renal allograft following transplantation is often difficult, and the allograft biopsy may play a critical role in the posttransplant management of the renal allograft patient. Until recently, however, there had been no standardized approach to histopathological evaluation of renal allograft biopsies or to grading of severity of rejection-related changes in those biopsies. An international schema for histological evaluation and grading of rejection-related changes in renal allograft biopsies was drafted in Banff, Canada, and this Banff schema was published in August, 1993 (1); this working formulation has subsequently been modified based on a meeting in Banff in 1995 (2). The schema is currently in use for central slide review of allograft biopsies in a number of large international trials of immunosuppressive agents, as well as in many medical centers worldwide. In this chapter, the classification system is reviewed and reproducibility results and clinical validation of the schema are discussed. The most recent modification of the schema, the Banff 1997 working formulation, is also described. Additional histological features that may prove useful in graft assessment are also discussed, as is the chronic allograft damage index (CADI), which has been applied to evaluation of later allograft biopsies. Other non–rejection-related pathological findings seen in renal allografts are described as well. The chapter concludes with a discussion of future directions in pathological evaluation of the renal allograft.

II. THE BANFF SCHEMA

A. Specimen Adequacy and Processing

Definition of specimen adequacy and recommendations for processing of specimens were put forward in the initial description of the Banff schema. A reasonable sample of cortex should be obtained to grade or rule out rejection with a significant degree of certainty. Therefore, an adequate sample is defined as one containing seven or more glomeruli and at least one artery. Specimens with between one and six glomeruli and at least one artery would be marginally adequate. If there are no glomeruli or no arteries in the biopsy specimen, the specimen is inadequate to rule out rejection or to adequately grade an ongoing rejection process, although it may be possible to establish a diagnosis of rejection. The number of glomeruli and arteries should be indicated.

It is recommended that each specimen be mounted with multiple sections on each of a total of seven slides, three stained with hematoxylin and eosin (H&E), three with periodic acid–Schiff reagent (PAS) and/or silver stains, and one with a trichrome stain. The PAS and/or silver stains are particularly useful for defining tubular basement membranes (TBM) to accurately assess severity of tubulitis, in which intraepithelial inflammatory infiltrates must, by definition, breach the TBM and for evaluating glomerulitis, chronic glomerulopathy, and tubular atrophy. The PAS stain is also useful for rapid identification of atrophic tubules and for identifying hyalinosis lesions in renal arterioles, lesions which are graded in the Banff schema. Trichrome stains aid in assessing chronic fibrosing changes in the interstitium and in vessel walls.

B. Semiquantitative Assessment of Histological Changes—Acute

The criteria for rejection diagnosis and grading of severity of rejection in the Banff schema are based on inflammatory infiltration of tubules or vessel walls. Even though interstitial inflammatory infiltrates are a constant feature in renal allograft rejection, such infiltrates must be regarded as not specific for rejection, because they have not infrequently been demonstrated in protocol biopsies in well-functioning grafts (3–7). This point has been reinforced in recent studies. Moreover, there is no correlation between degree of interstitial inflammation and response to antirejection therapy (8–10). Failure of the extent of inflammatory infiltrate on biopsy to correlate with either renal dysfunction or response to therapy is likely caused, at least in part, by sampling error. The relatively small needle biopsies that are typically obtained from these allografts may lead to overestimation or underestimation of extent of inflammation in the entire graft. Indeed, sampling error has been recognized as a problem since the very early days of allograft biopsy (11). In addition, benign infiltrates may develop in allografts without concomitant overt tissue injury and without clinical dysfunction. A few features of such infiltrates, however, including marked edema and presence of activated lymphocytes, may raise the index of suspicion that there is a rejection in a given biopsy (12); these features are less prominent after steroid therapy.

For semiquantitative assessment of histological changes, each of the major parenchymal components of the kidney—glomeruli, interstitum, tubules, and vessels—is assessed for both acute and chronic changes. Acute and chronic histological findings are graded on a semiquantitative scale. A numerical code is then developed, which, for acute changes, combines g (glomerulus), i (interstitium), t (tubules), and v (vessels) with a numerical score for each. Thus, a completely normal biopsy would be graded g0, i0, t0, v0. Chronic changes are coded similarly, with a "c" prefix for each category, and a numerical score for each. A biopsy lacking any chronic change, therefore, would be graded cg0, ci0, ct0, cv0.

Semiquantitative assessment of histological changes in the Banff schema provides a relatively objective way of recording a shorthand summary of these findings. Regardless of subsequent changes in summary classification of rejection, the criteria for which may evolve over time, or the narrative wording used at individual centers to describe biopsy findings, the numerical scores would remain unaltered. Although coding is not a mandatory feature of the system for general use, it is mandatory for allograft assessment in international and multicenter trials. Some investigators have developed sum scores, based on these numerical semiquantitative grades, which they have shown to correlate with clinical features and outcome.

1. Glomeruli

Acute inflammatory changes may occur in the glomerulus after transplantation, so-called glomerulitis, defined as an increase in number of mononuclear cells in the glomerular capillary lumina, often accompanied by reactive changes and swelling of endothelial cells, a feature that may be striking. Moderate or severe glomerulitis occurs in about 13% of graft biopsies from the early posttransplantation period (13). The light microscopic picture resembles that of acute immune complex glomerulonephritis, but neutrophils are usually not present in the glomeruli and, on electron microscopy and immune fluorescence microscopy, no immunodeposits are seen (13,14). There is some correlation between glomerulitis and acute rejection, but many biopsies with glomerulitis have no rejection and most biopsies with rejection have no glomerulitis (13). Graft function seems not to be influenced by its presence and even severe glomerulitis is compatible with a functioning graft. An adverse effect on graft survival has been presumed (15), but, in our material, graft survival did not seem to be influenced by the presence of glomerulitis per se (13) if acute rejection in the early posttransplantation course was taken into consideration. Transition from endocapillary glomerulitis to chronic glomerular transplant disease has also been suggested (16,17). We did not find such an association (13), but a larger number of patients and a longer observation period are necessary to determine whether glomerulitis has any prognostic impact on graft survival. Early posttransplantation endocapillary glomerulitis may be a particular pattern of rejection caused by other pathogenic mechanisms than conventional rejection, and in particular is seen in antibody-mediated rejection (18). Therefore, it is evaluated but not included as a diagnostic criterion for classic cell-mediated rejection. A summary of criteria for semiquantitative grading of glomerulitis is shown in Table 1. A glomerulus with glomerulitis graded g3 is shown in Figure 1. Immunostaining for T cells can be used to confirm and quantitate the presence of these cells in the glomeruli (see Fig. 1B).

2. Interstitium

Interstitial inflammation is graded by the extent of parenchyma infiltrated by mononuclear cells (Table 2). As discussed, mild to moderate interstitial inflammation must be regarded as not specific for rejection, because such infiltrates may be seen in autografts (19) as well

Table 1 Quantitative Criteria for the Early Type of Allograft Glomerulitis ("g") (0 to 3+)

Accumulation of monocytes and lymphocytes in glomerular capillaries with endothelial swelling
0 = No glomerulitis
1 = Glomerulitis in a minority of glomeruli
2 = Segmental or global glomerulitis in about 25% to 75% of glomeruli
3 = Glomerulitis (mostly global) in all or almost all glomeruli

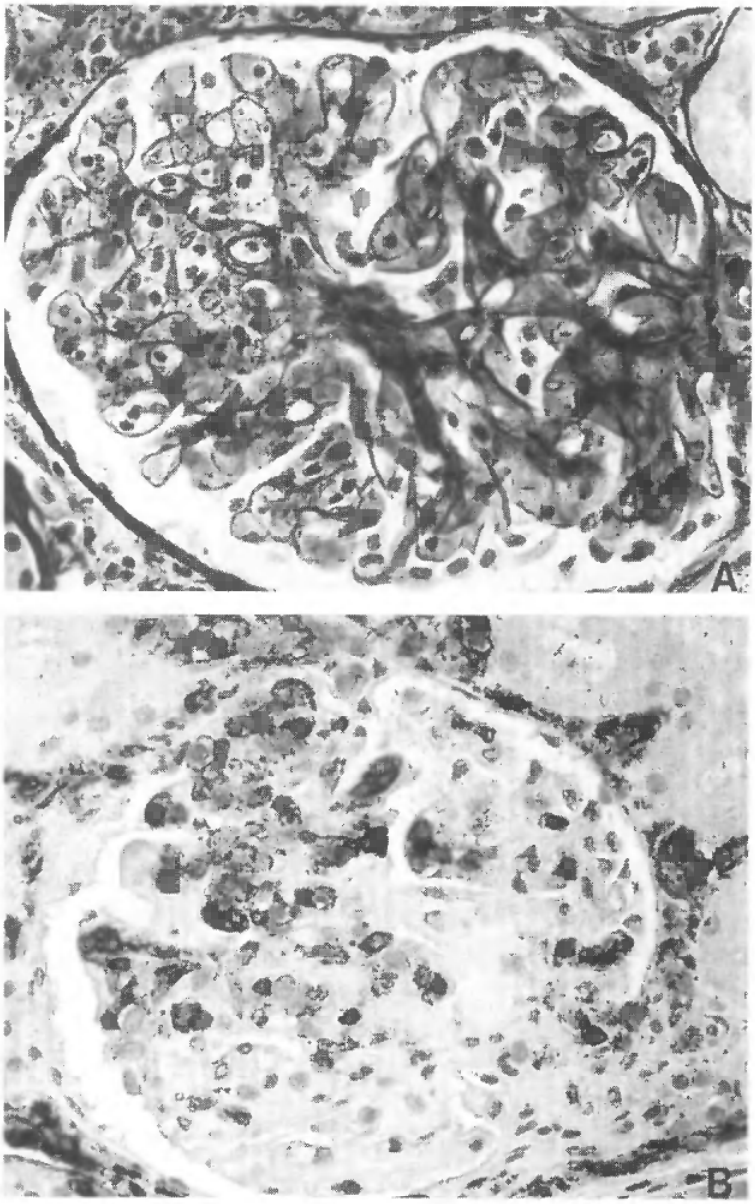

Figure 1 A. Glomerulus with severe glomerulitis (g3). Most capillary loops contain lymphocytes, macrophages, and/or swollen endothelial cells. (PAS; ×400.) B. Immunoperoxidase stain for common leukocyte antigen in the same case, demonstrating numerous leukocytes in the glomerulus and surrounding interstitium. (×400.)

Table 2 Quantitative Criteria for Tubulitis ("t") score (0 to 3+)

0 = No mononuclear cells in tubules
1 = Foci with 1 to 4 cells per tubular cross section or 10 tubular cells
2 = Foci with 5 to 10 cells per tubular cross section
3 = Foci with >10 cells per tubular cross section or t2 with foci of destruction of tubular basement membranes

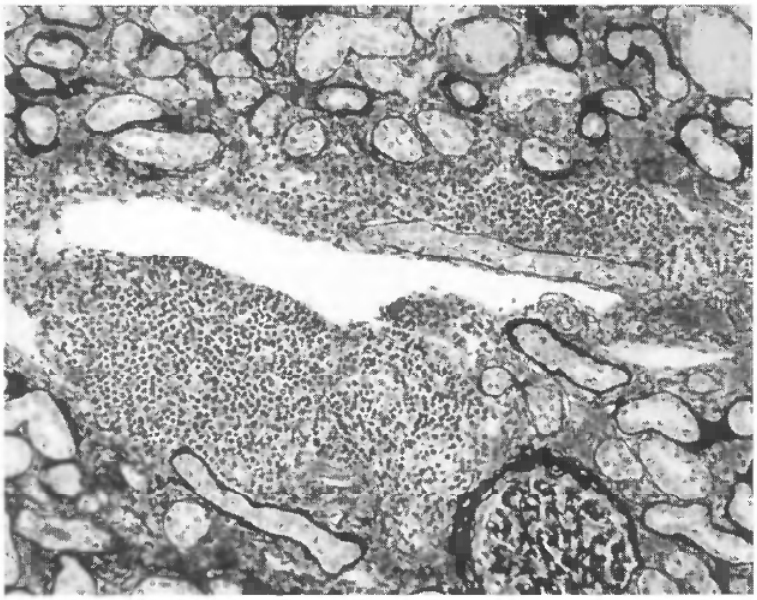

Figure 2 Large perivenous aggregate of bland lymphocytes. (PAS/MS, ×160.)

as in well-functioning transplants (3–7). If severe or diffuse, however, it is certainly suggestive if not diagnostic of rejection. Relatively large perivenous aggregates may be seen in renal allograft biopsies (see Fig. 2). In general, these aggregates do not appear to be as significant as extension of the infiltrate into the renal parenchyma. These large aggregates, as well as inflammatory infiltrates in the subcapsular cortex related to handling and "healing in" of the allograft (see Sec. 1V.B), should not be weighted as heavily in the scoring as infiltrates into renal parenchyma and away from the immediate subcapsular zone.

The mononuclear cellular infiltrate in renal allograft rejection has been evaluated by light and electron microscopy (20) and immunohistochemistry. It typically consists of approximately 50% lymphocytes, 25% macrophages, and 12% plasma cells. Large blast-like lymphocytes as well as plasma cells may be particularly numerous in early rejection and, when present, may be a useful diagnostic feature (11). The majority of cells infiltrating the graft express T-cell markers, generally a mixture of $CD4^+$ and $CD8^+$ cells. There is some evidence for a predominance of CD8 and CD45R0 cells in acute rejection of the renal allograft (21), but the ratio of CD4 to CD8 cells may vary in early versus later acute rejection (22,23). Leu-7–positive natural killer cells and macrophages have also been identified in rejecting renal allografts (24,25); the presence of Leu-7–positive cells infiltrating the tubular epithelium is a reliable marker of rejection (24) (Fig. 3). The presence of B cells in substantial numbers may indicate inflammation related to infection rather than rejection; when associated with clusters of atypical plasma cells, postransplant lymphoproliferative disorder must be ruled out (see Chap. 20). Although not yet formally incorporated into the rejection schema, immunophenotyping is an ancillary technique that may be useful in equivocal cases.

Immunophenotyping of mononuclear infiltrates may eventually prove to be a reliable and widely applicable technique, enabling more precise diagnosis and grading of rejection. A recent study by Strehlau et al. (26) utilized quantitative RT-PCR for quantitative detection of immune activation transcripts for 15 genes, including interleukins (ILs) 2, 4, 7, 8, 10, and 15, perforin, granzyme B, Fas ligand, RANTES, and interferon (IFN)-γ. In a group of 60 transplant biopsies (15 with rejection, 12 with nonrejection causes of dysfunction, 22

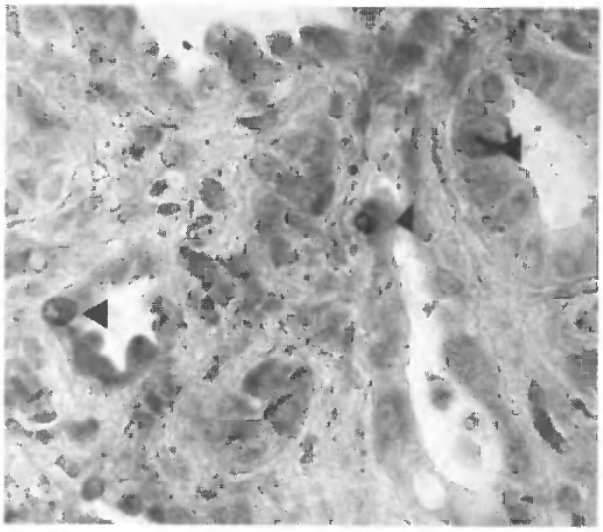

Figure 3 Several Leu-7 + intraepithelial lymphocytes in renal tubules (arrowheads). (Immunoperoxidase stain; ×500.)

surveillance biopsies, and 11 with recurrent disease or other complications), expression of IL-7, IL-10, perforin, granzyme, and Fas ligand were significantly elevated. A simultaneous RT-PCR determination for perforin, granzyme B, and Fas ligand showed 100% sensitivity and 100% specificity for rejection. Increased expression of RANTES and IL-8 genes were sensitive but nonspecific for rejection. If confirmed in larger trials, this approach would lead to rapid and accurate diagnosis of rejection, which may supercede histological rejection diagnosis.

Polymorphonuclear leukocytes and/or eosinophils may be present in the inflammatory infiltrate of acute rejection. When these cells are numerous, the rejection may be aggressive and respond poorly to therapy (27). Numerous polymorphonuclear leukocytes may be seen, typically marginating in glomerular or peritubular capillaries, in cases of antibody-mediated rejection (28,29) and are commonly seen in ABO-incompatible grafts. Infiltrates of polymorphs also occur around recent infarcts and may, therefore, be numerous in cases of antibody-mediated rejection or acute rejection with arteritis and infarction. An interstitial infiltrate with more than occasional polymorphs and/or eosinophils, or those with large numbers of typical or atypical plasma cells are designated with an asterisk in the coding system, for example i2*.

Some degree of interstitial edema almost invariably accompanies the inflammatory infiltrate and is not graded separately, although it may be noted in the narrative description. Steroid therapy rapidly reduces interstitial edema, compromising its usefulness as a diagnostic feature on subsequent biopsies. In centers in which biopsies are performed before antirejection therapy is begun, however, edema may be a useful feature (12), although it is nonspecific.

3. Tubules

The tubules in the renal allograft are a major target of the rejection process. Inflammatory infiltration of the tubular epithelium, or tubulitis, is a typical feature of acute rejection (11, 30–32). Even though mild tubulitis (1–4 lymphocytes per most affected tubules) may occur in allografts with stable function or acute tubular necrosis (4), more severe tubulitis appears to be quite specific for rejection (1). Criteria for semiquantitative grading of tubulitis based on severity of infiltrate in the most involved tubules, are listed in Table 2. Tubulitis is

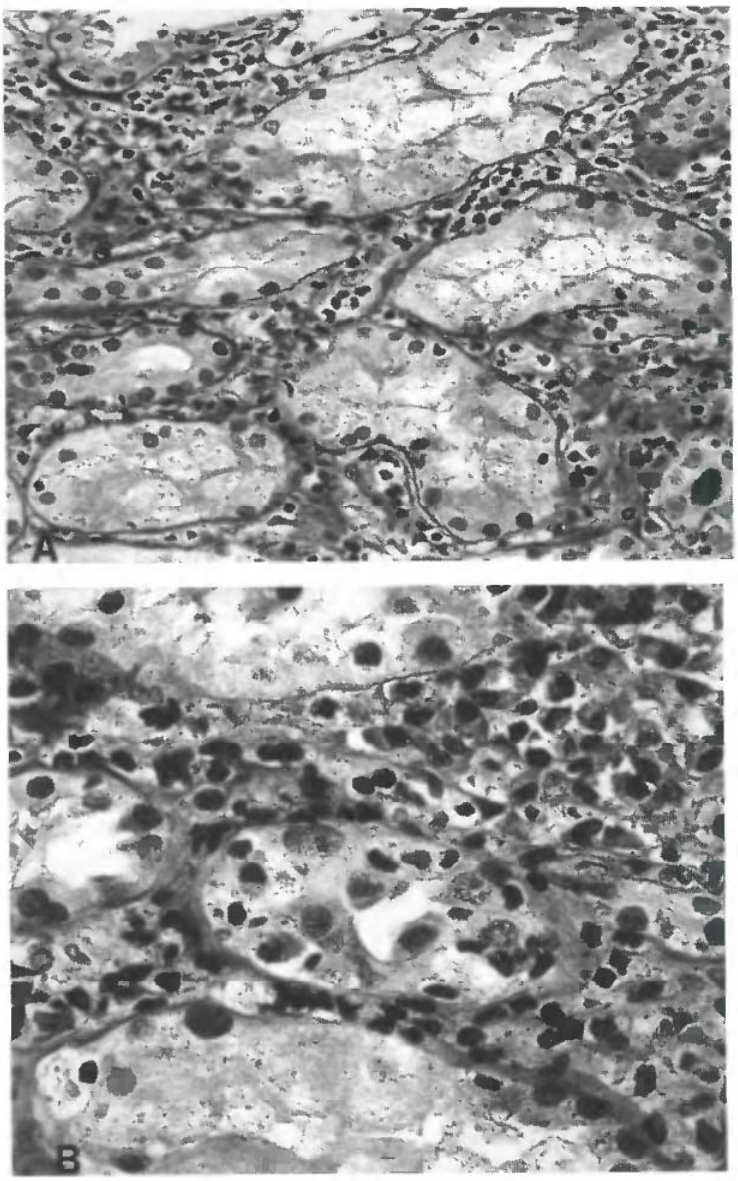

Figure 4 Tubulitis with intraepithelial lymphocytes that have crossed the tubular basement membrane. A. Mild, t1. (PAS; ×300.) B. Severe, t3 (PAS ×600.)

defined as invasion of mononuclear cells across the tubular basement membrane; these cells generally lie below or between the tubular epithelial cells (Fig. 4). Because localization of inflammatory cells may be difficult in hematoxylin and eosin–stained sections, definition of true tubulitis may be markedly enhanced by PAS or silver stain, which highlight tubular basement membranes. The infiltrating cells typically have a small clear area around them and can be differentiated from the surrounding tubular cells morphologically. They are morphologically similar to inflammatory cells in surrounding interstitium. Criteria for grading are based on severity in the most inflamed tubule; extent of tubulitis is less reliable because the process is often patchy.

In general, significant tubulitis tends to occur in areas of interstitial inflammation and should be sought in those areas, although in occasional cases tubulitis is more extensive than interstitial inflammation. With very aggressive inflammation, tubular basement membranes may be disrupted—this change can be defined with PAS or silver stains. Inflamed disrupted tubules are graded t3, severe tubulitis. Recent studies on segmental localization of tubulitis in allograft biopsies have demonstrated that tubulitis is most severe in distal

convoluted tubule and the cortical collecting system, followed by proximal tubules, and distal straight tubules (33). Because there is typically a "tubulitis" in atrophic tubules in native as well as allograft kidneys, tubules that are reduced by 50% or more from normal size should not be used to diagnose or grade tubulitis.

Immunohistochemistry may enhance identification and quantitiation of tubulitis. Staining for common leukocyte antigens serves to mark infiltrating leukocytes. Immunophenotyping of the lymphocytes invading the tubule may provide greater specificity in diagnosing a rejection process. As discussed, the presence of Leu-7–positive cells infiltrating the tubular epithelium is a fairly reliable marker of rejection (24) (see Fig. 3). Identification of cytotoxic cells using antibodies to granzyme B and/or perforin (34–36), or identification of cytotoxic T lymphocyte–specific gene transcripts (26,37) may ultimately also be useful adjuncts in identifying effector cells of the rejection response in the allograft biopsy. Other potentially useful markers include enhanced tubular cell expression of human leukocyte antigens (HLA-DR) (38,39) and adhesion molecules such as VCAM-1 and ICAM-1 (40–42), which may contribute to targeting of the tubular epithelium for immunological attack.

4. Vessels

a. Arteries

Intimal arteritis is the pathognomic lesion of acute rejection and is highly specific for that process in the allograft. This finding was recognized in several early studies of human renal allograft pathology (43–46) and continues to be an important finding in renal allograft biopsies. The lesion is characterized by intimal thickening resulting from edema and an inflammatory infiltrate, with mononuclear cells beneath the endothelium (Figs. 5 and 6). Criteria for quantitation of intimal arteritis/arteritis in the Banff 93–95 formulation are based on severity and extent of the inflammatory changes in the intima in the most severely involved artery, with v3 expanded to include transmural arteritis and/or mural necrosis and interstitial hemorrhage. Despite its importance as a marker of rejection, however, arteritis is the lesion probably most vulnerable to being missed because of sampling error. Multiple sections must be carefully examined to confidently rule out this process. The number of arteries in the specimen and the number involved in the rejection process should be enumerated.

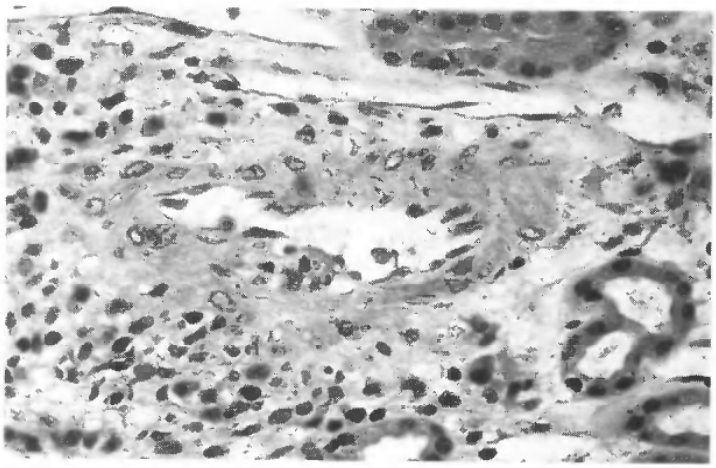

Figure 5 Mild intimal arteritis (v1). Note the focal accumulation of inflammatory cells beneath the intima of this small artery. (H&E, ×400.)

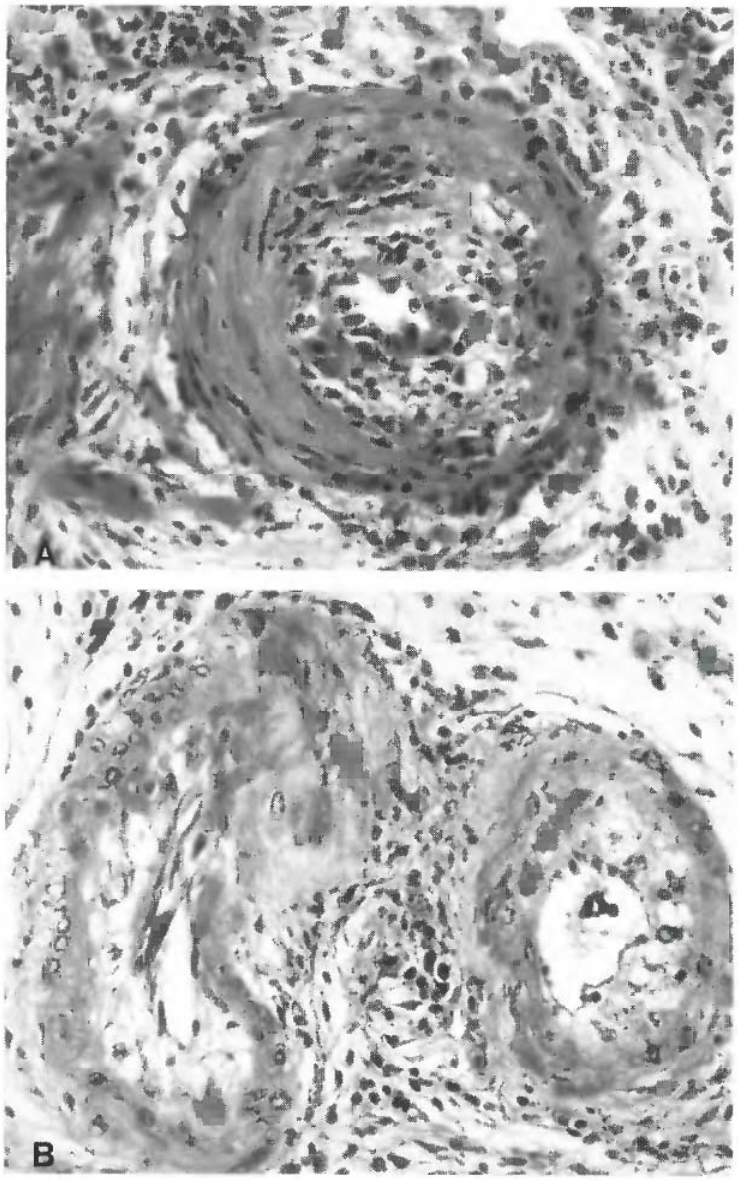

Figure 6 A. Severe intimal arteritis (v3). (H&E, ×400.) B. Fibrinoid necrosis in adjacent vessel. (H&E, ×400).

With intimal arteritis, endothelial cells are generally enlarged and vacuolated, and may be partially or completely detached from the intima. The intima contains edema fluid, matrix material, fibrin, and cells, including infiltrating inflammatory cells and myofibroblasts. Infiltrating cells include lymphocytes, plasma cells, and macrophages. With more severe involvement, cells of the media may become necrotic as the inflammatory process extends to become transmural. Vascular necrosis and thrombosis may be seen in severe lesions (see Fig. 6).

Whereas many of these vascular changes are quite specific, thrombosis and fibrinoid necrosis in arterioles and small arteries have also been reported as a manifestation of cyclosporine toxicity (47,48) (see Chap. 23) and have occasionally been reported without clinical evidence of rejection in renal allograft patients not receiving cyclosporine, including some series of patients treated with OKT3 (49). We have also seen these changes in kidneys harvested from individuals with severe hypertension before harvesting; the changes gradually resolved in the allografted kidney. Plasma levels of cyclosporine, careful investigation

of donor history, evaluation of baseline biopsy of the graft, and presence or absence of other features of allograft rejection are helpful in assessing these differential considerations. Capillary and arteriolar thrombosis may also be seen in antibody-mediated rejection (28,29).

Focal hyaline change is occasionally observed in arteries. It appears to have an entirely different significance from similar changes in arterioles. It is sometimes seen in continuity with healing intimal arteritis lesions, and, at least in some instances, focal arterial hyaline lesions appear to be a sequel of prior rejection episodes. However, this finding may also reflect donor disease or be seen with hypertension. Whatever the cause, this change may precede some of the chronic lesions discussed in Sec. IV. Special stains are useful in differentiating severe hyaline change (PAS-positive) and fibrinoid necrosis (highlighted by fibrin stains).

Studies of immunohistochemical localization of the vascular cell adhesion molecules VCAM-1 and intracellular adhesion molecules (ICAM-1) have shown that endothelial cell expression of these molecules may define sites of acute vascular inflammation in renal allograft rejection (41). However, it is not clear whether this finding enhances the diagnosis of rejection above detection of intimal arteritis by light microscopy alone, because expression may be increased essentially only in areas with a venulitis or arteritis (50).

b. Arterioles

Hyaline arteriolar thickening may be detected in renal allograft biopsies. These lesions are typically PAS-positive and are graded according to severity and extent (Table 3). When they are of new onset, and especially when they occur in a nodular pattern at the periphery of arterioles (Fig. 7), arteriolar hyalinosis lesions are highly suggestive of cyclosporine toxic-

Table 3 Quantitative Criteria for Arteriolar Hyaline Thickening ("ah") (0 to 3+)

0 = No PAS-positive hyaline thickening
1 = Mild to moderate PAS-positive hyaline thickening in at least one arteriole
2 = Moderate to severe PAS-positive hyaline thickening in more than one arteriole
3 = Severe PAS-positive hyaline thickening in many arterioles

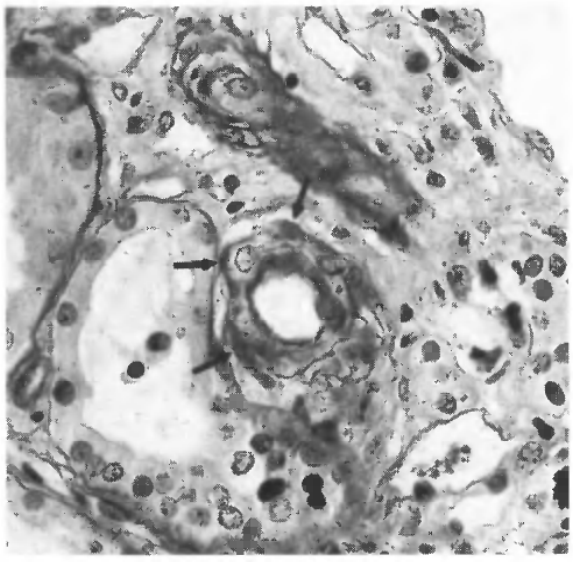

Figure 7 Arteriole with peripheral hyaline nodules (arrows). (PAS, ×300.)

ity (47). These changes may also develop in allografted kidneys, however, as a consequence of hypertension or diabetes in the recipient. In native kidneys, hyaline charge in arterioles is a relatively non-specific finding, which commonly develops as a result of hypertension, diabetes, or aging. Donor kidneys should be biopsied before or at the time of implantation to provide a baseline assessment of this and other chronic changes as well as to rule out hyperacute rejection or other preexisting disease or injury that may at least initially compromise graft function.

Occasionally, inflammatory changes seen in arteries in acute rejection may also be seen in arterioles. It is not known whether this arteriolitis has the same significance as intimal arteritis and it is not used to diagnose or grade rejection.

5. Sum Scores of Semiquantitative Grading

The semiquantitative grading of compartmental changes in the Banff schema allows generation of sum scores for both acute and chronic changes. The former has been designated the Banff Score for Inflammatory Changes (BSI), the latter the Banff Chronic Index (BCI). Both of these have been assessed for their utility in clinical studies. A discussion of the clinical validation of sum scores follows in subsequent paragraphs.

C. Semiquantitative Assessment of Histological Changes—Chronic

Acute rejection–related changes in the allograft, while interfering with function, are usually treatable with the array of immunosuppressive agents available, and renal allograft loss is largely due to chronic changes that develop in the grafted organ. These chronic changes are also assessed semiquantitatively in the Banff schema, and are regarded as manifestations of "chronic allograft nephropathy," which may result from a number of processes, including "chronic rejection." Chronic histopathological changes have been detected in systematic biopsies of well-functioning long-term renal allografts as well as in biopsies of grafts with functional impairment; renal functional reserve would presumably be compromised in these kidneys, and these changes are predictive of later graft dysfunction (51). The grading of "chronic allograft nephropathy," regardless of underlying causes for the nephropathic changes, is most important for prediction of long-term graft function and is often important in the decision whether or how to treat any accompanying acute changes in the older allograft. Here, assessment of chronic changes using the Banff schema is discussed; the Chronic Allograft Damage Index developed by Hayry and colleagues is discussed in a subsequent section.

1. Glomeruli

Chronic glomerular changes that develop in renal allografts have been designated as "chronic transplant glomerulopathy." Changes in the glomerulus include subendothelial widening, mesangial expansion, swollen endothelial cells, peripheral mesangial interposition, and occasional cellular crescents (Fig. 8) (16,51–53). Adhesions may form between the glomerular tuft and Bowman's capsule. The PAS and silver stains may be useful in quantitating mesangial matrix increase and peripheral interposition. Immunofluorescence microscopy may reveal IgM, C_3, and IgG in peripheral capillary loops and mesangial regions, and weak reactions for IgA have also been reported. On electron microscopy, flocculent electron-lucent material may be seen in the widened subendothelial space. This subendothelial widening is also seen in acute rejection, but in chronic glomerulopathy in transplants, there is a more complex picture, with several types of basement membrane

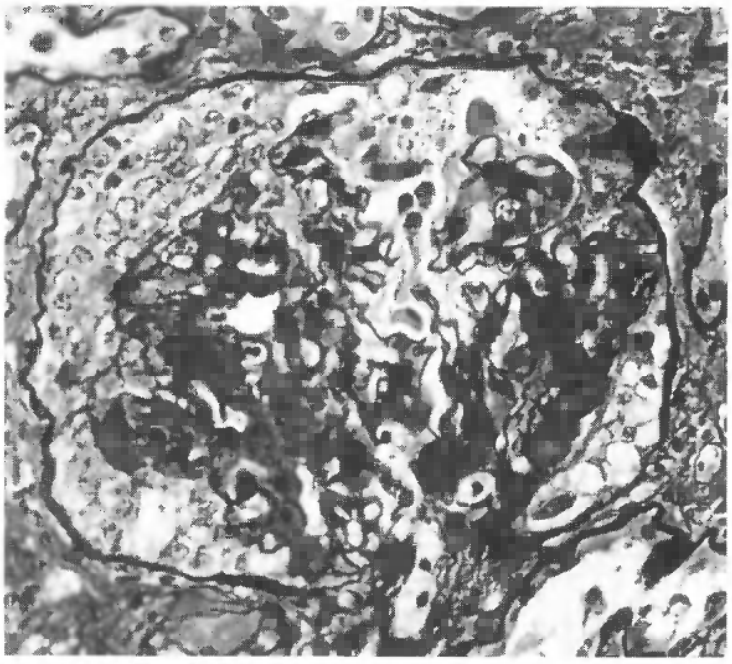

Figure 8 Glomerulus with mesangial interposition, increased mesangial matrix, and crescent formation. (×400.)

abnormalities. There may also be fine granular electron dense deposits that may be subendothelial, intramembranous, subepithelial, or mesangial; these are similar in appearance to dense deposits of immune complexes (54). If the deposits are numerous, the possibility of a de novo or recurrent glomerulonephritis must be considered (see Sec. V.5.B).

In the Banff schema, these glomerular changes, which involve most or all of the glomeruli, are graded as mild, moderate, or severe chronic transplant glomerulopathy. Even though they have been assumed to result from chronic immunological injury and provide evidence for "chronic rejection," the pathogenic mechanisms of these changes are not clear.

Other chronic glomerular changes may be present in the renal allograft. These are most often due to hypertension or ischemic changes resulting from compromise of allograft vessels. With the use of increasingly older donors, preexisting glomerular sclerosis and chronic vascular disease may be present in the donor kidney at the time of grafting (Fig. 9). This glomerulosclerosis persists in the allograft. Many centers do pretransplant biopsies in older donors and those with vascular disease to assess the degree of irreversible chronic change. In general, kidneys are considered suitable for transplantation if less than 25% of glomeruli are sclerotic on pretransplant biopsy. In addition to these preexisting changes, late ischemic glomerulopathy with thickening, wrinkling, and collapse of glomerular capillary walls associated with accumulation of extracapillary fibrotic material may develop in the allografts as occlusive changes develop in allograft vessels owing to hypertension, drugs, or "chronic rejection." These ischemic glomerular changes are not directly immune mediated. While these changes are not chronic transplant glomerulopathy in the strictest sense, they are real changes that have implications for allograft function; bland glomerulosclerosis should be quantitated by expression as percentage of glomeruli sclerosing or sclerotic.

Glomerular changes tend to correlate with tubulointerstitial scarring and chronic vascular lesions, but they may not. Some grafts have chronic glomerular changes with no demonstrable vascular lesions, or vice versa—this dissociation may be due in part to sampling error. Moreover, tubular atrophy and interstitial scarring may also be out of propor-

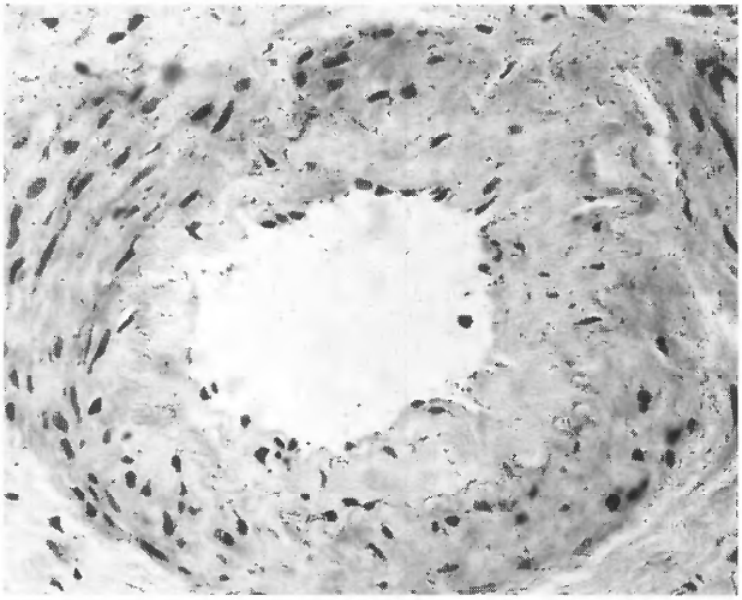

Figure 9 Moderate intimal thickening in a small artery in a biopsy sample obtained immediately after implantation. (H&E, ×400.)

tion to chronic glomerular changes, probably due in part again to sampling error and to the existence of "atubular glomeruli," which persist intact despite atrophy and loss of their accompanying nephron. It is the tubulointerstitial changes that are used to grade chronic transplant nephropathy, because these latter changes are least susceptible to sampling error and appear to be the best predictor of long-term outcome.

2. Tubulointerstitium

The degree of interstitial fibrosis and of tubular atrophy, which almost always correlate closely, are the critical factors in grading chronic transplant nephropathy. They are coded semiquantitatively by the extent of chronic changes, varying from mild (ci1, ct1) to severe (ci3, ct3). A mild grade is assigned when interstitial fibrosis or tubular atrophy involves less than 25% interstitium or tubules, moderate with 25% to 50% involvement, and severe when more than 50% of the parenchyma shows these chronic changes. Special stains may be very useful in the quantitation of these changes. In particular, trichrome stains that highlight collagen may help in assessment of interstitial fibrosis, and PAS or silver stains, which preferentially stain tubular basement membranes, may be used to define the thickened and sometimes reduplicated basement membranes that surround atrophying tubules (Fig. 10). With tubular chronic changes, actual loss of tubules may occur as atrophy progresses, and loss of tubules should be included in quantitation of chronic tubular changes. As with more acute changes, superficial cortical changes reflecting scarring related to perioperative subcapsular injury should be identified as such if possible and not graded; such changes have not been shown to compromise graft survival.

As with glomerular lesions, these chronic tubulointerstitial changes may evolve in the graft secondary to chronic immunologically mediated vascular injury ("chronic rejection"). However, they may also be secondary to hypertension-induced or drug-induced vascular changes, or secondary to primary tubulointerstitial processes including infection, reflux, and drug reactions. More rarely, they may be due to primary glomerular disease in the allograft. In addition, they may be present in the donor kidney, once again emphasizing the

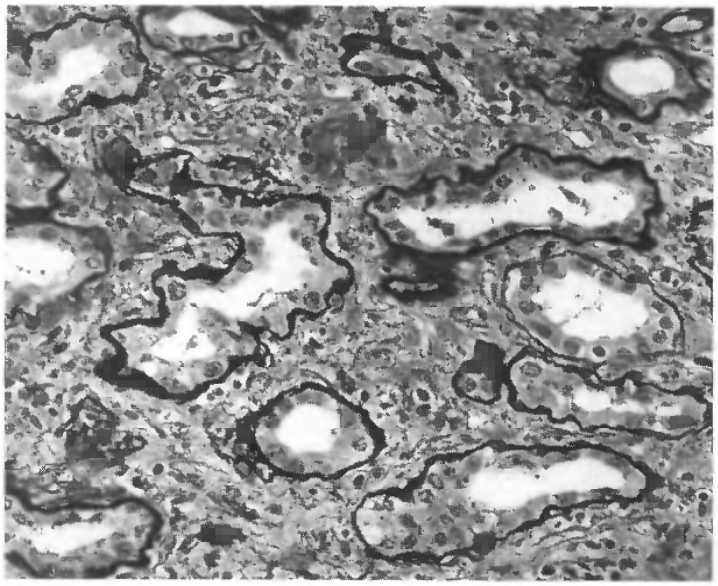

Figure 10 Thickened basement membranes around atrophic tubules. (PAS/MS, ×250.)

importance of a peri-implantation biopsy of the allograft. However, regardless of the cause, these chronic changes have significant implications for graft survival.

3. Vessels

Chronic vascular changes in the renal allograft may be caused by a variety of mechanisms. Repeated episodes of acute rejection, especially with a prominent vascular component, are presumed to lead to chronic vascular damage resulting from immunological injury, so-called chronic vascular rejection (55–57). In particular, new-onset fibrous intimal thickening with disruptions in the internal elastica is suggestive of chronic rejection (58). However, hypertension must be ruled out, since it may produce identical changes. V-CAM-1 expression in peritubular capillaries may be a good marker (41).

Accelerated arteriosclerosis may be seen in renal allografts although this lesion is more common in cardiac grafts. In these cases, the intima becomes rapidly thickened with numerous foam cells; occasionally, chronic inflammatory cells may be seen (Fig. 11). These changes may evolve quickly, within a few months, and lead to relatively rapid graft loss. The origin of this lesion is unclear, but is likely different than the usual mechanisms of arteriosclerosis because the lesion in kidney, as well as in other organs, is typically concentric and diffuse (59).

D. Banff Working Classification—1993–1995

1. Rejection Diagnosis

Diagnostic categories for renal allograft rejection in the 1993 to 1995 Banff formulation are outlined in subsequent paragraphs. As discussed, the cardinal features used for diagnosis of acute allograft rejection are tubulitis and vasculitis. For chronic transplant nephropathy, severity is graded on the basis of tubulointerstitial changes, although glomerular and vascular lesions help define type and potential etiology of the chronic changes. Each of these categories is discussed briefly. This formulation is currently in use in ongoing clinical trials. A somewhat modified schema, the Banff 1997 working formulation, is described in the subsequent section.

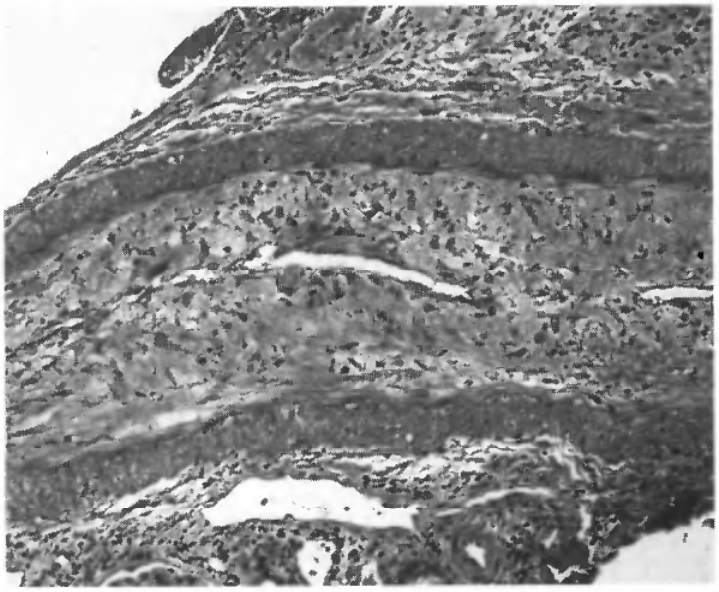

Figure 11 Accelerated atherosclerosis in a biopsy sample obtained less than 4 months after transplantation. There are numerous foam cells and occasional inflammatory cells in the markedly thickened intima. H&E, ×100.

a. Normal

A normal biopsy is one showing no change or trivial inflammation or scarring. This category is most often applied to postimplantation biopsies or to protocol biopsies performed in the absence of specific clinical indications.

b. Hyperacute Rejection

Hyperacute rejection occurs rapidly in the allograft, and morphological changes related to this process are typically seen in the postimplantation biopsy. This form of rejection results from preformed antibody in a previously sensitized patient and is characterized by accumulation of polymorphonuclear leukocytes in glomerular and peritubular capillaries (Fig. 12). These vessels may be congested, and small thrombi form rapidly as endothelial cell injury progresses. Infarcts of the renal parenchyma then develop, and most of these grafts are lost.

Immunofluorescence stains can demonstrate antibody and complement components in vessels (47,60,61). IgM isohemagglutinins are deposited with ABO incompatibility, whereas antibodies from presensitization to HLA-A or ALA-B antigens are largely IgG (47). Rarely, preexisting antibodies to class II MHC antigens (HLA-DR, HLA-DQ) have been reported to cause hyperacute or accelerated acute rejection, with deposition in arterioles in some of these cases. Activation of complement can occur even with low levels of antibody and may be more reliably identified in allograft vessels than antibody itself (Fig. 13) (29,62,63). Fibrinogen may also be detected in capillaries in this setting.

c. Borderline Changes

Synonyms for this category include "very mild acute rejection" and "consistent with early acute rejection." By definition, no intimal arteritis is present. Biopsies in this category show a mild tubulitis (t1) accompanied by a mild or moderate focal interstitial inflammatory infiltrate. Because well-functioning grafts may show mild tubulitis (t1), this biopsy finding is not in itself an indication for antirejection therapy. However, in the presence of suggestive

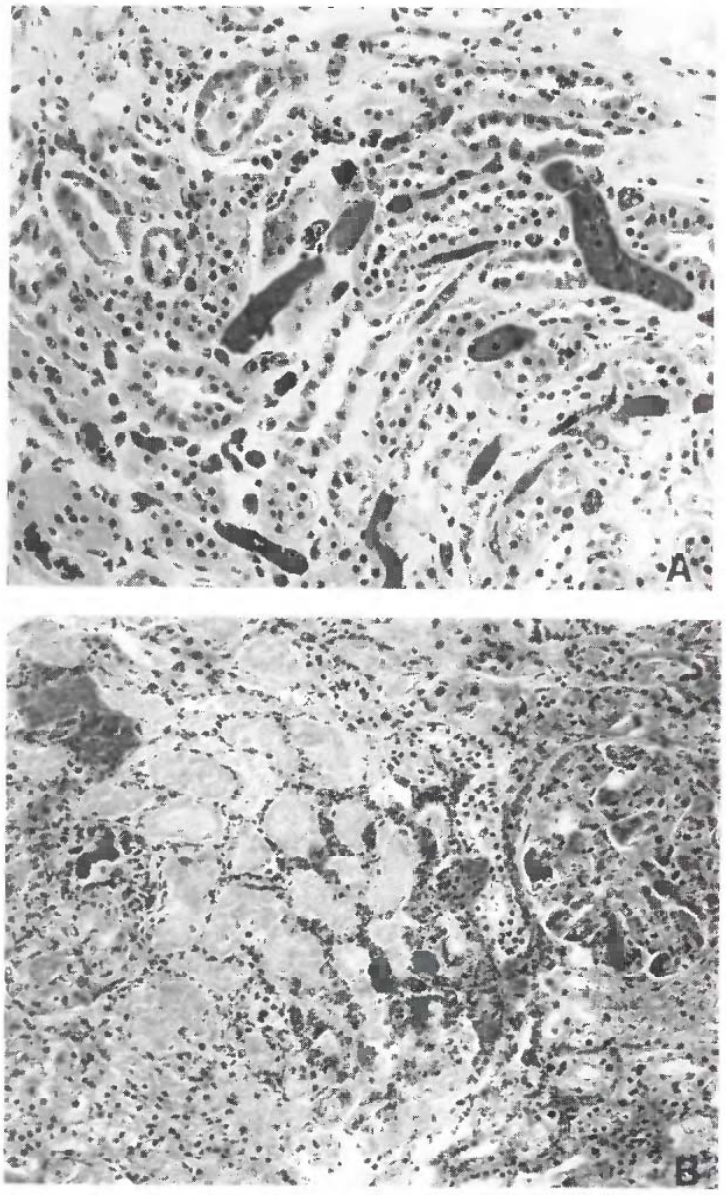

Figure 12 Hyperacute rejection. A. Congestion and numerous polymorphonuclear leukocytes in peritubular capillaries in implantation biopsy. (H&E, ×250.) B. Biopsy sample obtained several days later showing parenchymal infarction. (H&E, ×160.)

clinical features of rejection, therapy may be warranted in individual cases. Several investigators are studying the response of "borderline" lesions to antirejection therapy.

d. Acute Rejection

The changes of acute rejection are assigned to grades I, II, or III (mild, moderate, or severe rejection), respectively.

In grade I acute rejection, there is a moderate tubulitis (t2) accompanied by a significant interstitial infiltrate (i2 or i3). The likely clinical response to this finding is to treat rejection, unless there are absolutely no clinical signs of rejection.

Moderate acute rejection is divided into grade IIA and IIB, depending on the absence or presence of arteritis. In grade IIA, there is severe tubulitis (t3) with a significant interstitial infiltrate; in grade IIB there is mild to moderate intimal arteritis (v1 or v2). With these

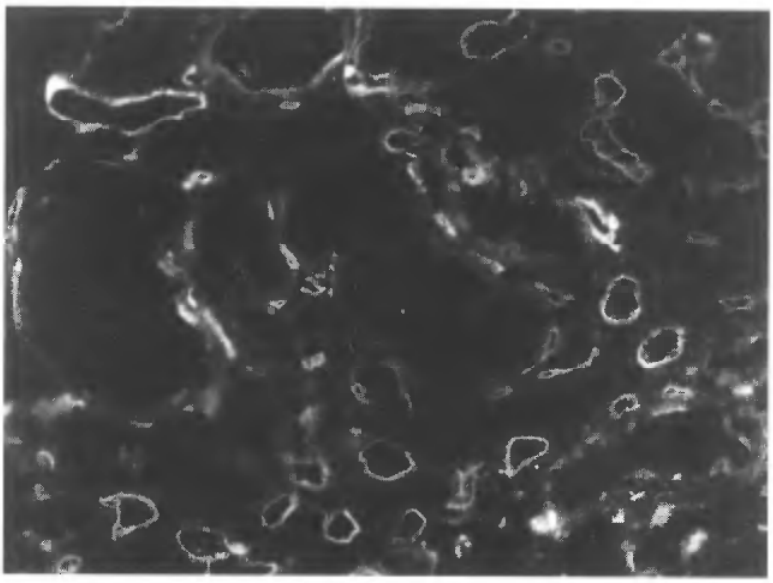

Figure 13 Complement in antibody-mediated rejection.

findings on biopsy, rejection should be treated. If the case is refractory to steroids, OKT3/ALG should be considered.

When the biopsy reveals severe intimal arteritis or transmural arteritis, with or without fibrinoid change or medial smooth muscle necrosis (v3), the changes are classified as severe acute rejection. There may be patchy infarction or interstitial hemorrhage as well, and occasionally these latter changes are found without identifying a vascular lesion. In the presence of severe acute rejection, the patient must undergo aggressive treatment unless the clinical course suggests that the rejection may be irreversible. It has been shown that if infarcts, glomerular thrombosis, or arterial or arteriolar thrombosis was found in a graft biopsy as a new finding not dating from the time of transplantation, then 100% of affected grafts were lost within 1 year (64), so long-term prognosis may be compromised even if therapy is initiated. These latter changes may also reflect an antibody-mediated rejection.

e. Chronic Allograft Nephropathy

As discussed, 90% or more of renal allografts survive for 1 year, despite occasional episodes of acute rejection. It is the chronic changes, which appear to occur inevitably in most allografts over time, that produce graft loss (65–67). Sometimes, chronic changes evolving in the graft are superimposed on preexisting chronic changes present in the donor kidney at the time of transplantation. Implantation biopsy helps define these baseline changes, enabling better determination of progression in the allograft recipient.

In native kidney diseases of various causes, including primary glomerular diseases, it is the degree of interstitial scarring and tubular atrophy on biopsy that determines long-term prognosis. This strong correlation probably is due to the fact that biopsies generally capture a great deal of tubulointerstitium, with relatively few glomeruli and even fewer vessels. These same considerations are important in the renal allograft, and chronic transplant nephropathy is, therefore, graded on the basis of extent of interstitial fibrosis and tubular atrophy, changes that almost invariably correlate strongly with each other.

In grade I, mild chronic allograft nephropathy, there are mild tubulointerstitial changes (ct1, ci1). Moderate chronic change, grade II, shows intermediate changes (ct2, ci2), with 25% to 50% of the parenchyma showing interstitial fibrosis and tubular atrophy.

With extension to greater than 50% of the biopsied parenchyma (ct3, ci3), this process i graded as severe, grade III.

As discussed, a number of processes may converge to produce scarring in the rena allograft. These include vascular disease (immunological or nonimmunological), hyperten sion, drug toxicity, and reflux or obstruction. Because the origin is often, and perhaps usu ally, multifactorial, these changes are designated "chronic allograft nephropathy" rathe than "chronic rejection." However, there are a few changes that may lead to a specific diag nosis of chronic rejection.

E. Banff 1997 Working Formulation

The meeting at Banff in 1997 led to further defining and refining of the Banff Working For mulation. Alterations were made in both quantitative criteria and schema categories Changes were made in part to make this grading system more compatible with the simpli fied system being used in National Institute of Health (NIH)-sponsored collaborative clin ical trials (CCTT) (12). The CCTT criteria for rejection diagnosis are based on presence o vasculitis or on numbers of tubules inflamed in a maximally involved area in those case with only tubulointerstitial inflammation. The CCTT schema divides rejection into thre types: (1) tubulointerstitial, (2) vascular with intimal arteritis, and (3) vascular with fibri noid necrosis or transmural arteritis. Because vasculitis clearly has a negative impact or prognosis (68,69), such a delineation seems warranted. Despite these recent modifications for the near future, Banff 1993 to 1995 will continue to be used in ongoing clinical trials.

Quantitative criteria for glomerulitis and arteriolar hyaline thickening remair unchanged. Criteria for mononuclear interstitial inflammation, expressed as percentage o unscarred parenchyma involved, have been altered slightly (Table 4), so that i0 "trivial' inflammation is defined as up to 10% of parenchyma, with il being 10% to 25% o parenchyma. Severe tubulitis (t3) is defined by foci with greater than 10 cells per tubula cross section or 10 tubular cells, or the presence of at least two areas of tubular basemen membrane destruction accompanied by t2 elsewhere in the biopsy sample and moderate t severe interstitial inflammation.

Quantitation of arteritis was also modified (Table 5). v1 is defined as mild to moder

Table 4 Quantitative Criteria for Mononuclear Cell Interstitial Inflammation ("i") (0 to 3+)

0 = Less than 10% of parenchyma inflamed
1 = 11% to 25% of parenchyma inflamed
2 = 26% to 50% of parenchyma inflamed
3 = >50% of parenchyma inflamed

Table 5 Quantitative Criteria for Intimal Arteritis ("v") (0 to 3+)

0 = No arteritis
1 = Mild to moderate intimal arteritis in at least one arterial cross section
2 = Moderate to severe intimal arteritis in at least one arterial cross section
3 = Severe intimal arteritis in many arterial cross sections and/or "transmural" arteritis, fibrinoid change, and medial smooth muscle necrosis, often with patchy infarction and interstitial hemorrhage

ate intimal arteritis in at least one artery; v2 is applied to cases with severe intimal arteritis with at least 25% loss of luminal area in at least one arterial cross section. Finally, v3 is defined by arterial fibrinoid change and/or transmural arteritis with medial smooth muscle necrosis. The number of arteries present in the biopsy and the number of affected vessels should be designated. An asterisk should be added to the v score if there is infarction or interstitial hemorrhage in the biopsy.

The current numerical codes for semiquantitative assessment of acute and chronic changes in the allograft are listed in Table 6.

Diagnostic categories have also been modified somewhat in the Banff 1997 schema. These changes are as follows:

1. Normal—no change.
2. *Antibody-mediated rejection* replaces *hyperacute rejection.* Histological features include extensive margination of polymorphonuclear leukocytes, glomerulitis, capillary thrombi, and, in some cases, infarcts. This category is further divided into "immediate" (within 72 hours) or "delayed" (greater than 72 hours).
3. *Suspicious for rejection* (formerly "borderline changes"), defined by mild tubulointerstitial inflammatory changes (less than i2t2; includes i1t1 and i2t1). This category has been changed in part because of early reports that many of these patients respond to antirejection therapy (68,70).
4. *Acute rejection* is categorized into three types: type A "tubulointerstitial," type B "vascular," and type C "transmural" rejection. Type A is further categorized into A1 with

Table 6 Numerical Codes for Semiquantitative Assessment of Acute and Chronic Changes in the Allograft Biopsy

Acute Changes[a]		
g	0,1,2,3—	No mild, moderate, severe glomerulitis. g3 = mononuclear cells in capillaries of all or nearly all glomeruli with endothelial enlargement and luminal occlusion.
i	0,1,2,3—	Trivial, mild, moderate, severe mononuclear cell infiltration. In rejection, edema and lymphocyte activation usually accompany mononuclear cell infiltration; i3 = >50% of parenchyma inflamed.
t	0,1,2,3—	No, mild, moderate, severe tubulitis. t3 = >10 mononuclear cells per tubule or per 10 tubular cells in several tubules, or destruction of basement membrane.
v	0,1,2,3—	No, mild, moderate, severe arteritis. v3 = fibrinoid necrosis/transmural arteritis.
ah	0,1,2,3—	No, mild, moderate, severe nodular hyaline afferent arteriolar thickening suggestive of cyclosporine toxicity. ah3 = severe PAS-positive thickening in many arterioles.
Chronic Changes[a]		
cg	0,1,2,3—	No, mild, moderate, severe chronic transplant glomerulopathy.
ci	0,1,2,3—	No, mild, moderate, severe interstitial fibrosis, often with mononuclear cell inflammation.
ct	0,1,2,3—	No mild, moderate, severe tubular atrophy and loss.
cv	0,1,2,3—	No, mild, moderate, severe fibrous intimal thickening often with elastica fragmentation. cv3 indicates severe occlusion. cg and cv lesions suggest the presence of chronic rejection.

[a]Both acute and chronic codes can be used together if the situation warrants.

moderate tubulitis (t2) or A2 with severe tubulitis (t3). Both require i2 or i3. Type B is divided into two categories, B1 and B2, depending on the severity of intimal arteritis; B1 designates those with mild arteritis, B2 those with moderate arteritis.

5. *Chronic allograft nephropathy* remains essentially unchanged, with severity defined by tubulointerstitial changes. However, the presence of specific vascular signs of chronic rejection should be indicated (e.g., breaks in the elastica, inflammatory cells in fibrous intima). The current Banff 1997 Working Formulation is shown in Table 7.

III. CHRONIC ALLOGRAFT DAMAGE INDEX

Hayry et al. have developed a numerical scoring system for chronic allograft pathological appearance based on observations in a rat allograft model. They performed DA to WF renal allografts, immunosuppressed the animals for several weeks, and sacrificed the animals at 3 months after transplantation at a time when there was significant allograft dysfunction.

Table 7 Diagnostic Categories for Renal Allograft Biopsies—Banff 1997 Working Formulation

1. Normal
2. Antibody-mediated rejection
   A. Immediate
   B. Delayed
3. Suspicious for acute rejection
   No intimal arteritis is present; only mild or moderate focal mononuclear cell infiltration with foci of mild tubulitis (1 to 4 mononuclear cells per tubular cross section) is present.
4. Acute rejection
   Type A—"tubulointerstitial" without documented vasculitis
      1. Mild to moderate—cases with significant interstitial infiltration (>25% of parenchyma affected) and foci of moderate tubulitis (t2)
      2. Moderate to severe—cases with significant interstitial infiltration and foci of severe tubulitis (t3)
   Type B—"vascular" with intimal arteritis
      1. Mild—cases with v1
      2. Moderate–cases with v2
   Type C—severe acute rejection
      Cases with fibrinoid change and/or "transmural" arteritis and necrosis of medial smooth muscle cells (v3)
5. Chronic allograft nephropathy
   (Glomerular and vascular lesions help define type of chronic nephropathy. New-onset arterial fibrous intimal thickening, disruption of internal elastica, and/or transplant glomerulopathy may be interpreted as evidence of chronic rejection).
   Grade I
      Mild chronic transplant nephropathy
      Mild interstitial fibrosis and tubular atrophy
   Grade II
      Moderate chronic transplant nephropathy
      Moderate interstitial fibrosis and tubular atrophy
   Grade III
      Severe chronic transplant nephropathy
      Severe interstitial fibrosis and tubular atrophy and tubular loss
6. Other (changes not considered to be caused by rejection)

Thirty histopathological variables were assessed and compared with those in control kidneys from syngenic allografts treated with comparable immunosuppression. Eight variables were significantly more severe in allografts than in control subjects. These included interstitial inflammation, interstitial fibrosis, interstitial pyroninophilia, mesangial matrix increase, glomerular basement membrane thickening, glomerulosclerosis, vascular intimal proliferation, and obliteration (51).

These variables were subsequently applied in human allograft biopsies. In the initial biopsy study, these parameters were analyzed in patients with renal function ranging from normal to severely impaired. Eight variables correlated with serum creatinine: interstitial inflammation, interstitial pyroninophilia, interstitial fibrosis, mesangial matrix increase, glomerulosclerosis, arterial intimal proliferation, tubular atrophy, and tubular dilatation. The CADI was then developed using all of these features except pyroninophilia and tubular dilatation. The remaining six features are graded from 0 to 3, for a minimum sum score of 0 and a maximum of 18.

IV. PATHOLOGICAL FINDINGS NOT RELATED TO REJECTION

A. Posttransplantation Lymphoproliferative Disorder

Although it is uncommon posttransplantation, posttransplant lymphoproliferative disorder (PTLD) is an important entity to be distinguished from rejection, and it is placed first among differential diagnoses. Early identification of PTLD is critical for patient management. Previously, PTLD was considered not to occur until months to years after transplantation (64). Recently, however, it has been reported considerably earlier, sometimes within weeks of allografting (71–73), and it is often associated with particularly aggressive immunosuppression.

Histologically, PTLD is characterized by a monomorphous or polymorphous infiltrate containing plasma cells, which are usually atypical. The interstitial infiltrate is often diffuse (i3) in the absence of concomitant rejection, that is, in the absence of significant tubulitis or vasculitis. However, the two processes, PTLD and rejection, may coincide. In PTLD, cells in the interstitial infiltrate express B-cell markers, and in situ hybridization or other molecular probes for Epstein-Barr virus (EBV) antigen are almost invariably positive (Fig. 14).

B. Nonspecific Changes

There are a variety of changes in renal allograft biopsies that may accompany rejection but in themselves are not diagnostic of, or even strongly suggestive of, rejection. These include nonspecific inflammatory changes and nonspecific vascular changes. As discussed, focal nodular inflammatory infiltrates and perivascular aggregates of inflammatory cells, especially near the corticomedullary junction, are common findings in allograft kidneys. Infiltrates involving less than 10% of the interstitium are regarded as nonspecific. Several vascular changes may also be seen in well-functioning grafts, and must be considered nonspecific. These include endothelial reactive changes without true endothelialitis, that is, without invasion of inflammatory cells beneath the endothelial cells into the intima, vacuolization of smooth muscle cells in the vessel wall, and venulitis.

C. Acute Tubular Necrosis

Acute tubular cell injury is common in the allografted kidney (Fig. 15) and is a major cause of primary nonfunction of the allograft. Tubular injury may result from in situ injury in the

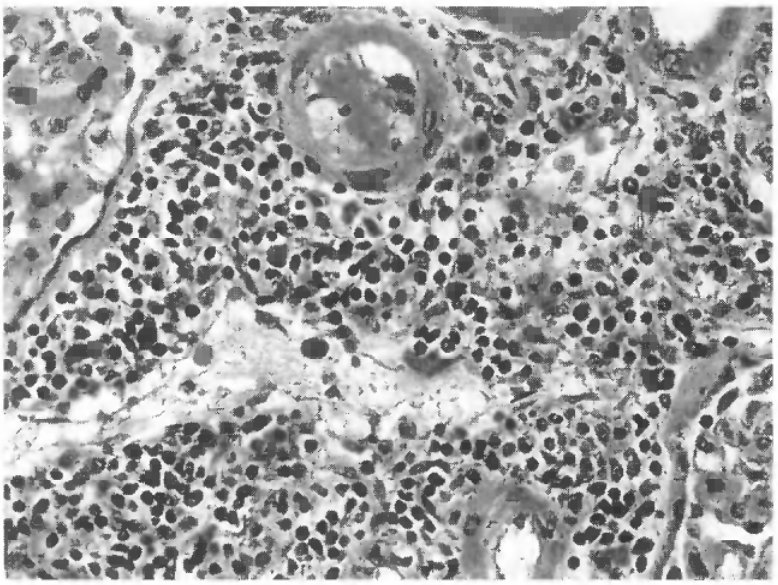

Figure 14 In situ for Epstein-Barr virus in a case of posttransplant lymphoproliferative disorder.

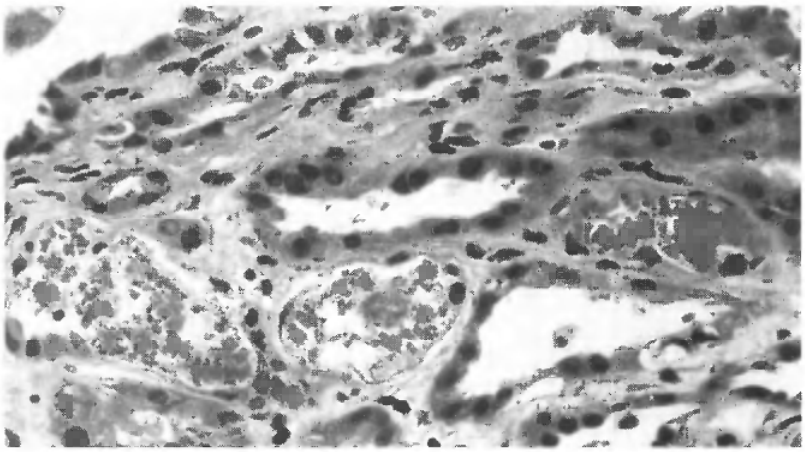

Figure 15 Early posttransplant biopsy showing frank necrosis of tubular cells (H&E, ×400.)

donor; ischemia during harvesting, storage, and transport of the organ; or ischemic injury incurred perioperatively in the recipient. Some morphological features of acute tubular injury in native and allograft kidneys are similar, but there are also some differences. We have found more extensive tubular cell necrosis, less loss of apical microvilli and basolateral infoldings, and more numerous deposits of calcium oxalate in allograft tubular cell injury compared to native kidney (4). Differences are probably caused by important differences in blood flow, temperature during ischemia time, and other factors.

D. Acute Interstitial Nephritis

Non–rejection-related interstitial nephritis may occur in allografts and may be difficult to differentiate from the rejection response. Neutrophils in an interstitial inflammatory infiltrate, especially if they are present in tubular casts as well, suggest the possibility of infection, and eosinophils could signal a hypersensitivity reaction, perhaps because of a drug reaction. Neutrophils or eosinophils may be numerous in the infiltrates of acute rejection,

however. The presence of coexistent significant tubulitis or vasculitis strongly suggests rejection but does not necessarily rule out infection or drug reactions, which may occur concomitantly with rejection. It is best to consider these differential diagnoses if the interstitial inflammatory infiltrate is not largely mononuclear.

E. Cyclosporine-Associated Changes

Tubular cells changes associated with cyclosporine administration are not necessarily a manifestation of cyclosporine *toxicity*, because they may be seen with administration of the parenteral vehicle for cyclosporine in experimental animals (74). Tubular cell changes include vacuolization and especially isometric vacuolization, eosinophilic inclusions in tubular cells, and microfilaments aggregated in the cytoplasm.

There is a range of vascular changes described with cyclosporine (reviewed in 47,74), changes that are important in the pathogenesis of cyclosporine toxicity. These include marked vasoconstriction, which may result in near occlusion of arterioles and smooth muscle vacuolization. Progressive injury to the vessel wall leads to necrosis and hyalinosis of individual muscle cells, and nodular hyaline afferent arteriolar deposits, which have recently been shown to be renin-rich. These hyaline changes in their most typical form are nodular and peripheral in the arteriole (see Fig. 7), but the entire arteriolar wall may be replaced by hyaline material; hyaline arteriolar change related to cyclosporine, can usually be confidently diagnosed if an early peri-implantation biopsy is available to rule out preexisting arteriolar hyaline change. A mucoid intimal thickening in arterioles may occasionally be seen. Finally, cyclosporine may rarely produce a thrombotic microangiopathy that may be devastating to the allograft (48).

Interstitial fibrosis has been described as a late sequel of cyclosporine therapy (47, 75–77), probably caused by ischemia resulting from chronic vasoconstriction. Glomerular sclerosis or ischemic collapse and juxtaglomerular apparatus hyperplasia may also be seen with chronic cyclosporine therapy.

F. Subcapsular Injury

Subcapsular injury may occur at the time of transplantation of the allograft (78) and is related to ischemia with loss of capsular blood supply. This process results in acute inflammatory changes and later chronic scarring. Recognition of localization of these changes to the superficial cortex and absence of other evidence of rejection should enable differentiation from rejection-related changes.

G. Pretransplant Acute Endothelial Injury

Pretransplant endothelial injury was historically seen in kidneys perfused before transplantation; with current preservation methods, this lesion is rarely seen in severe form. The histological changes seen included capillary thrombosis, ischemic change, and ultimate infarction in severe cases. This form of injury historically led to reduced graft function and, in some cases, graft loss (79).

H. Papillary Necrosis

Rarely, papillary necrosis may be seen in allografted kidneys. If it is extensive, graft loss invariably ensues.

I. De Novo Glomerular Disease

Various types of de novo glomerular diseases occur in allografted kidneys; a few occur with significantly increased frequency in allografts.

The most common is membranous nephropathy, reported in approximately 1% to 2% of grafts (80,81). Histological changes may reflect both the thickened capillary loops and increased mesangial matrix seen in membranous nephropathy and associated rejection or vascular changes. IgG and C_3 are seen in capillary loops by immunofluroscence and subepithelial dense deposits are seen on electron microscopy (Fig. 16). It has been postulated that this immune complex glomerulopathy is produced by a humoral immune response to allograft antigens (82).

Other de novo glomerular diseases that may occur in special circumstances in allografts are anti-GBM disease and acute immune complex glomerulonephritis secondary to administration of antilymphocyte serum. Anti-GBM disease may occur when patients with

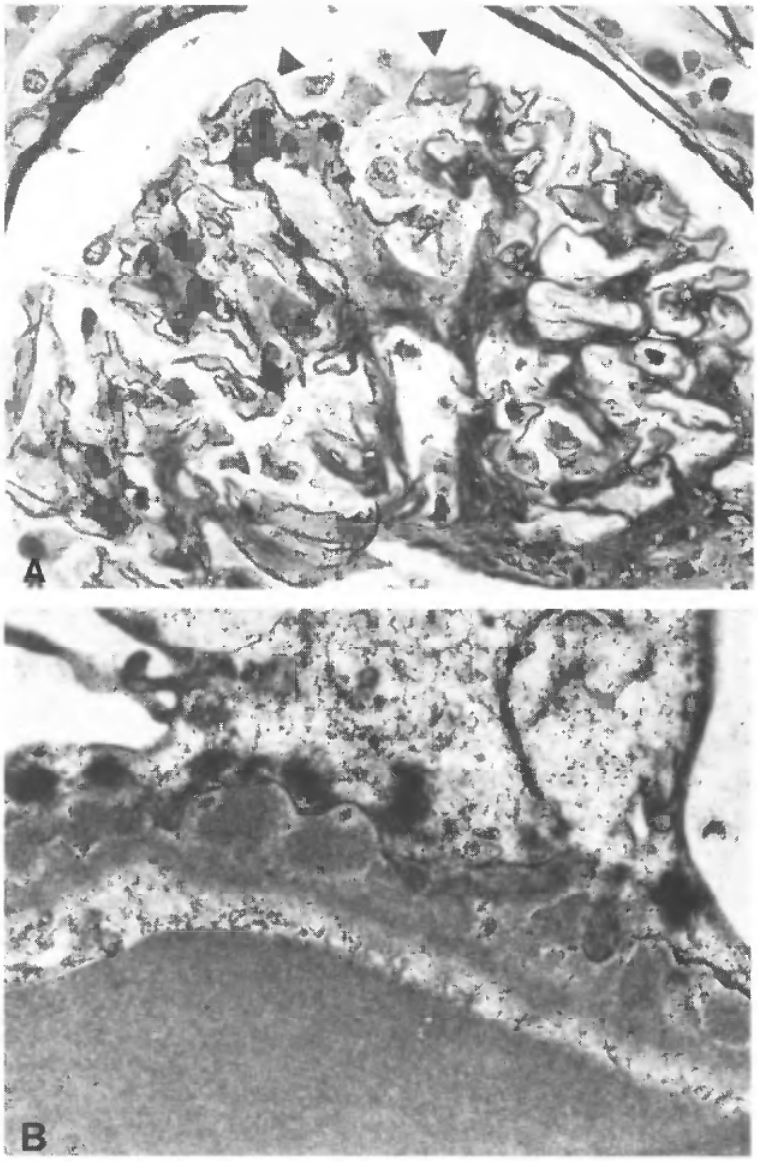

Figure 16 A. De novo membranous glomerulopathy in a patient 3 years after renal transplantation. The patient's original disease was IgA nephropathy. Note silver-positive "spikes" (arrowheads). (PAS/MS, ×500.) B. Subepithelial deposits on electron micrograph. (×26,470.)

Alport's disease, who develop glomerular disease because a normal collagen constituent is absent in the glomerular basement membrane (83,84), receive allografted normal kidneys that contain this antigen. In some of these individuals, a humoral response to this antigen may develop. Anti-GBM antibodies may be detected in the serum of these patients; the renal allograft in these cases may continue to function normally, but a typical crescentic glomerulonephritis with graft loss may occur (85,86), and recurrent anti-GBM disease has been reported in a subsequent allograft (87). We have also seen diabetic nephropathy occur de novo in older renal allografts, apparently a result of steroid-induced diabetes.

The glomerulonephritis produced by antilymphocyte serum protein/antibody produces a picture analogous to acute serum sickness. There is glomerular hypercellularity, and subepithelial immune deposits are seen by immunofluorescence and electron microscopy.

J. Recurrent Disease

Recurrent disease is a significant cause of late allograft dysfunction and should be separated from rejection-related changes. Recurrent disease can only be diagnosed with confidence when the patient's original disease has been well documented.

Immune complex glomerulonephritis may recur in allografted kidneys. Of these cases, type II membranoproliferative glomerulonephritis (MPGN) and IgA nephropathy have the highest recurrence rate (88). Membranoproliferative glomerulonephritis and chronic transplant glomerulopathy may be difficult to differentiate by light microscopy. Immunofluorescence microscopy, however, should reveal peripheral glomerular staining for C_3 and characteristic deposits by electron microscopy in type I MPGN. However, the subepithelial deposits may be sparse, and because rare densities may be seen in transplant glomerulopathy, the differential diagnosis may be difficult; it has been suggested that peripheral staining for C_3 on immunofluorescence should be used to diagnose MPGN in this setting (17). With type II MPGN, the distinctive ribbonlike intramembranous deposits are easily identified. Membranous nephropathy recurs in renal allografts in 3% to 7% of cases, but it is less common than de novo membranous nephropathy (89,90).

Focal segmental glomerulosclerosis with hyalinosis (FSGSH) recurs commonly in renal allografts, with a frequency of from 20% to 100% (88). Recurrence is most likely to occur in younger patients who had rapid progression to end stage (less than 3 years) in their native kidney (91,92). If recurrence occurs in an initial allograft, there is a substantially increased risk of recurrence in subsequent allografts (93,94). Light microscopy of recurrent FSGSH has demonstrated an early focal segmental proliferation that may have a predominance of epithelial cells, followed by typical segmental sclerosis with hyalinosis (see Fig. 17) (95,96).

Anti GBM disease recurs with clinical symptoms in approximately 25% of patients, but graft loss is rare. Pauci-immune crescentic glomerulonephritis may also recur and lead to graft loss (97).

There is a variety of systemic diseases that produce pathological changes in the kidney and that may produce recurrent disease in the renal allograft. These include Henoch-Schönlein purpura, hemolytic uremic syndrome, amyloidosis, Wegener's granulomatosis, cryoglobulinemia, monoclonal gammopathies, lupus nephritis, and diabetes. Most of these recur at rates of 5% to 30% and lead to graft loss relatively rarely. Hemolytic uremic syndrome is an exception, with an incidence of graft loss of 40% to 50%. Lupus nephritis recurs rarely (<1% of cases). Even though diabetic nephropathy occurs in essentially all patients with time, the disease rarely leads to graft loss. In general, the histology of this disease in the allograft is similar to that in native kidneys. However, diabetic changes progress

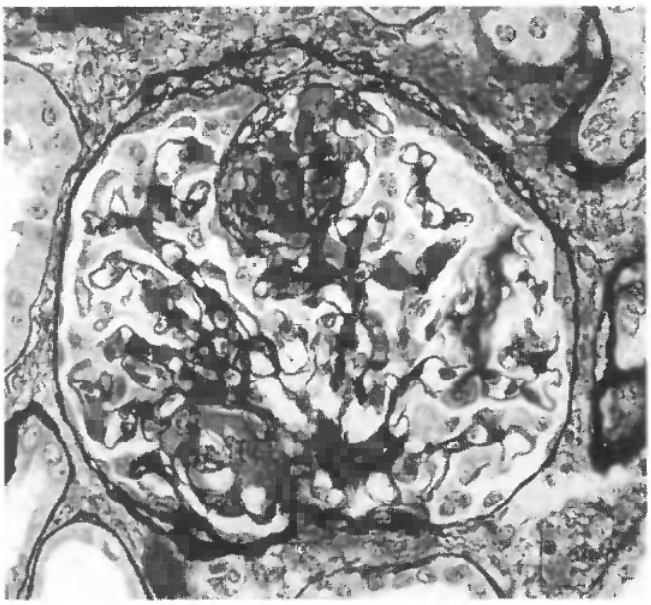

Figure 17 Focal segmental glomerulosclerosis with hyalinosis (FSGSH) 6 months after transplantation in a patient with a rapidly progressive FSGSH in his native kidney. At transplant nephrectomy 18 months after transplantation, many glomeruli showed characteristic lesions of FSGSH. (PAS/MS, ×400.)

slowly, so that even though thickened glomerular basement membranes, increased mesangial matrix, and arteriolar hyaline change may be seen, full-blown Kimmelseil-Wilson nodules are rare in renal allografts. The microangiopathic changes of hemolytic uremic syndrome may be mimicked or exacerbated by both cyclosporine and ALG, as well as by malignant hypertension and severe acute allograft rejection, and these factors should be considered in interpreting microangiopathic changes in the allograft.

Nonglomerular diseases may also recur in the allograft. Most of these, including Alport's syndrome, progressive systemic sclerosis, sickle cell nephropathy, and Fabry's disease, recur with low incidence. Oxalosis, however, historically has recurred in 90% to 100% of grafts and led to graft loss in many; current strategies to deplete oxalate stores or provide the absent enzyme or cofactors to prevent oxalate overproduction and deposition in the allograft (98) have improved this bleak prognosis.

K. Preexisting Disease

Disease in the donor kidney may have important effects on allograft function following grafting, and such disease needs to be defined to accurately interpret pathological changes in the allografted kidney. In particular, chronic changes may preexist in kidneys from older donors (99), which are being used more and more frequently for transplantation. These changes, which include glomerulosclerosis, intimal fibrous thickening in arteries and hyaline arteriolar change, and associated interstitial fibrosis and tubular atrophy need to be assessed in a peri-implantation biopsy so that pathological changes in subsequent biopsies can be interpreted adequately. Kidneys from diabetic patients are also being harvested and transplanted at some centers, and these, too, should be evaluated histologically for baseline assessment at the time of engraftment.

Other preexisting diseases, and especially glomerular diseases, may also be detected in the allograft. This is a particular problem in areas where certain diseases are endemic, such as IgA nephropathy and hepatitis B–associated glomerulonephritis in Asia. We have

also seen a few cases in which there was unsuspected pyelonephritis or vascular changes of malignant hypertension in the allograft.

L. Other

A variety of processes may have important effects on the allograft and may be diagnosed, or at least suggested, by pathological findings on biopsy of the graft.

Bland infarction on biopsy early after transplantion and in the absence of evidence of rejection may be the result of arterial or venous thrombosis in the graft caused by mechanical injury or postoperative hypotension; the size of the infarct determines impact on allograft function. Severe congestion or hemorrhagic infarction suggests venous occlusion. It has been reported that fresh infarcts or thrombosis of vessels on biopsy predicts early graft loss (98). Infarcts or thrombosis in very early biopsies, which are unlikely to be rejection-related, however, do not preclude a good graft outcome (100). Absence of collateral circulation in the allograft may modify the typical appearance of infarcts in this setting (101).

These immunosuppressed patients are vulnerable to viral infections, which may involve the allografted kidney. Of these, cytomegalovirus (CMV) is the most common. Most commonly, CMV is associated with focal mononuclear infiltrates, with some evidence for a preponderance of $CD8^+$ T lymphocytes (102). Cytomegalovirus inclusions may occasionally be seen within tubular cells or interstitial cells. Rarely, there is associated tubular damage and atrophy (103). Occasionally, CMV inclusions have been reported in glomerular endothelial and epithelial cells and vascular endothelium as well (104). We have also seen CMV in the wall of the allograft ureter. The EBV-associated B-cell lymphoproliferative disorder may be detected as a graft infiltrate; this entity is described previously. Finally, stricture of the graft may result from infection with polyomavirus which may also infect the kidney (105). Detection of virus in the allografted kidney or ureter is enhanced by immunostaining and in situ hybridization techniques. Cytomegalovirus and EBV infection are discussed in detail in Chap. 20.

Finally, ureteric complications in the transplanted kidney may produce pathological changes. With obstruction and reflux, tubular dilation, extravasated Tamm-Horsfall protein, interstitial edema, and a bland inflammatory infiltrate may be seen. It is likely that, in occasional patients, slowly developing strictures may contribute to the chronic tubulointerstitial changes denoted as chronic transplant nephropathy in the Banff classification scheme.

V. REPRODUCIBILITY OF THE BANFF CLASSIFICATION

As with any classification system, the value of the Banff schema hinges on reproducibility among pathologists, and ultimately on biological significance and clinical relevance.

Even before the Banff classification was finalized, preliminary studies were underway to assess the reproducibility of the schema. This early study was expanded to an international group of five pathologists who had helped to develop the schema. These individuals scored early renal allograft biopsies for acute histopathological findings according to the Banff classification, assessing interobserver and intraobserver variability over a range of specimens. The results of this study revealed generally adequate agreement (106), but need for improvement in definition of several changes; these improvements have since been incorporated into the schema. For vasculitis scoring, the best reproducibility was seen for v0 and v3, with probabilities of 0.86 and 0.51 and corresponding kappa values of 0.64 and 0.46. Probabilities of agreement of rejection grading varied from 0.29 (kappa 0.24) for grade I to 0.68 (kappa 0.51) for grade II. Weighted kappa values, calculated for vasculitis

scoring and rejection grade, were 0.58 and 0.55 respectively. As might be expected, intraobserver agreement was generally higher than interobserver agreement. For both interobserver and intraobserver agreement, the best reproducibility was obtained for v and i scores, with less good values for g and t scores. There was good correlation between semiquantitative scoring of interstitial inflammation and morphometric assessment of the volume fraction of inflammatory infiltrate in the cortex.

Other studies of reproducibility are ongoing and should be relevant to most aspects of the newly modified schema.

VI. CLINICAL VALIDATION STUDIES

A number of clinical validation studies of the Banff 1993 to 1995 schema have been performed. Hansen et al. have published a study assessing rejection grading using the schema and corresponding half-year and one-year renal allograft survivals (107).

Dittmer et al. also reported a small study of 23 allograft biopsies obtained in patients, comparing existing in-house diagnostic criteria to the Banff criteria. They found that the Banff criteria correlated more closely to clinical rejection and outcome (108).

Rush et al. used a sum score (the Banff Score for Inflammatory changes or BCI) to assess the predictive value of Banff scoring in protocol biopsies in predicting histology and function at one year (109). The histological changes were graded on 1-, 2-, 3-, 6-, and 12-month protocol biopsies and a cumulative sum calculated at 1 year. A significant number of these biopsies showed borderline inflammatory changes or subclinical rejection. When BSI scores were summed for each patient, normal histology and excellent function were seen only in patients with a low BSI; the difference was highly significant.

In a study of 51 biopsies from pediatric and young adult recipients with clinical evidence of rejection, Corey et al. also used Banff grading and sum score to evaluate allograft rejection and graft survival (68). They found that the diagnosis of rejection (grade I-III) and sum score greater than 5 correlated with relative steroid resistance and allograft loss. In patients with rejection, Banff grade I, II, or III, antirejection therapy was successful in 74% of those with sum less than 5, but in only 8% of those with a sum greater than or equal to 5. All six patients with vasculitis lost their allografts despite aggressive therapy. Patients with borderline changes often responded rapidly to antirejection therapy.

Gaber et al. reported on analysis of 56 biopsies obtained from cadaveric or living donor allografts with rejection to identify correlations between renal rejection scores and

Table 8 Severity of Rejection Grade Related to Duration of Graft Survival

		Graft Function	
Banff Class	N	½ year	1 year
Grade 0	52	47	46
Grade I	8	8	8
Grade II	37	26	25
Grade III	4	2	2
Not classified	4	4	4

reversal of rejection (69). Borderline biopsies showed a mean sum of 1.6 ± 0.5, grade I 3.3 ± 0.4, grade II 4.2 ± 0.3, and grade III 8.5 ± 0.4. They found sum scores useful for distinguishing borderline and grade III rejections, but found that the sum did not distinguish grades I and II rejection. Both grade and sum score correlated with complete reversal in 93% of patients with grade I rejection, but only 53% of those with grade III rejection. Vasculitis scores were significantly higher in those with irreversible rejection, as were mean scores for tubulitis and interstitial inflammation. Efficacy of steroid therapy in reversing rejection correlated with vasculitis score and sum score. Finally, the mean sum score for complete reversal of rejection was significantly lower than for partial or no reversal (110).

In a recent study focused on macrophages and chronic renal allograft nephropathy, Croker et al. correlated both the BSI and the BCI with renal survival in the 6 months after biopsy and in the entire posttransplant interval (111). Both indices predicted survival in the postbiopsy interval.

Seron et al. evaluated 3-month protocol biopsies in 98 patients, with subsequent follow-up for a mean of 58 months (112). Patients with features of chronic transplant nephropathy had an increased incidence of acute rejection and higher mean cyclosporine level before biopsy, and a significantly lower actuarial graft survival.

In important studies that led to a rethinking of the significance of subclinical histological changes for long-term survival, Hayry et al. found that the CADI score derived from protocol biopsies in largely asymptomatic patients had predictive value for the development of chronic allograft dysfunction (51).

VII. FUTURE DIRECTIONS

Assessment of renal allograft biopsies to detect rejection, define the nature of the process, or predict outcome remains an evolving process. There are a number of areas that need to be more carefully defined, and some new observations may need to be integrated into the diagnostic schema.

The Banff working classification for diagnosing and grading rejection has been recently modified and is currently undergoing testing and reassessment. The significance of borderline lesions, the differential significance of v1 versus v2 lesions, and differential grades of tubulitis to assess "severity" of rejection and responsiveness to therapy are under investigation as well.

Immunohistology or molecular biology may ultimately prove to be pivotal in defining a rejection process in the renal allograft. As discussed, identification of cytotoxic effector cells, by cell surface markers or expression of characteristic enzymes or other gene products (24,35–41), already has been shown to correlate with acute rejection in the graft. Detection of expression of growth factors, cytokines, and their receptors will probably ultimately prove to be important tools in diagnosing rejection (113). Enhanced expression of adhesion molecules in tubules and vascular endothelium has been detected in acute rejection (40,41,50), although it remains unclear whether expression of ICAM or VCAM, which correlate with inflammatory changes in the biopsy, adds significantly to histological observations made on routinely prepared sections. Moreover, expression of these adhesion molecules may be seen not only in rejection but with ischemic injury, for example, and may not be as specifically useful for ruling in rejection in the early posttransplant setting.

Defining the mechanisms underlying a specific rejection process is an area not addressed by the Banff classification in its current form. It is hoped that further studies of the molecular biology of humoral, cellular and antibody-mediated cell cytotoxicity-medi-

ated rejection processes will enable confident identification of morphological and molecular correlates of these processes. In the current formulation, the emphasis is on a "typical" cell-mediated process, but the presence of intracapillary or intratubular polymorphonuclear leukocytes, glomerular and vascular thrombi with or without infarction, and vascular deposition of C_3 with or without immunoglobulin suggests an antibody-mediated component of rejection.

Whereas acute rejection grading is being rigorously tested for reproducibility and clinical validity, assessment of chronic changes awaits testing. It is clearly chronic changes that will determine ultimate outcome of most allografts. It is hoped that the relatively straightforward semiquantitative assessment used in the schema to grade "chronic transplant nephropathy" will provide clinically useful prognostic information. Using a more complex "chronic allograft damage index," Isoniemi et al. have found that chronic changes at 2 years in normally functioning grafts were predictive of functional impairment at 4 years after transplantation (51).

Newly recognized lesions that may have prognostic significance in the renal allograft involve the peritubular capillaries. Peritubular capillary basement membrane splitting and lamination, detectable by light and electron microscopy, have been described an average of 2 years after transplantation (114,115). These changes correlated with the presence of transplant glomerulopathy, and it is assumed that both are the result of immune-mediated endothelial injury. Changes in the peritubular capillaries precede, and may be predictive of, glomerular changes (115). Potentially related observations have been made in biopsies done 1 to 12 weeks after transplantation in which significant increases in endothelial thickness and cross-sectional area, and increased adherence and passage of lymphocytes and monocytes through the peritubular capillary endothelium were seen by electron microscopy (116). Whether these alterations will have prognostic significance greater than other chronic changes routinely evaluated remains to be seen.

As newer immunosuppressive agents are introduced, pathological changes and their significance must be reassessed. Some changes, such as the presence in the graft of large numbers of eosinophils as a feature auguring poor prognosis (27), were studied before the era of cyclosporine immunosuppression and require reassessment. Although many changes are likely to be the same, important differences may emerge as clinical experience with these new agents grows. The field of pathological assessment of renal allografts remains a fertile area for investigation.

ACKNOWLEDGMENTS

The authors thank Ms. Marion Eggleston for typing the manuscript. Dr. Racusen was supported in part by grant R01 DK4381103 from the NIH.

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12

Fine-Needle Aspiration Cytology

CYNTHIA C. NAST
UCLA School of Medicine, UCLA Medical Center, and Cedars-Sinai Medical Center, Los Angeles, California

I. INTRODUCTION

The evaluation of renal dysfunction in the transplanted kidney often requires core biopsy, which is considered the diagnostic "gold standard." Renal allograft fine-needle aspiration is another, less invasive means of obtaining renal tissue for diagnostic purposes. This technique was initially described by Pasternack (1) in human allografts, wherein he identified particular leukocytes that appeared to be associated with acute rejection. Subsequently, evaluations of aspirated material from human transplant nephrectomies and various experimental models confirmed the morphological appearance of acute rejection within fine-needle aspirates (2–4). In this setting, characteristic cell infiltrates, including transformed lymphocytes, plasma cells, and macrophages, were observed consistently. Many studies have since reproduced these findings in aspirates from viable human grafts (5,6). As the interpretation of allograft aspiration cytology has become more refined, its diagnostic use has expanded to include most abnormalities associated with renal dysfunction in the first 3 to 6 months after engraftment.

Fine-needle aspiration is a safe procedure, with little associated morbidity and virtually no mortality. It may be performed on a daily basis in the outpatient setting with quick turnaround time, because processing and interpretation take approximately 3 hours. Thus,

aspiration provides a rapid and safe method for sequentially following up intragraft events, compared with the single "snapshot in time" method of a core biopsy. In addition to clinical uses, aspiration biopsy provides a unique research tool within transplant medicine. Viable intragraft cells are obtained by this method for culture or molecular biological studies, opening avenues for exploration of transplant immunology. This chapter discusses the processing and interpretation of allograft aspirates, the current use of this technique, and a range of research applications.

II. SPECIMEN HANDLING

A. Laboratory Requirements

The collection, processing, and interpretation of allograft fine-needle aspirates require few specialized tools. Collection of the samples is performed with a 22- to 25-gauge clear-hubbed spinal needle, syringes, and an aspiration gun (7). Specimens are collected into sterile aspirate medium, which is prepared in the laboratory, composed of RPMI-1640 culture medium supplemented with heparin, albumin, and HEPES. The medium must be kept refrigerated and should never be at room temperature for more than 30 minutes during the aspiration procedure. A cytocentrifuge is used to make the slides for routine cytology and immunochemical staining. If samples are to be collected for polymerase chain reaction, sterile plasticware must be used.

B. Sample Collection

Renal allograft fine-needle aspirates are obtained at the bedside in outpatient or inpatient settings, without the need for prior assessment of coagulation parameters. The patient is supine and rotated contralateral to the allograft with the ipsilateral leg straight and the contralateral leg bent, making the graft more prominent. The transplant kidney is localized by palpation or with ultrasound, and the area is sterilized; local anesthesia is usually not given. The needle is inserted into the renal cortex, trochar removed, and the needle attached to a 10-mL syringe containing 4 mL of cold sterile aspirate medium. The syringe may be fitted into an aspiration gun, which provides more suction and better samples, but this is not necessary. Suction is applied, and the needle is moved back and forth to sample a larger intrarenal area until clear or slightly blood-tinged fluid is observed within the hub. The needle then is removed rapidly, and the specimen is immediately and thoroughly washed into a sterile 10-mL tube using the aspirate medium in the syringe. Two or three aspirate samples are obtained and each is placed into a separate tube. A finger-stick blood sample is then collected into 2 to 3 mL of aspirate media in a 5-mL syringe and placed into an additional tube for separate processing. The specimens are kept at 4°C to prevent cellular degeneration and must be processed within 24 hours.

C. Sample Processing

The peripheral blood sample is always processed separately from the aspirate specimens. All samples are centrifuged at 600 rpm for 7 to 10 minutes, and the supernatant is decanted and discarded. The cells are resuspended in aspirate medium; at my institution all aspirate samples that appear to have similar degrees of blood contamination are pooled to increase the material available for various studies. Three or four drops (30–35 μL) of each pooled sample and the peripheral blood are added to separate centrifuge cups of a loaded cytocentrifuge and spun at 500 rpm for 7 minutes. The monolayer cytospin preparations are air-dried, and one or two slides from each sample are stained with May-Grunwald-Giemsa.

Additional slides are used routinely for immunochemical staining; results are best when slides are kept in a desiccator overnight before staining. A three-antibody immunoalkaline phosphatase method is used to obtain maximal staining while preventing interference from endogenous tubular cell peroxide (8). Cytospin preparations are incubated with the primary antibody for 30 to 40 minutes and then rinsed. A secondary linking antibody is added for 15 minutes, followed by washing and application of the tertiary alkaline phosphatase–antibody complex for 15 minutes. The slides are then incubated with the color development agent and counterstained. At my institution we routinely stain aspirate samples for major histocompatibility complex (MHC) class II antigen and cytomegalovirus (CMV) immediate-early nuclear proteins.

Aspirate and peripheral blood specimens may be stored for later assessment by polymerase chain reaction. After resuspending the cells in aspirate medium and appropriate pooling of samples, aliquots are snap frozen. The samples remain intact for up to 1 year when maintained at –70°C. At the time of processing, cells are thawed, pelleted, suspended in dimethylpyrocarbonate-treated water and boiled for 5 minutes. Samples are centrifuged and the supernatant containing RNA is extracted for routine reverse transcription polymerase chain reaction (9). In this way, cells infiltrating the graft and intrinsic renal cells can be assessed for gene expression in clinical and experimental settings.

III. SPECIMEN EVALUATION

The evaluation of renal allograft aspirates includes the assessment of all elements within the kidney and peripheral blood. Tubular epithelial cells, and intragraft and peripheral blood leukocytes are the most important components for interpretation. Additionally, endothelial cells and all other cell types that may be present contribute to the ultimate diagnostic accuracy of this technique.

A. Sample Adequacy

The May-Grunwald-Giemsa stained aspirate cytospins are initially screened at low magnification to assess cellularity. The slide with the most numerous renal parenchymal cells, including tubular epithelial and endothelial cells, is further assessed (10,11). An adequate sample is one of cortical origin with minimal blood contamination. Adequacy is determined by the number of parenchymal cells and defined as greater than or equal to seven tubular and/or endothelial cells per 100 leukocytes within the aspirate (10,12). If there is excess blood contamination, there is a lower concentration of parenchymal cells and the specimen is inadequate. The presence of glomeruli indicates a cortical sample, but glomeruli are observed infrequently (Fig. 1). A cortical origin also can be determined by the presence of proximal tubular cells; at my institution, we have evaluated tubular cells from different nephron segments for this purpose. Proximal tubular cells have a unique appearance, being large (350 μ^2) with a low nuclear to cytoplasm ratio of 1:4 and occurring singly or in small clusters (13). In contrast, distal cells are smaller (171 μ^2) with a 1:2 nuclear cytoplasmic ratio and occur in aggregates of 10 to 20 cells. Collecting duct cells are even smaller (99 μ^2) and cluster in groups of 2 to 7 cells. Small numbers of proximal cells (<50% of tubular cells) indicate a predominately noncortical sample, which is also inadequate.

B. Parenchymal Cells

Tubular and endothelial cells are evaluated qualitatively as well as quantitatively as part of the overall interpretation of allograft fine-needle aspirates (6,10,12,14). Within an aspirate,

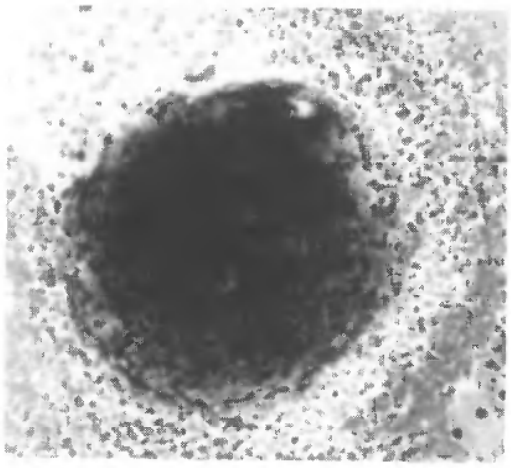

Figure 1 A glomerulus without well-defined capillary or mesangial structures. (May-Grunwald-Giemsa ×125.)

tubular cells appear normal or damaged, with differing morphology, dependent on the extent and origin of injury (Fig. 2). Normal tubular cells have round to oval nuclei with variable amounts of cytoplasm and intact cell borders (13). Injured tubular cells first display cytoplasmic swelling followed by irregular cytoplasmic vacuoles. Tubular cells damaged by cyclosporine contain small isometric cytoplasmic vacuoles, and, with more severe injury, phagocytize erythrocytes while in the aspirate medium before processing (15). Degenerated tubular cells are small with condensed cytoplasm and pyknotic nuclei. Necrotic cells are even smaller with markedly pyknotic to absent nuclei and more condensed cytoplasm.

Endothelial cells are present less frequently in aspirates, and they do not have the range of changes observed in tubular cells. Normal endothelial cells have rounded nuclei with a single eccentric prominent nucleolus and round cytoplasmic borders (Fig. 3). When activated, endothelial cells have swollen cytoplasm. These cells are predominantly used for determining adequacy and have limited utility in the interpretation of renal allograft aspiration cytology.

C. Leukocytes

Leukocytes are assessed quantitatively in the aspirate and peripheral blood specimens. A wide spectrum of normal and activated white blood cells is evaluated; these include lymphocytes, large granular lymphocytes (natural killer cells), activated (early transformed) lymphocytes, immunoblasts (fully transformed lymphocytes), monocytes, macrophages, monoblasts, plasma cells, neutrophils, basophils, and eosinophils (Fig. 4). In experimental and human studies, an intragraft leukocytic infiltrate of immunoblasts, activated lymphocytes, plasma cells, and macrophages was found to be the hallmark of acute rejection occurring in the transplanted kidney (2–5,12,16). This process is termed immune activation. Evaluation of allograft aspiration is based on the presence of immune activation and the identification of particular cell types in the aspirate and peripheral blood.

Immune activation is assessed in two ways: by the absolute number of blasts and plasma cells and by the increment method. In the former method, the entire aspirate cytospin preparation is screened and the total sum of immunoblasts and plasma cells tabulated. At my institution, immune activation is present when this number is greater than 4 (17). The increment method was developed by von Willebrand as a means to eliminate the effect of blood contamination in allograft aspirates and enhance diagnostic accuracy (18). In this method, 100-cell differential leukocyte counts are performed separately on the aspirate and

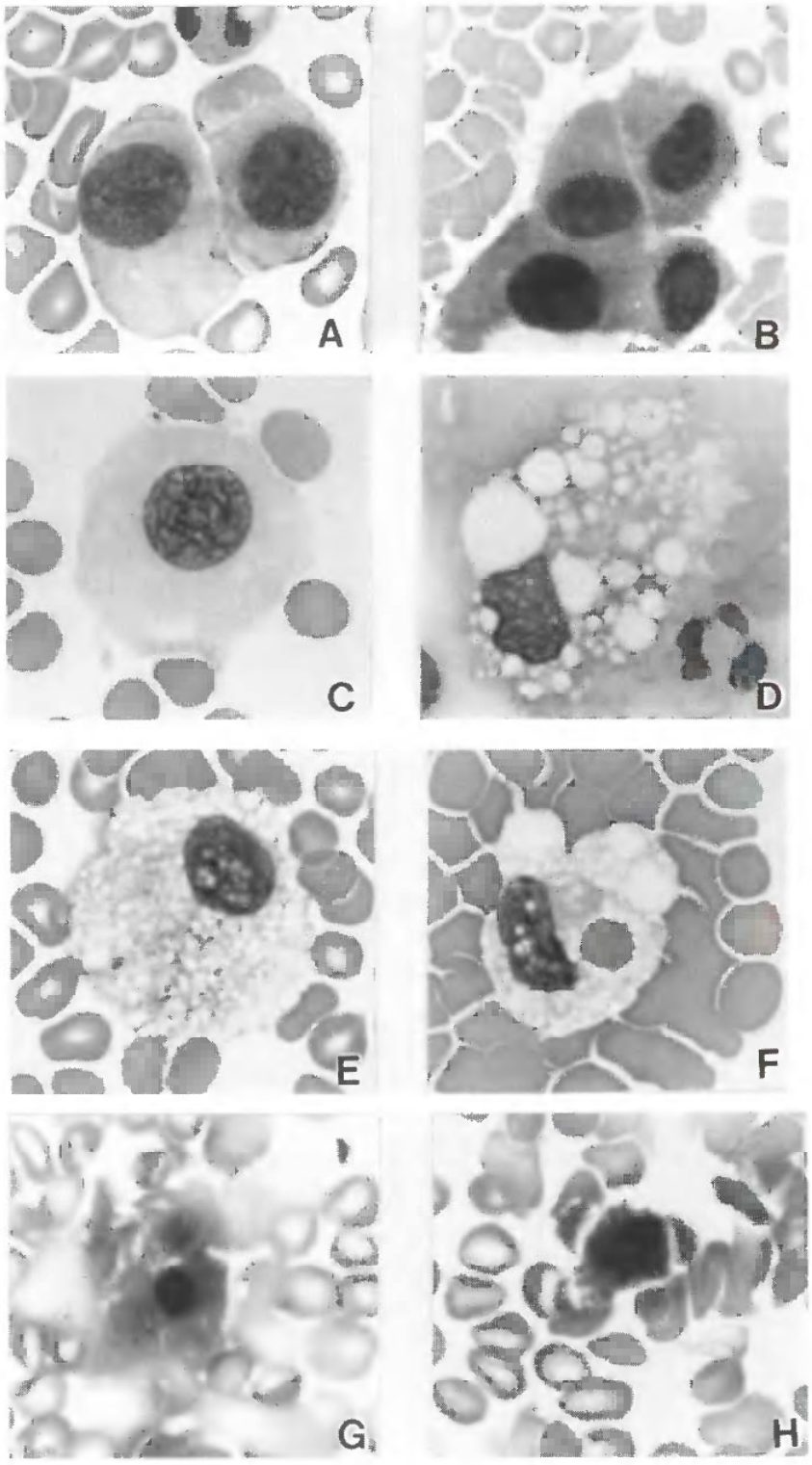

Figure 2 Range of tubular cell appearances in increasing severity of injury. A. Normal proximal cells. B. Normal distal cells. C. Swollen cytoplasm. D. Swollen cytoplasm containing irregular vacuoles. E. Isometric vacuoles in swollen cytoplasm. F. Swollen vacuolated cytoplasm with a phagocytized erythrocyte. G. Degeneration with condensed cytoplasm and nucleus undergoing pyknosis. H. Necrosis with markedly pyknotic nucleus and condensed cytoplasm. (May-Grunwald-Giemsa, ×760.)

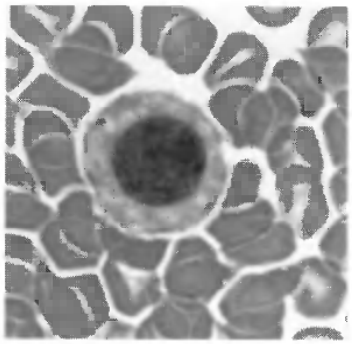

Figure 3 Endothelial cell. The cell and nucleus are round with a prominent eccentric nucleolus. (May-Grunwald-Giemsa, ×760.)

peripheral blood cytospin preparations at ×40 magnification. The peripheral blood differential count is subtracted from that of the aspirate, resulting in increments for each leukocyte type counted (Table 1). Only positive increments are subsequently used, and these are multiplied by correction factors that more heavily weight those cell types most closely associated with acute rejection. Immunoblasts and plasma cells in the peripheral blood are added to, not subtracted from, the aspirate count because they reflect more widespread immune activation. The corrected increments are then added to give the total corrected increment (TCI), a numerical quantitation of intragraft inflammation. In initial studies at my institution, we determined a TCI of greater than 3.0 indicates immune activation (17); this number varies somewhat between laboratories. Therefore, we define immune activation as an immunoblast and plasma cell count greater than 4 and/or a TCI of greater than 3.0. In practice, however, a TCI between 2.8 and 3.2 should be considered borderline, with clinical correlation and ancillary immunostaining required to make a firm diagnosis.

IV. INTERPRETATION

The interpretation of renal transplant fine-needle aspiration cytology is based on the presence of immune activation, qualitative assessment of aspirate and peripheral blood leukocytes, parenchymal cell morphology, and immunochemical findings. It is necessary for the person interpreting the samples to have a working knowledge of transplant medicine and preferably of routine transplant pathology as well; the ability of the reader to correlate aspirate and core needle biopsy specimens considerably enhances diagnostic accuracy and identification of minor abnormalities reflected in the aspirates. As with all biopsy specimens, clinical correlation is required for optimal interpretation.

Aspirates from well-functioning normal grafts have no immune activation (TCI <3.0 and four or fewer immunoblasts and/or plasma cells), tubular cells that are normal or mildly swollen with only infrequent coarse vacuoles, and unremarkable peripheral blood (Fig. 5). (17,19,20)

A. Acute Rejection

Aspirates from patients with acute rejection demonstrate immune activation and variable degrees of tubular cell injury (Fig. 6) (10,12,14). Early in the course of rejection, there is a marked infiltrate of immunoblasts and plasma cells (>4) with an elevated TCI (>3.0); often, the immunoblast count is disproportionately elevated. More established or partially treated rejection episodes have fewer blasts and plasma cells with a greater number of intragraft

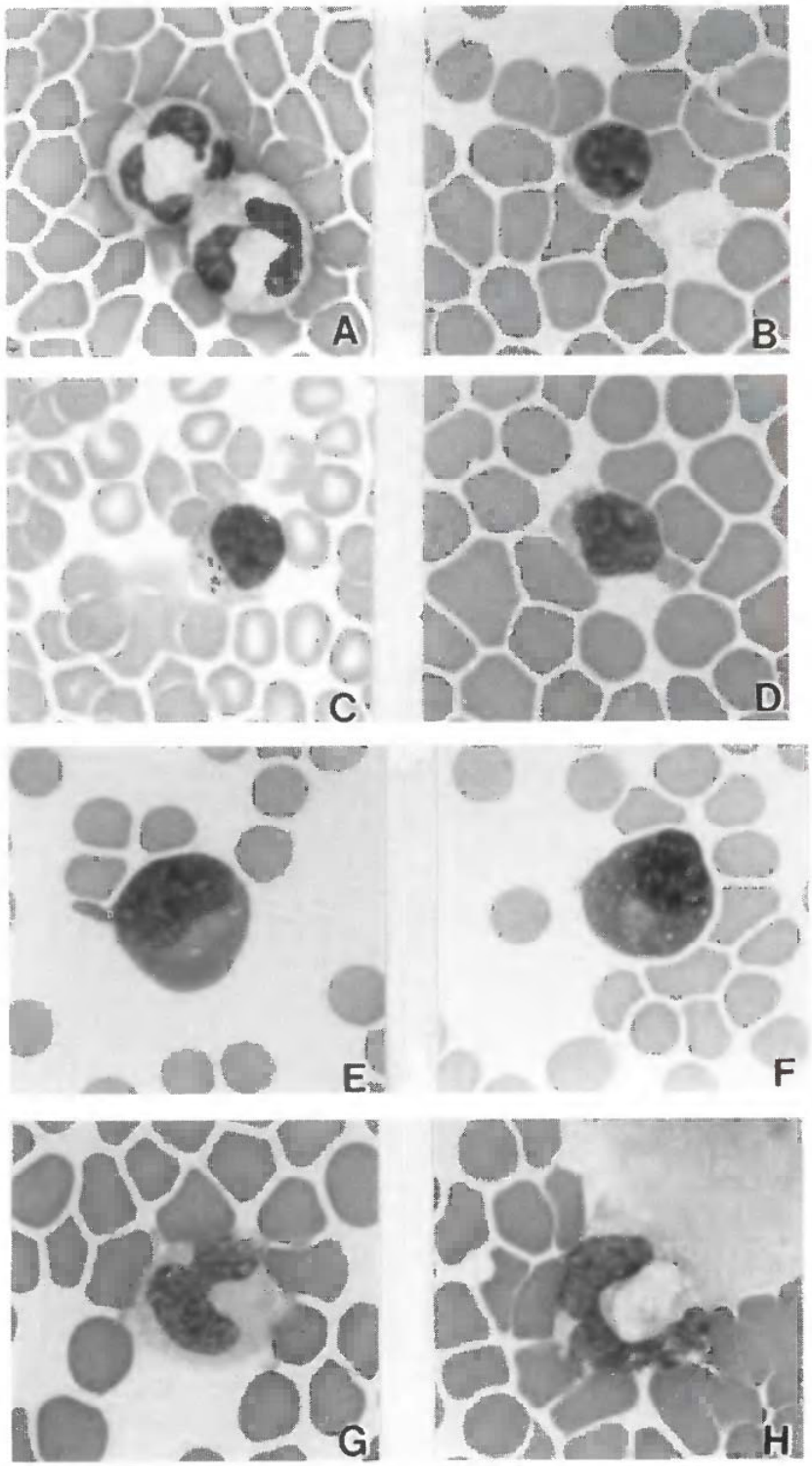

Figure 4 Leukocytes observed within allograft fine-needle aspirates. A. Neutrophils. B. Mature lymphocyte. C. Large granular lymphocyte (natural killer cell) containing cytoplasmic granules. D. Activated (early transformed) lymphocyte, which is larger than a mature lymphocyte with more open nuclear chromatin. E. Immunoblast (fully transformed lymphocyte) with abundant cytoplasm, prominent golgi, and immature nucleus containing several nucleoli. F. Plasma cell with the characteristic clumped nuclear chromatin, eccentric nucleus, and prominent golgi. G. Monocyte. H. Macrophage with eccentric elongated nucleus and cytoplasmic vacuoles, which often contain phagocytized material. (May-Grunwald-Giemsa, ×760.)

Table 1 Aspirate Worksheet for a Patient with Acute Transplant Rejection[a]

Leukocytes	Aspirate (%)		Peripheral Blood (%)		Increment		Correction Factor		Corrected Increment
Immunoblast	3	+	1	=	4	×	1.0	=	4.0
Plasma cell	1	+	0	=	1	×	1.0	=	1.0
Macrophage	0	–	0	=	0	×	1.0	=	0
Activated lymphocyte	4	–	2	=	2	×	0.5	=	1.0
Large granular lymphocyte	2	–	1	=	1	×	0.2	=	0.2
Monocyte	6	–	3	=	3	×	0.2	=	0.6
Lymphocyte	28	–	6	=	22	×	0.1	=	2.2
Neutrophil	53	–	85	=	0	×	0.1	=	0
Eosinophil	3	–	2	=	1	×	0.1	=	0.1
Basophil	0	–	0	=	0	×	0.1	=	0
					Total Corrected Increment				9.1

[a]One hundred cell differential leukocyte counts are performed on the aspirate and peripheral blood cytospin preparations. These are recorded and the blood count is subtracted from the aspirate count (except for immunoblasts and plasma cells) giving the increments; negative increments are not used. The increments are multiplied by correction factors weighted for the cell type association with acute rejection. The corrected increments are added to give the total corrected increment.

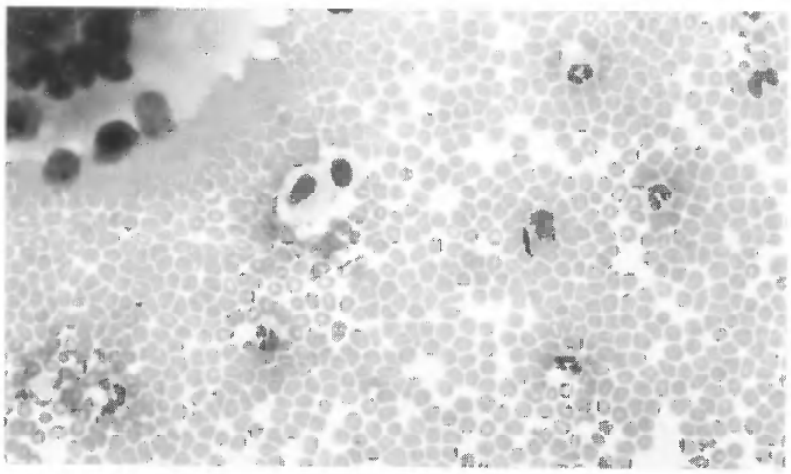

Figure 5 Aspirate from well-functioning allograft. There are ample normal tubular cells and the inflammatory infiltrate is similar to that in the peripheral blood. (May-Grunwald-Giemsa, ×250.)

lymphocytes, monocytes, activated lymphocytes, and possibly macrophages. Macrophages are more prominent in long-standing, severe, or irreversible rejection episodes (5,10,11). In severe rejection episodes, there may be blasts and plasma cells within the peripheral blood. Tubular cell findings include swelling, irregular vacuolization, degeneration, and necrosis.

Immunochemical staining is of great use in fine-needle aspiration. Analysis of MHC class II antigens reveals positive tubular cell staining in more than 30% of all tubular cells present at the time of rejection (Fig. 7) (8,21,22). Class II antigens are not constitutively expressed on tubular cells but these antigens are upregulated by interferon (IFN)-γ which is

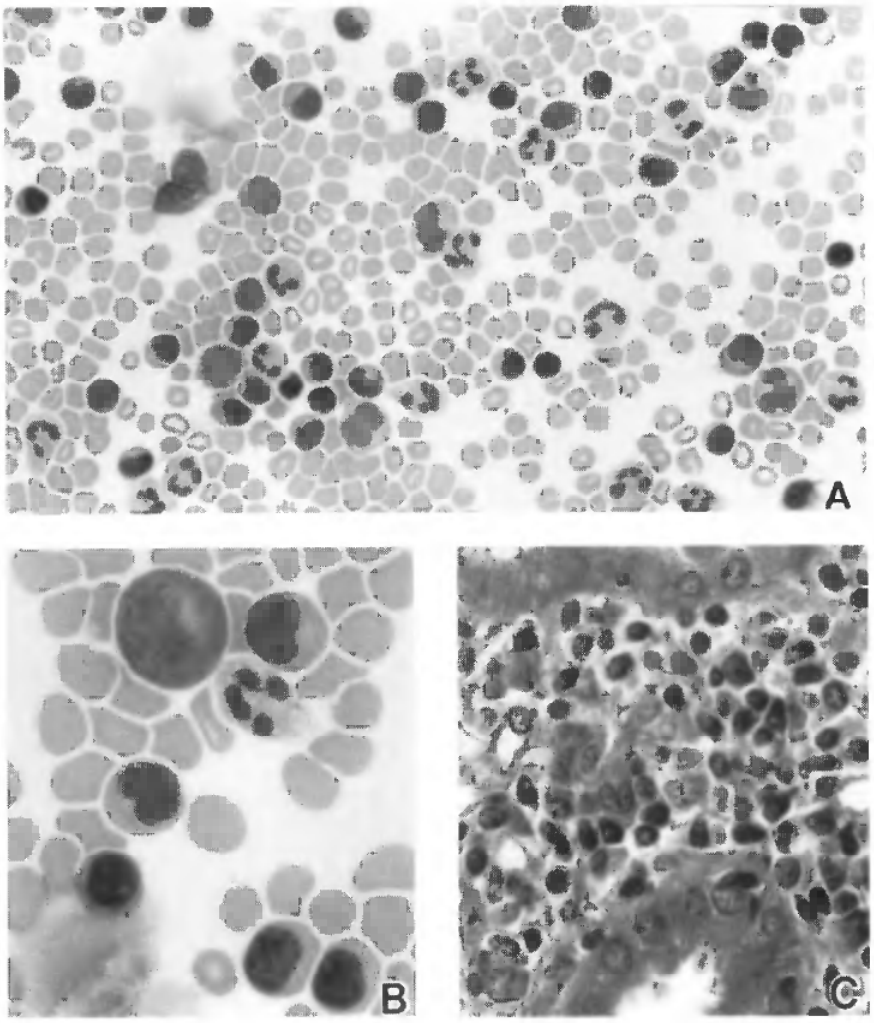

Figure 6 Acute transplant rejection. A. There are many lymphocytes and activated lymphocytes with immunoblasts and monocytes in the aspirate. (May-Grunwald-Giemsa, ×250.) B. The mononuclear cells are in much higher concentration than in peripheral blood and an immunoblast is present (large cell). (May-Grunwald-Giemsa, ×500) C. Compare to the infiltrate in the corresponding core biopsy (Hematoxylin and eosin, ×500.)

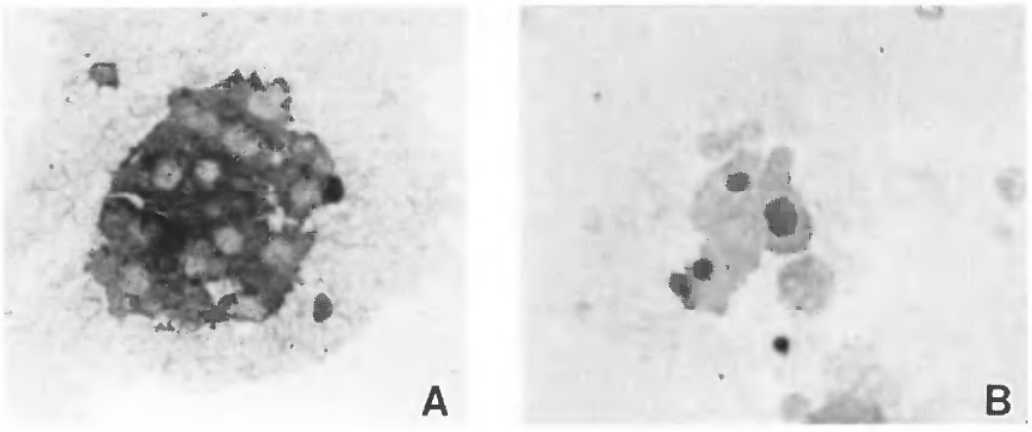

Figure 7 A. Positive tubular cell cytoplasmic major histocompatibility complex class II immunostaining in acute transplant rejection. B. Negative tubular cell staining in aspirate from patient with acute tubular necrosis without rejection. (×250.)

present during the onset of acute rejection (9). With successful antirejection therapy, blasts disappear within 48 hours and immune activation subsides over 2 to 5 days. Interferon-γ rapidly disappears after successful therapy, whereas tubular cell class II antigen staining abates more slowly over a 2-week time period (9). The types of infiltrating lymphocytes have been evaluated to assess usefulness in identifying acute rejection episodes. However, studies examining the subtypes of graft-infiltrating leukocytes (CD4, CD8) have had conflicting results (26–26). Therefore, we do not evaluate leukocyte subtypes routinely. In summary, using routine and MHC class II immunochemical stains, the sensitivity and specificity of aspiration approaches 95% for the diagnosis of acute rejection in an adequate sample.

B. Viral Infection

Viral infection of the allograft induces immune activation, with increases in the blast and plasma cell count and TCI (8,10). However, there are subtle differences between the infiltrate in viral infection as compared to that observed in acute rejection. Viral disease is associated with a more pronounced increase in intragraft plasma cells relative to immunoblasts and with elevated numbers of large granular lymphocytes in the graft and the peripheral blood (Fig. 8).(27,28) A hallmark of viral infections is the disproportionately large number of blasts or plasma cells in the peripheral blood compared with the graft; this is because viral disease is a systemic process moving from blood into the kidney, whereas severe rejection is an intragraft process spilling over into the peripheral blood. Atypical monocytes and activated lymphocytes also may be observed in the peripheral blood. Tubular cells demonstrate variable injury, similar to acute rejection; viral inclusions are virtually never found.

Immunochemical staining is useful in establishing a diagnosis of CMV infection. In active infection, CMV immediate-early proteins are identified within greater than 35% of the tubular cell population (Fig. 9). In contrast, we have found that MHC class II antigen staining is not associated with viral infection but is specific for acute rejection, and is the best means to distinguish between CMV infection and acute rejection (8). In situ hybridization has been used to identify viral genome in renal tissue (29). However, we have found immunochemical techniques easier to perform and just as sensitive as in situ hybridization in aspirated samples.

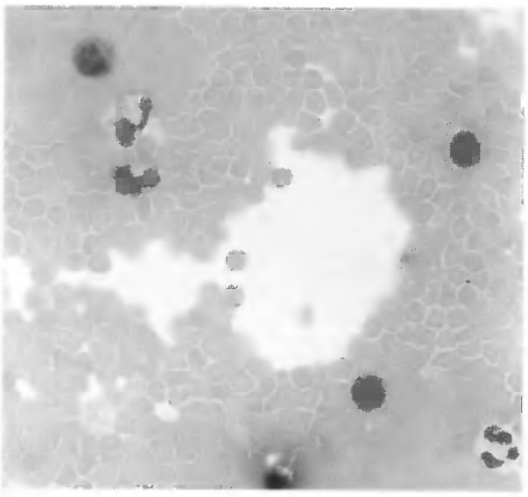

Figure 8 Cytomegalovirus infection. The aspirate shows immune activation with an immunoblast, activated lymphocyte, and monocyte. (May-Grunwald-Giemsa, ×250.)

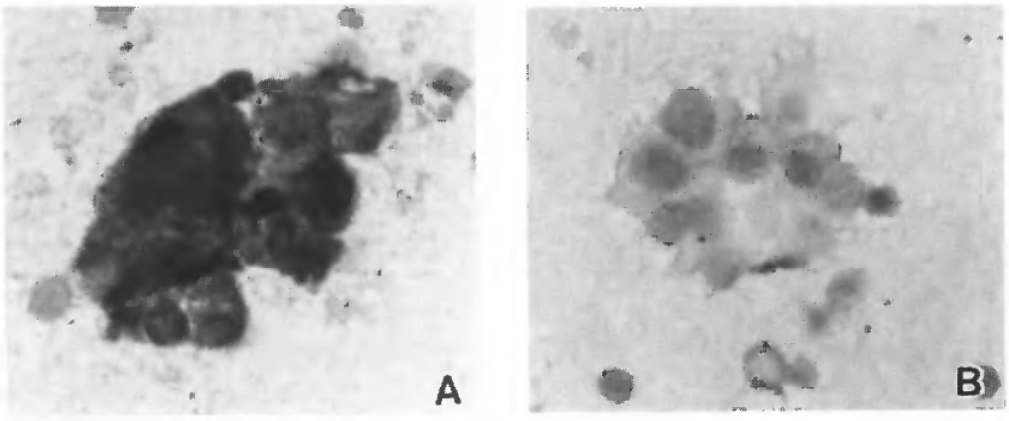

Figure 9 A. Tubular cells staining for cytomegalovirus (CMV) immediate-early proteins showing nuclear and cytoplasmic staining. B. Negative control from a patient without active CMV infection. (×250.)

C. Acute Tubular Necrosis

Aspirates from patients with acute tubular necrosis contain injured tubular cells, ranging from swollen and irregularly vacuolated to degenerated and necrotic cells (Fig. 10). There is no immune activation. Immunochemical stains for MHC class II antigens and CMV proteins are negative. The severity of tubular cell damage correlates well with the severity of clinical renal dysfunction and with tubular necrosis on core biopsy. As the tubular necrosis resolves, there are fewer necrotic cells and an increase in cells undergoing mitotic activity (Fig. 11).

D. Acute Cyclosporine Toxicity

Cyclosporine induces a number of characteristic changes in renal allograft fine-needle aspirates. Small numbers of tubular cells (<15%) contain cytoplasmic small isometric vacuoles in the majority of patients receiving cyclosporine. However, the presence of isometric vacuoles in greater than 50% of the tubular cell population is diagnostic for cyclosporine toxicity (15). Tubular cell phagocytosis of erythrocytes, and cytoplasmic isometric vacuoles in aspirate neutrophils and monocytes are also features of cyclosporine toxicity (Fig. 12) (15,17). Vacuoles are not present in the peripheral blood leukocytes. There is a variable degree of tubular cell degeneration and necrosis, usually without immune activation; MHC class II antigen and CMV protein stains are negative. There are few reports of cyclosporine inducing lymphocytic infiltrates in the kidney, which may result in immune activation resulting solely from nonactivated lymphocytes in an allograft aspirate (30,31). We have not observed this form of immune activation in patients given the initial oral formulation of cyclosporine; however, patients receiving the longer acting micromulsion formulation do appear to have more frequent infiltrates of lymphocytes and monocytes causing immune activation without activated lymphocytes, immunoblasts, or plasma cells. In this setting, the presence of other typical features of cyclosporine toxicity with negative MHC class II staining aids in making the correct diagnosis.

Tubular cell isometric vacuoles are observed much more frequently in aspirates than in core biopsies, making aspiration a more sensitive test for this abnormality. However, tubular cells may contain small cytoplasmic vacuoles within the first 5 days after transplantation as a nonspecific form of injury. Therefore, cyclosporine nephrotoxicity cannot be assessed accurately until after this time. As with core biopsies, there are some cases of

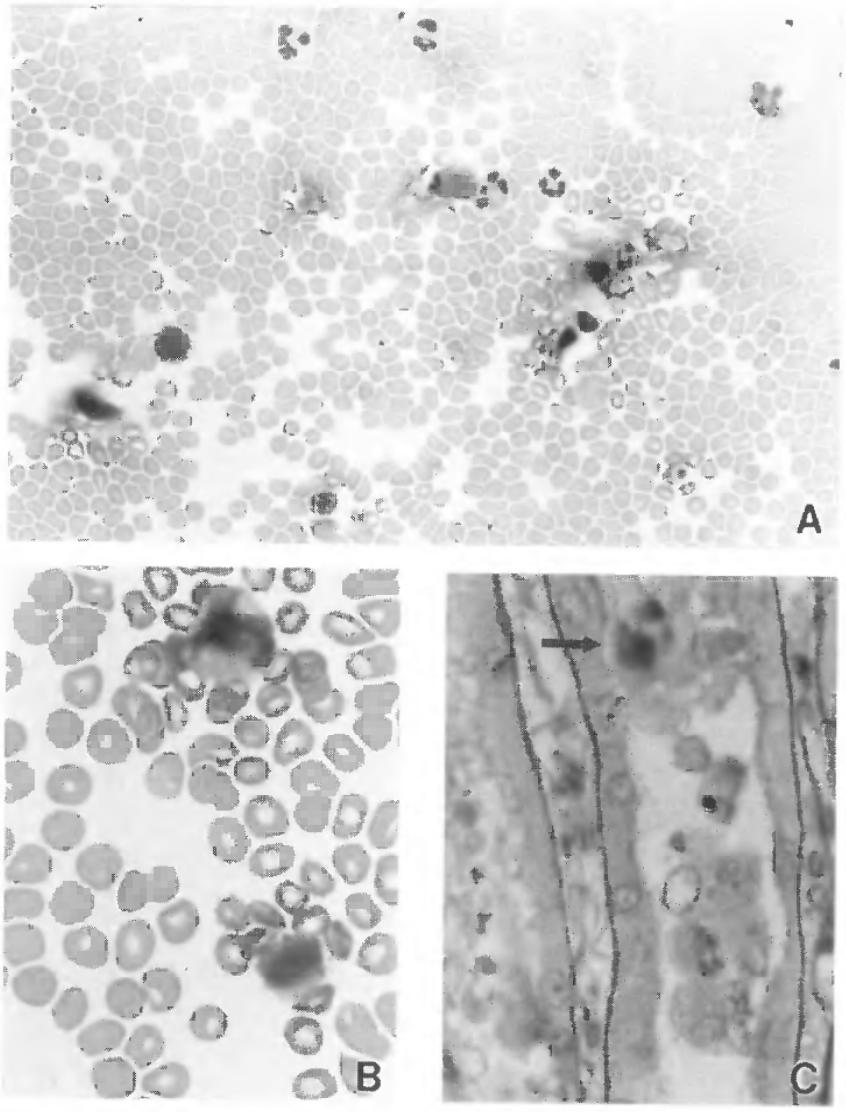

Figure 10 Acute tubular necrosis. A. The aspirate contains several necrotic tubular cells without immune activation. (May-Grunwald-Giemsa, ×250.) B. Higher magnification of necrotic tubular cells (May-Grunwald-Giemsa, ×500), which are similar to C. C. Necrotic tubular cells (arrow) in a core biopsy from a patient with acute tubular necrosis. (Jones-methenamine silver, ×500.)

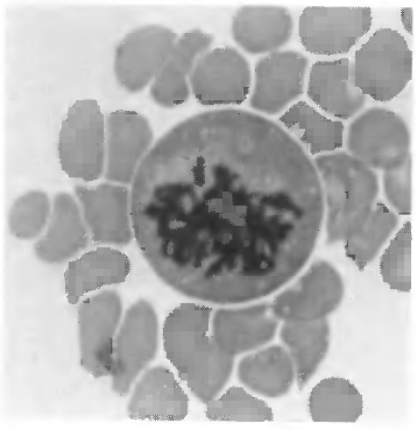

Figure 11 Mitotic figure in a fine-needle aspirate. (May-Grunwald-Giemsa, ×760.)

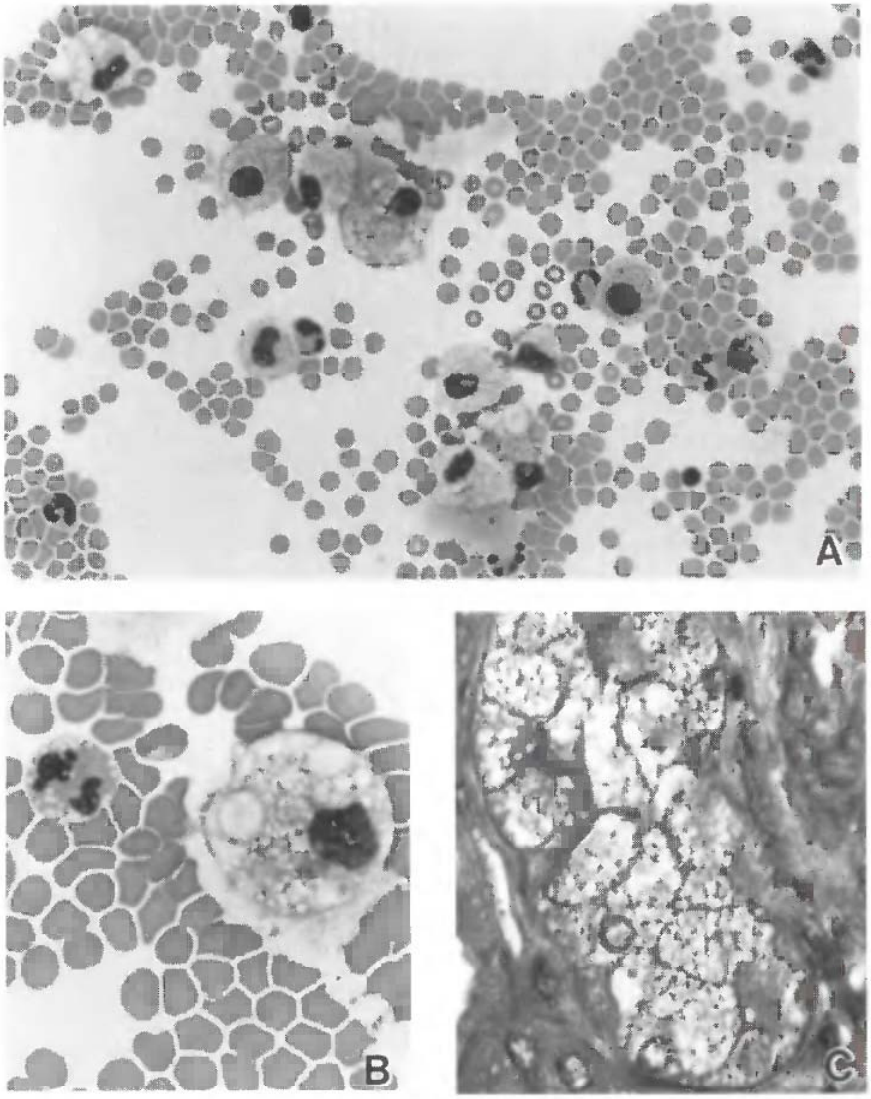

Figure 12 Acute cyclosporine toxicity. A. There are many isometrically vacuolated tubular cells without immune activation in the aspirate. (May-Grunwald-Giemsa, ×250.) B. Tubular cell containing isometric and large vacuoles; cells often contain both types in cyclosporine toxicity. A neutrophil also has isometric cytoplasmic vacuoles. (May-Grunwald-Giemsa, ×500.) C. Compare the vacuolated aspirate tubular cell to those in a core biopsy from a patient with cyclosporine toxicity. (Masson's trichrome, ×500.)

cyclosporine toxicity with only mild tubular cell damage, requiring clinical correlation to distinguish between acute tubular necrosis and cyclosporine toxicity. After reduction of the cyclosporine dose, the number of isometric vacuoles within tubular cells slowly declines over a period of weeks (32).

E. Bacterial/Fungal Infection (Acute Pyelonephritis)

Acute infection is readily identified in fine-needle aspirates. The number of neutrophils is similar to, or increased from, that in the peripheral blood. Microorganisms are easily observed with the May-Grunwald-Giemsa stain and are found intracellularly and extracellularly (Fig. 13) (33,34). There is a variable amount of tubular necrosis and often a background of cellular debris. There is no immune activation. The MHC class II antigen and

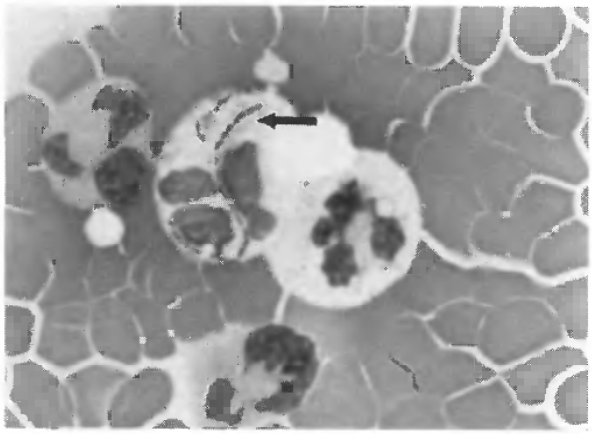

Figure 13 Aspirate neutrophils from a patient with acute infectious interstitial nephritis (acute pyelonephritis). One neutrophil contains engulfed bacteria (arrow). (May-Grunwald-Giemsa, ×760.)

CMV protein stains are negative. The aspirate medium must be examined to determine whether there was contamination of the medium before aspiration to avoid false-positive results. If acute bacterial or fungal infection is suspected, the aspirate sample may be cultured as it is obtained in a sterile medium.

F. Infarction

Infarction results in extensive cellular necrosis with a background of debris within the aspirate (Fig. 14). There are scattered neutrophils, monocytes, and possibly macrophages with fragments of these and other renal cells. No microorganisms are identified, and there is more cellular destruction than in acute bacterial infection. There may be large numbers of neutrophils if the area of inflammation adjacent to the infarct is aspirated. There is no immune activation, and immunostains are negative. If infarction is diagnosed, a core biopsy is usually recommended for confirmation.

G. Other Abnormalities

1. FK506 (Tacrolimus) Toxicity

FK506 is used increasingly in renal transplantation. Acute toxicity has a picture similar to cyclosporine toxicity on core biopsy examination (35) and in fine-needle aspirates. There are isometric vacuoles within the cytoplasm of tubular cells and intragraft leukocytes, although the vacuoles may be somewhat larger than those seen in patients receiving cyclosporine (Fig. 15). Tubular cell degeneration and necrosis occur to varying degrees, with tubular cell phagocytosis being evident. No immune activation is present, and immunostains for MHC class II antigens and CMV proteins are negative. As with cyclosporine, diminishing numbers of tubular cell vacuoles may persist for days to weeks following reduction of the FK506 level.

2. Posttransplant Lymphoproliferative Disorder

There are few reports of posttransplant lymphoproliferative disorder (PTLPD) diagnosed by allograft aspiration cytology (36). I have observed two cases. Features include typical and atypical plasma cells and abnormal large immunoblasts containing convoluted nuclei

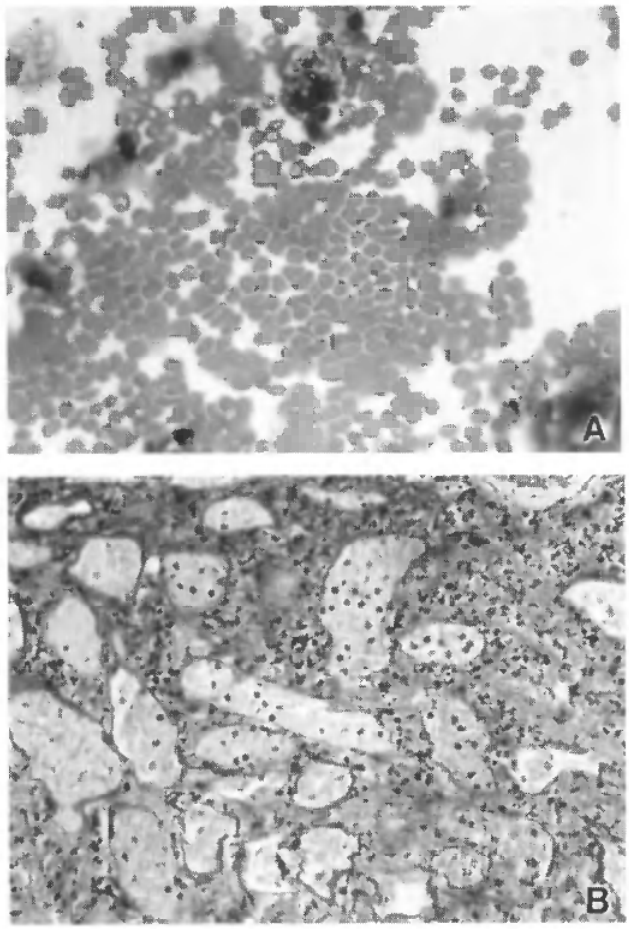

Figure 14 A. Aspirate from a patient with renal infarction. There are degeneration and necrosis of most cellular elements. (May-Grunwald-Giemsa, ×250.) B. Compare with the corresponding renal core biopsy sample showing similar cellular necrosis. (Periodic acid–Schiff, ×250.)

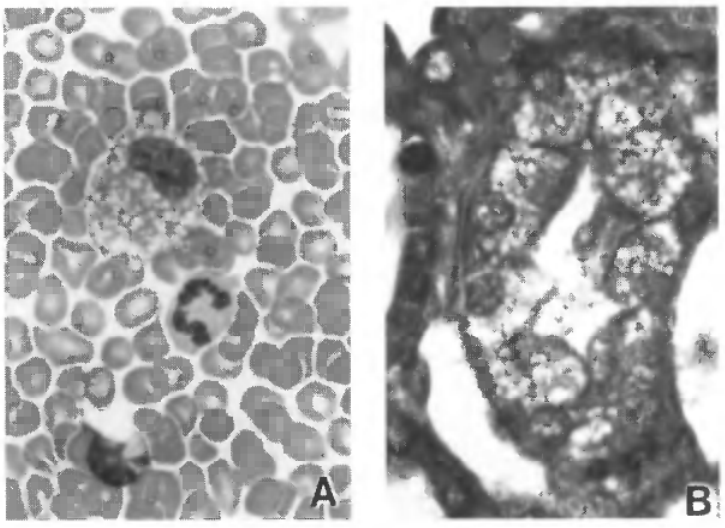

Figure 15 Acute FK506 toxicity. A. An aspirated tubular cell contains cytoplasmic isometric vacuoles. (May-Grunwald-Giemsa, ×500.) B. Core biopsy specimen from a patient with FK506 toxicity showing similar tubular cells. (Masson's trichrome, ×500.)

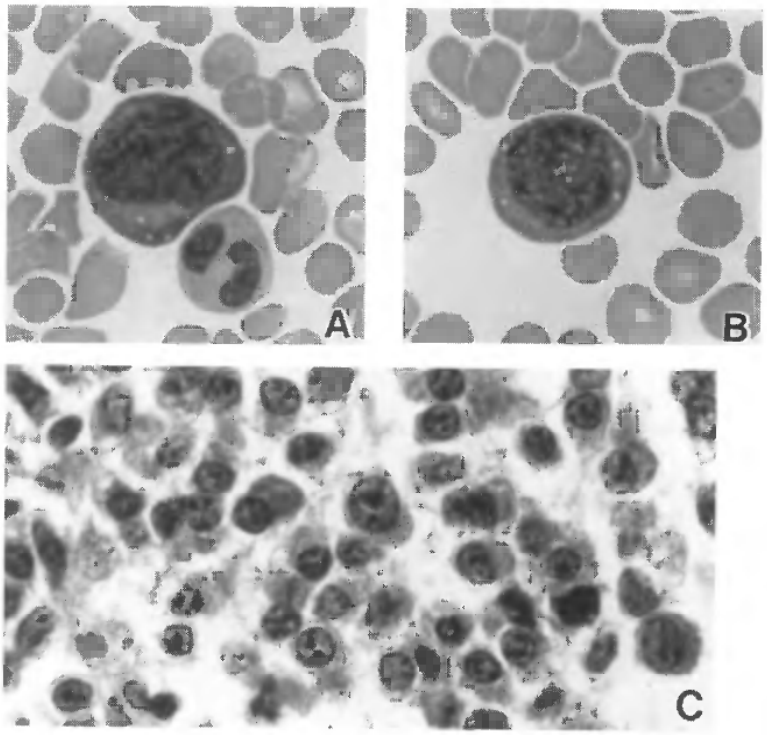

Figure 16 Posttransplant lymphoproliferative disorder. A and B. Aspirated atypical immunoblasts with large nuclear to cytoplasmic ratio, very immature nuclear chromatin, nucleoli, and small vacuoles. (May-Grunwald-Giemsa, ×760.) C. Compare to core biopsy specimen from the same patient. (Hematoxylin and eosin, ×760.)

with prominent numerous nucleoli and moderate to scanty cytoplasm with small vacuoles (Fig. 16). There are also atypical activated lymphocytes with nuclear folding, and scattered monocytes. The MHC class II antigens and CMV protein staining are negative. If there is adequate material, kappa and lambda immunoglobulin staining should be performed to determine monoclonality, a poor prognostic indicator. Staining for Epstein-Barr virus latent membrane protein or EBER is also helpful in confirming PTLPD because most cases are EBV positive (37). If there is active viral infection at the time, the peripheral blood also contains plasma cells and atypical immunoblasts. A diagnosis of PTLPD is an indication for core biopsy, for confirmation and further immunological workup.

3. Acute Interstitial Nephritis

Drug-induced or allergic acute interstitial nephritis can be difficult to differentiate from acute rejection in aspirate material and core biopsies. However, there are some subtle features within fine-needle aspirates allowing for diagnosis in these cases. There is immune activation with normal or only mildly increased numbers of immunoblasts and plasma cells. Pronounced increases in intragraft eosinophils and activated or immature monocytes are found, with a mild to moderate intragraft lymphocytosis (Fig. 17).

In an unpublished study, my colleagues and I reviewed 250 aspirates from 28 pediatric renal allograft recipients. Eleven aspirates from four patients with intrarenal eosinophilia were identified, with intrarenal eosinophilia defined as a corrected eosinophil count of greater than 5%. Review of the clinical course and medications demonstrated association with antibiotic use for all patients (10 of 11 aspirates) (Fig. 18). Two patients had CMV infection associated temporally with intragraft eosinophilia. No patients had acute rejec-

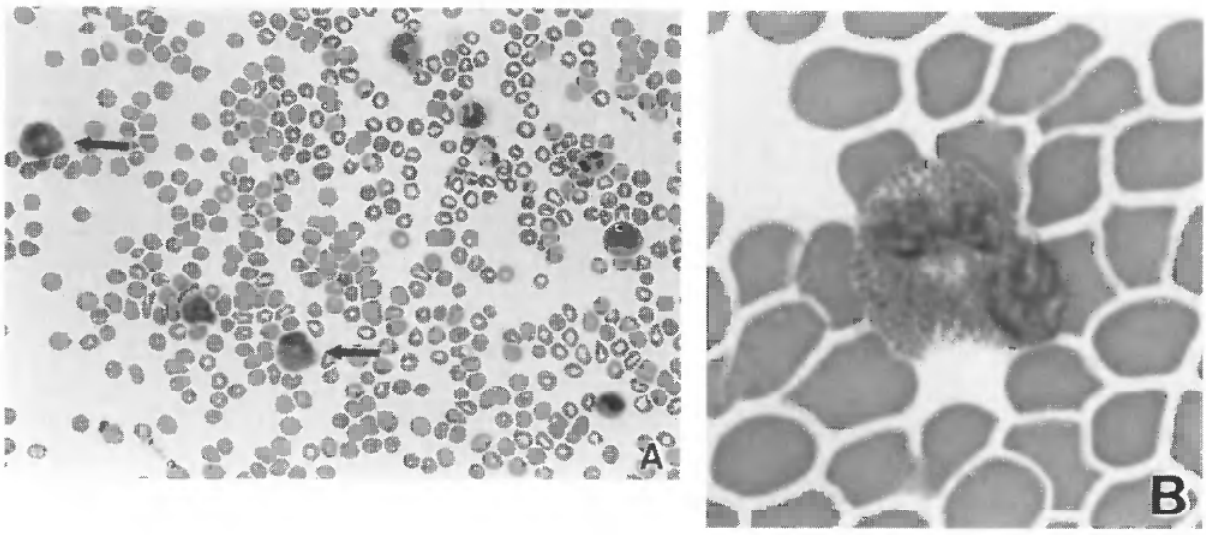

Figure 17 Acute interstitial nephritis. A. The aspirate contains increased eosinophils (arrows) and monocytes with one immunoblast. (May-Grunwald-Giemsa, ×250.) B. An eosinophil. (May-Grunwald-Giemsa, ×500.)

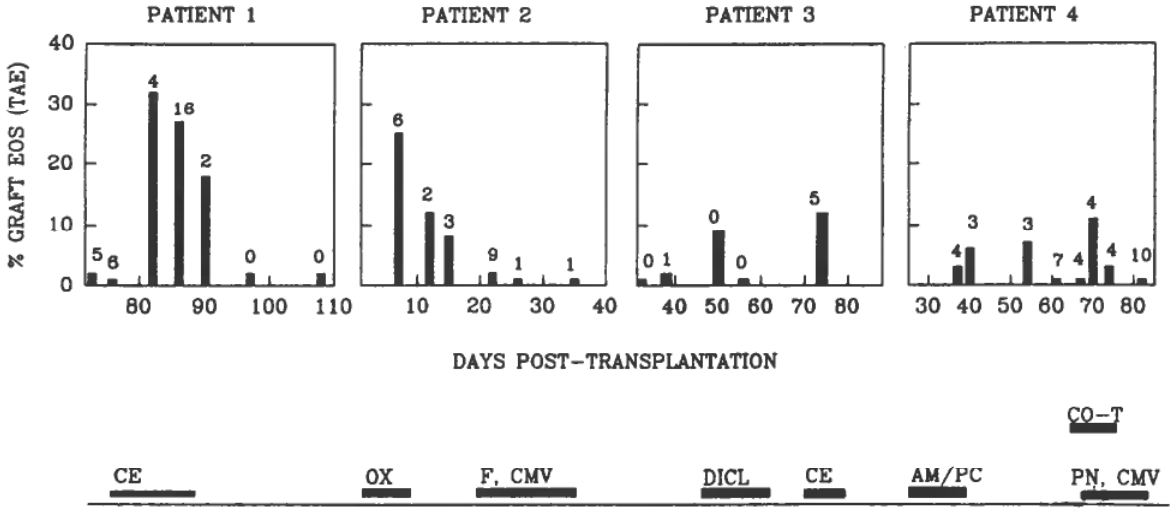

Figure 18 Time course of transplant associated eosinophilia (TAE) in four pediatric patients. The ordinate represents percent corrected graft eosinophilia. The abscissa represents the time course in days after transplantation. The bars below indicate the duration of treatment with antibiotic (CE = cephalexin, Ox= oxacillin, DICL = dicloxacillin, AM/PC = amoxacillin/potassium clavulinic acid, CO-T = co-trimoxazole) or clinical diagnosis (F = fever, CMV = cytomegalovirus infection, PN = pneumonia). The numbers above the vertical bars indicate the percent eosinophils in the peripheral blood as determined by differential.

tion, renal insufficiency, history of atopy, or systemic allergic reaction to the identified antibiotics (oxacillin, co-trimoxazole, cephalexin, amoxacillin/potassium clavulanate, dicloxacillin), or evidence of hepatitis or Epstein-Barr virus infection at the time of aspiration. Other investigators have identified eosinophils in the graft and peripheral blood in acute rejection (38–40), but we have found increased intragraft eosinophilia only with allergic interstitial nephritis. In acute interstitial nephritis, tubular cells exhibit some degeneration or necrosis. The MHC class II stains may be positive, so this test cannot distinguish this

entity from acute rejection. Often, the diagnosis can only be suggested, and clinical correlation with recent drug exposure is necessary; core biopsy may not contribute significantly to the diagnosis.

4. Lymphocele/Perinephric Fluid Collection

Lymphoceles are not uncommon in renal transplantation and may overlie the graft unbeknownst to the physician, because ultrasonography usually is not performed for fine-needle aspiration. When the needle is inserted for aspiration and manipulated, the lymphocele may be included in addition to material from the kidney, or the lymphocele alone may be aspirated. Therefore, tubular cells may or may not be present within the sample and the specimen may be erroneously considered to be adequate. Lymphocele aspirates contain large numbers of lymphocytes, which may all be mature or which may be a mix of normal and activated cells with immunoblasts and plasma cells similar to lymph node aspirates. The striking feature of a lymphocele aspirate is the disproportionate number of lymphoid cells almost to the exclusion of neutrophils and monocytes (Fig. 19). Even in a case of severe acute rejection, an aspirate virtually never contains 70% to 90% lymphoid cells as are present within a lymphocele sample.

Perinephric fluid collections contain numerous macrophages or mesothelial cells, which are usually vacuolated and often have phagocytized other cells. These are similar to cells identified in a variety of benign cysts. Scattered neutrophils and lymphocytes may also be present (Fig. 20). Class II antigen staining is negative or focally positive within monocytes and macrophages. Ultrasound studies should be suggested so an adequate aspirate or biopsy sample can be obtained away from the lymphocele or fluid collection.

5. Extramedullary Erythropoiesis

Extramedullary hematopoiesis is uncommon in the kidney after the fetal period. Erythropoiesis has been seen in aspirates of transplant kidneys from donors younger than 13 years of age in anemic recipients receiving human recombinant erythropoietin therapy (41). These aspirates contain the range of immature red cells including pronormoblasts, normoblasts, and nucleated erythrocytes with their characteristic features of round immature nuclei and round cytoplasm (Fig. 21). Red cell maturation is evident and cells at the appropriate stage contain iron or hemoglobin, a helpful adjunct in making the diagnosis. This phenomenon likely occurs when resident hematopoietic stem cells within the pediatric kid-

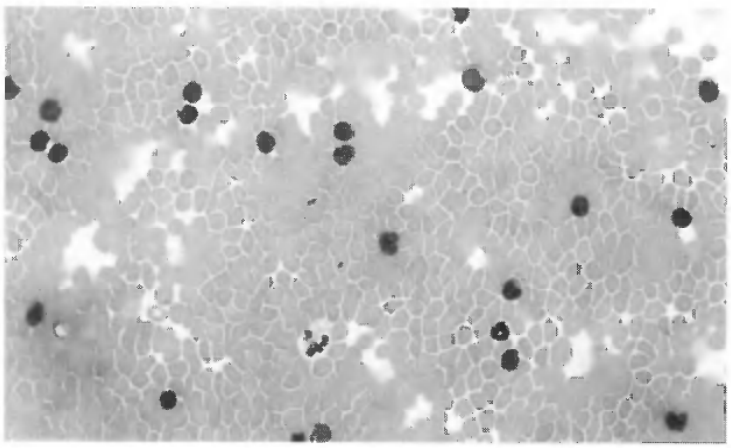

Figure 19 Lymphocele aspirate containing abundant mature and few activated lymphocytes with rare neutrophils. (May-Grunwald-Giemsa, ×250.)

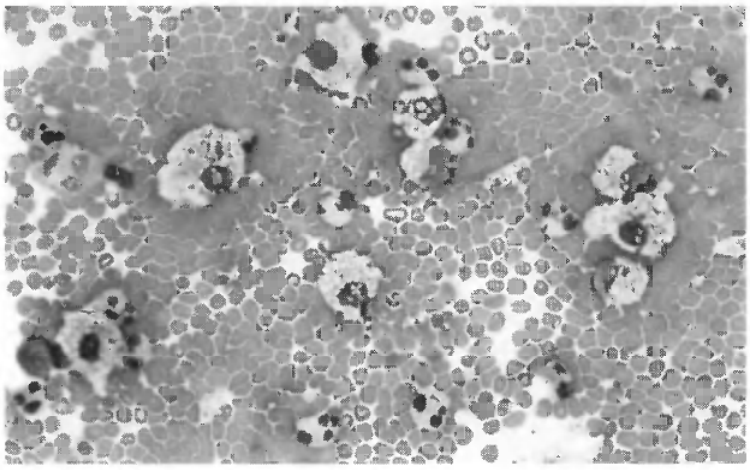

Figure 20 Aspirate of perinephric fluid collection. There are vacuolated macrophages or mesothelial cells with phagocytized material and many neutrophils with few lymphocytes. (May-Grunwald-Giemsa, ×250.)

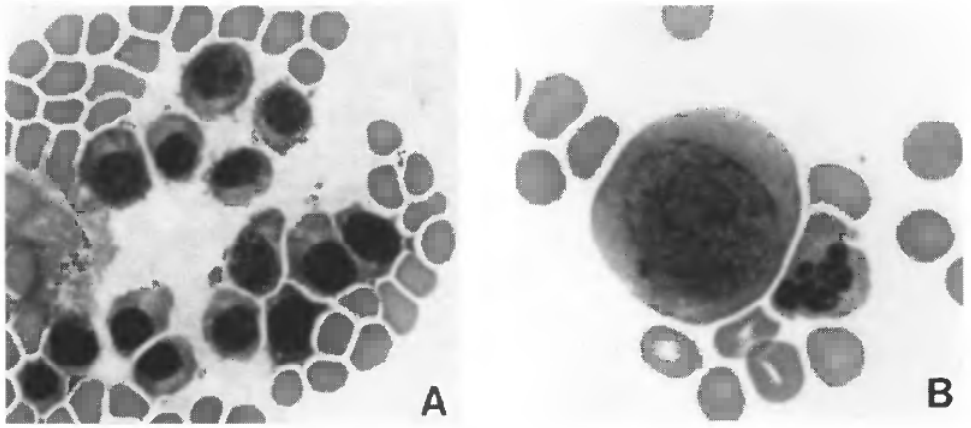

Figure 21 Aspirate with intrarenal extramedullary erythropoiesis. A. Cluster of normoblasts with characteristic rounded nuclei and clumped chromatin. (May-Grunwald-Giemsa, ×500.) B. Basophilic normoblast. (May-Grunwald-Giemsa, ×760.)

ney are stimulated by high endogenous and exogenous erythropoietin levels. The background is that of the underlying renal pathology for which the aspirate was performed (e.g., acute rejection, acute tubular necrosis). It is important for clinicians to recognize red cell precursors so as not to mistake them for malignant cells or immunoblasts.

V. ACCURACY AND USE OF ALLOGRAFT FINE-NEEDLE ASPIRATION

A. Accuracy

Fine-needle aspiration findings have a high degree of reproducibility when adequate samples are used, that is, when cortical aspirates have seven or more parenchymal cells per 100 leukocytes. Studies evaluating duplicate aspirate specimens have demonstrated correlations in the range of 95% (42–45). A high degree of consistency among different areas in the

same cytospin preparation has also been found. Early in the development of the aspiration technique, studies correlating aspirate findings with renal tissue in experimental models (43,46) and allograft nephrectomies (47) found strong correlations between the aspirate inflammatory component and tissue leukocytic infiltrates. Comparing renal aspirates with concomitant core biopsies in allograft recipients reveals sensitivities ranging from 50% to 100% (averaging 80%–85%) and specificities of 82% to 100%, with wide variation for the different diagnostic categories (19,45 48–54). There tend to be higher sensitivity levels for acute rejection and better specificities for cyclosporine toxicity. The studies cited previously have used only routine staining and interpretation of aspirates; the addition of immunostaining considerably improves diagnostic accuracy (6,8). There are numerous reasons for discrepancies between core biopsy and aspirate diagnoses (Table 2). These include mild immune activation with weakly positive MHC class II antigen staining identifying low-grade or "subclinical" rejection in the aspirate without adequate inflammation in the biopsy to support a similar diagnosis. In fact, aspiration may be a more sensitive technique for evaluating smoldering acute rejection episodes that may be a risk factor for early chronic rejection.

B. Clinical Use

Renal allograft fine-needle aspiration is best used within the first 3 to 6 months after engraftment. Material is readily removed from the kidney within this time frame, with a good percentage of adequate aspirates when obtained by an experienced individual. After that period of time, aspirates are often inadequate, probably because there is interstitial fibrosis with cell adherence, although occasionally adequate samples may be obtained with a late acute rejection episode in an otherwise well-functioning kidney with little chronic damage (45). The diagnostic utility of aspiration also enhances its use in the early posttransplantation period when the differential diagnosis includes acute tubular necrosis, acute cyclosporine nephrotoxicity, and acute rejection (Tables 3 and 4). There are a few settings in which aspiration is superior to core biopsy: (1) acute cyclosporine and possibly FK506 toxicity are more readily identified, as tubular cell isometric vacuoles are observed more easily in aspirate samples, (2) viral infection has more specific aspirate and peripheral blood features in aspirate material, with the added ability to perform CMV and EBV immunostains, and (3) low-grade rejection episodes may be underinterpreted by core biopsy.

One of the unique features of aspiration is its ability to safely monitor intragraft events as they unfold. Aspiration can be performed daily and is best used serially to monitor a patient's course (45,55). This is extremely beneficial in settings such as prolonged primary nonfunction to assess the healing of tubular epithelium and possible transformation to acute rejection, reversal of acute cyclosporine toxicity, and response to antirejection ther-

Table 2 Etiologies of Discrepant Fine-Needle Aspirate and Core Biopsy Findings

Medullary sample
Vascular-limited acute rejection
Spontaneous resolution of acute rejection
Low-grade or focal acute rejection
Cyclosporine-associated graft lymphocytosis
Aspirate at site of prior biopsy or injury
Erroneous aspirate or core diagnosis

Table 3 Etiologies of Graft Dysfunction Identified Using Fine-Needle Aspiration

Acute transplant rejection (tubulointerstitial)
Acute tubular necrosis
Acute cyclosporine nephrotoxicity
Acute FK506 nephrotoxicity
Viral infection
Acute infectious interstitial nephritis (pyelonephritis)
Infarction
Acute interstitial nephritis
Posttransplant lymphoproliferative disorder

Table 4 Fine-Needle Aspirate Findings in Common Causes of Acute Transplant Dysfunction

	Acute Rejection	Viral Infection	Acute Tubular Necrosis	Cyclosporine/FK 506 Toxicity
Total corrected increment (TCI)	>3.0	>3.0	<3.0	<3.0
Blast/plasma cell count	>4	>4 Plasma cells predominate	<4	>4
Tubular cells	Variable necrosis	Variable necrosis	Necrosis	Isometric vacuoles Phagocytosis Variable necrosis
Major histocompatibility complex class II	Positive	Negative	Negative	Negative
Other		Positive cytomegalovirus stains Peripheral blood activation	Variable mitoses	Vacuolated aspirate leukocytes May be TCI >3.0 with mature lymphocytes

apy (56,57). Aspiration is not only safe but also has a rapid turnaround time of 2 to 4 hours, and saves the additional costs associated with core biopsy such as those of ultrasound, laboratory tests, and postprocedure patient observation.

Fine-needle aspiration does have intrinsic limitations (Table 5). The location of the infiltrate cannot be assessed, and therefore it is not possible to definitively identify cases with vascular rejection or other vascular lesions. Chronic abnormalities result in dry taps with inadequate samples caused by fibrosis, and can only be assessed by biopsy. Glomeruli are occasionally obtained in aspirate samples, but with routine processing are not suitable for diagnostic evaluation. Abnormalities resulting in nonspecific or absent graft infiltrates such as obstruction, hemorrhage, and hematoma formation are not evaluable.

Aspiration and core biopsy are not competing or mutually exclusive means for assessing a poorly functioning renal allograft; they are complementary procedures to be used in the appropriate clinical setting. Aspiration may guide the use of core biopsy when the aspirate findings suggest an abnormality requiring confirmation by core biopsy or are discrepant

Table 5 Etiologies of Graft Dysfunction Not Discerned Using Fine-Needle Aspiration

Acute vascular rejection
Chronic transplant rejection
Chronic cyclosporine toxicity
Chronic FK506 toxicity
Graft rupture or hematoma
Obstruction
Thrombotic microangiopathy
Glomerulonephritides

with the clinical course. There are several causes of discrepant or nondiagnostic aspirate samples, ranging from medullary samples to incorrect clinical or core biopsy diagnoses. As with all morphological specimens, sampling variability must be taken into consideration.

C. Research Applications

Allograft fine-needle aspiration affords unique material for use in many areas of transplantation research. The aspirate samples are obtained in sterile cell culture-based media in which cells remain viable, so they can be used for culture to examine cell phenotypes and reactivities in various settings of allograft dysfunction and therapeutic response (58,59). Immunostaining, including double labeling and flow cytometry, can be applied to investigate cell phenotyping, protein synthesis, receptor expression and activation with the abundance of markers currently available (60–65). There are methods for handling aspirated material to enhance recovery and preservation of glomeruli, enabling assessment of glomerular lesions in transplant (and native kidney) settings (66). Aspiration also can be used in experimental models of transplantation (67,68).

Aspirate and peripheral blood samples can be collected and processed for reverse transcription polymerase chain reaction (see Sec. II). This method allows evaluation of intragraft immunological events during the course of rejection and other injuries to the graft. At my institution, aspirates have been subjected to PCR in various settings to examine the role of IFN-γ and interleukin-12 in acute rejection (9,69). We found that IFN-γ mRNA levels are upregulated before and during acute rejection, and are probably pathogenetically linked to the initiation of rejection. These levels become undetectable soon after the initiation of successful antirejection therapy. Tubular cell MHC class II expression is augmented by IFN-γ and disappears more slowly than IFN-γ after treatment. There does not appear to be any correlation between interleukin-12 mRNA expression and the onset or perpetuation of acute rejection. These findings illustrate the potential for further investigation of intragraft events with PCR. Complementary technologies that may be applied to aspirated samples include in situ hybridization and in situ PCR.

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13

Sonographic Evaluation of Acute Renal Transplant Rejection

SHEILA SHETH
The Johns Hopkins Hospital, Baltimore, Maryland

I. INTRODUCTION

With the large number of renal transplants performed in this country to treat irreversible renal failure, there is a pressing need for noninvasive diagnosis of potential complications that may jeopardize graft survival if not recognized and treated promptly. Clinical signs of fever, pain over the graft, and oliguria and laboratory findings indicating decrease in renal function are nonspecific. Sonography is often requested to help differentiate the various causes of graft dysfunction (1,2). Most surgical complications such as peritransplant fluid collections, ureteral obstruction and hydronephrosis, and occlusion or stenoses of major arteries and veins, are readily diagnosed with color and Doppler sonography. However, the value of sonography in evaluating parenchymal disturbances associated with poor graft function is controversial. This chapter reviews the physical principles governing gray scale, color, and duplex Doppler sonography, and discusses the role of ultrasound in assessing patients with suspected acute transplant rejection.

II. GRAY-SCALE AND COLOR DOPPLER SONOGRAPHY

Unlike conventional x-ray imaging, the sonographic image is created when high-frequency sound waves emitted by the piezoelectric crystals of a transducer interact with the different

tissues in the human body. The transducer acts as a sound emitter as well as a receiver of the sound energy returning from the body. Pulses of sound waves emitted by the transducer interact with the tissues in various fashions, including sound absorption, transmission, reflection, refraction, diffraction, and scattering. The type of interaction that predominates depends on the frequency of the emitted sound wave, the acoustic impedance, the physical properties of the tissues, and the various tissue interfaces. In between emission of pulses, the transducer acts as a receiver, the returning sound energy is converted into an electrical pulse and the amplitude information it contains is amplified, processed, and displayed on the video monitor as a two-dimensional gray-scale image.

Whereas high-resolution real-time sonography provides anatomical information, Doppler ultrasound is useful in analyzing the flow pattern from the renal transplant vasculature. The Doppler effect is the change in frequency of the returning sound waves, which occurs when the sound wave hits moving reflectors such as circulating red blood cells. The change in frequency, or Doppler shift, is a function of the velocity of the blood flow and the Doppler angle between the direction of the emitted sound wave and the blood vessel. The returning Doppler signal is analyzed, and a spectrum of the vascular flow pattern can be obtained.

With color Doppler sonography, a two-dimensional color-coded map of mean velocities of blood flow is superimposed on the gray-scale image. Moving blood within blood vessels is displayed in color, allowing rapid assessment of the blood supply to the entire transplant. Qualitative or semiquantitative analysis of blood flow pattern is performed by obtaining Doppler spectra from specific blood vessels.

III. TECHNIQUE OF EXAMINATION

The renal transplant is examined using high-resolution 3.5- and 5-MHz linear, curvilinear, or sector transducers with color and duplex Doppler capabilities. Gray-scale sonography provides morphological information regarding transplant size and echotexture. An oblique approach over the iliac fossa with the transducer oriented parallel and then perpendicular to the graft provides the best overall evaluation of the kidney and allows accurate measurement of the maximum sagittal and anteroposterior diameters.

Color and duplex Doppler modes provide invaluable information concerning the integrity of the main artery and vein supplying the transplant as well as the state of cortical perfusion. Doppler spectral patterns are recorded from the main renal artery and vein and at least two of the peripheral arteries (arcuate, interlobar, or segmental). Analysis of the Doppler waveform allows differentiation between arterial and venous signal as well as assessment of the vascular impedance in the allograft. The two Doppler indices most widely used to measure the pulsatility of arterial waveform in the renal transplant include the following (3,4):

$$\text{Resistive index: } \frac{\textit{peak systolic velocity} - \textit{end diastolic velocity}}{\textit{peak systolic velocity}}$$

$$\text{Pulsatility index: } \frac{\textit{peak systolic velocity} - \textit{end diastolic velocity}}{\textit{mean velocity}}$$

Pathological conditions associated with an increase in the peripheral vascular resistance in the transplant lead to elevation of these indices above normal values.

At my institution, an initial sonogram is obtained routinely within 24 to 48 hours after transplantation. This provides a baseline study for future comparison and greatly facilitates

interpretation of subsequent sonograms and detection of subtle pathological changes in the echotexture or flow pattern of the transplanted kidney (1,2).

IV. NORMAL RENAL TRANSPLANT

The normal renal allograft is elliptical in shape with a relatively hypoechoic renal cortex and a strongly echogenic renal sinus (Fig. 1A). Although the appearance of the healthy allograft is similar to that of a normal native kidney (2), fine morphological details are more readily visible because of the superficial location of the transplant and the ability to image with higher frequency transducers, allowing better spatial resolution. Thus, the renal pyramids are easily seen as triangular structures, slightly more hypoechoic than the surrounding cortex (Fig. 1A). The normal arterial flow pattern as demonstrated by Doppler spectral analysis is characteristic of a low impedance vascular bed: there is continuous forward flow throughout the cardiac cycle, with a rapid systolic upstroke followed by a gradual downslope in diastole (Fig. 1B) (4–7). Measurements of pulsatility indices (PIs) or resistive indices (RIs) allow semiquantitative analysis of renal arterial blood flow (3,4). An RI value of less than 0.7 in the main and peripheral renal arteries indicates normal peripheral resistance in the transplant (3).

V. MORPHOLOGICAL ABNORMALITIES ASSOCIATED WITH ACUTE REJECTION

The diagnosis of acute rejection, by either morphological criteria or Doppler criteria, is possible if the pathological changes associated with acute rejection are severe enough to translate into recognizable alteration of the sonographic appearance of the transplant. The sonographic abnormalities directly reflect the underlying histopathological process.

In acute interstitial rejection, the lymphocyte-mediated immune response is most prominent at the corticomedullary junction, although in severe cases the entire transplant is involved. The interstitium of the kidney is infiltrated with edema and inflammatory cells (8,9). The allograft suddenly increases in size compared to baseline study, and becomes more globular in shape (9–12). Because the lymphocyte-mediated immune response is most prominent at the corticomedullary junction, the edematous pyramids become larger, lose their triangular shape, and are more hypoechoic (9,12,13), and there is loss of the normal corticomedullary differentiation (9). The renal sinus echoes hypoechoic and indistinct as adipose tissue is infiltrated by edema and fibrosis (Fig. 2A) (8). In severe cases, areas of hemorrhage and infarction are found throughout the renal parenchyma and the echotexture of the renal cortex becomes inhomogeneous with scattered patchy areas of decreased echotexture, reflecting edema and infarction, and areas of hyperechogenicity caused by recent hemorrhage (Fig. 3) (9,13,14). Mild dilatation of the collecting system and thickening of the wall resulting from submucosal edema can occur (15,16). Peritransplant fluid collection may be seen secondary to small areas of cortical disruption along Brodee's line. The overall sensitivity of gray-scale sonography in the diagnosis of acute rejection has been reported to be as low as 52% (17) or as high as 85% to 90% (18,19). The presence of multiple sonographic abnormalities increases the specificity of the diagnosis at the cost of a reduced sensitivity (13,20); the combination of a sudden increase in transplant size, enlargement of the pyramids, and heterogeneity of the renal cortex has been found to be particularly helpful (21).

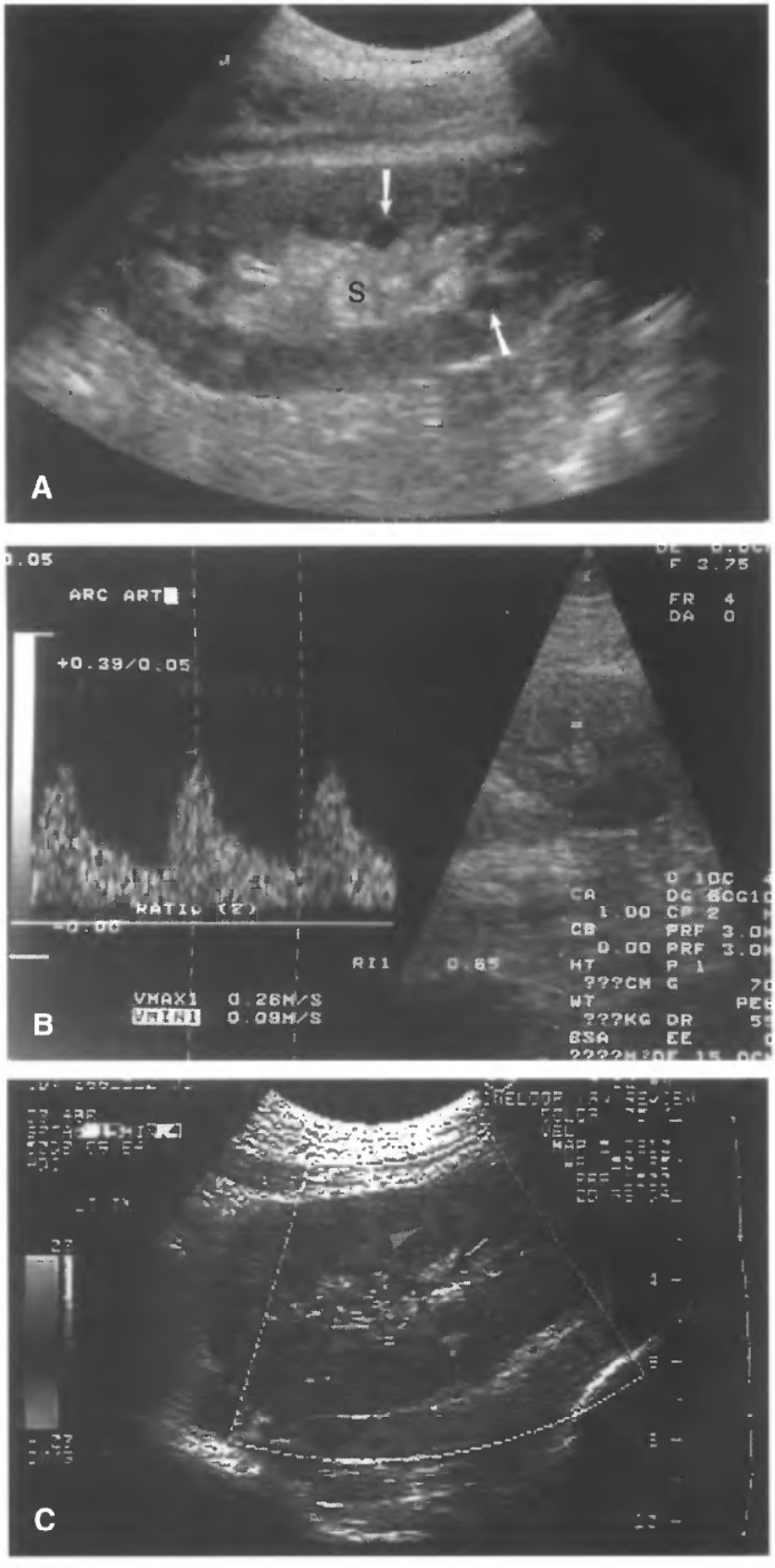

Figure 1 A. Normal renal transplant. Sagittal sonogram of a renal transplant shows excellent corticomedullary differentiation. The hypoechoic triangular pyramids are clearly visible (arrows). S = renal sinus. B. Doppler spectrum from an arcuate artery shows a normal flow pattern characteristic of a low impedance vascular bed. The resistive index was 0.65. (From Hamper, UM, Sheth S. Ultrasound evaluation of allograft rejection. In: Solez K, Racusen LC, Billingham ME, eds. Solid Organ Transplant Rejection: Mechanisms, Pathology, and Diagnosis. New York: Marcel Dekker, 1996:503.) C. Color Doppler sonogram of a normal transplant in another patient demonstrates the segmental and interlobar arteries (arrowheads)

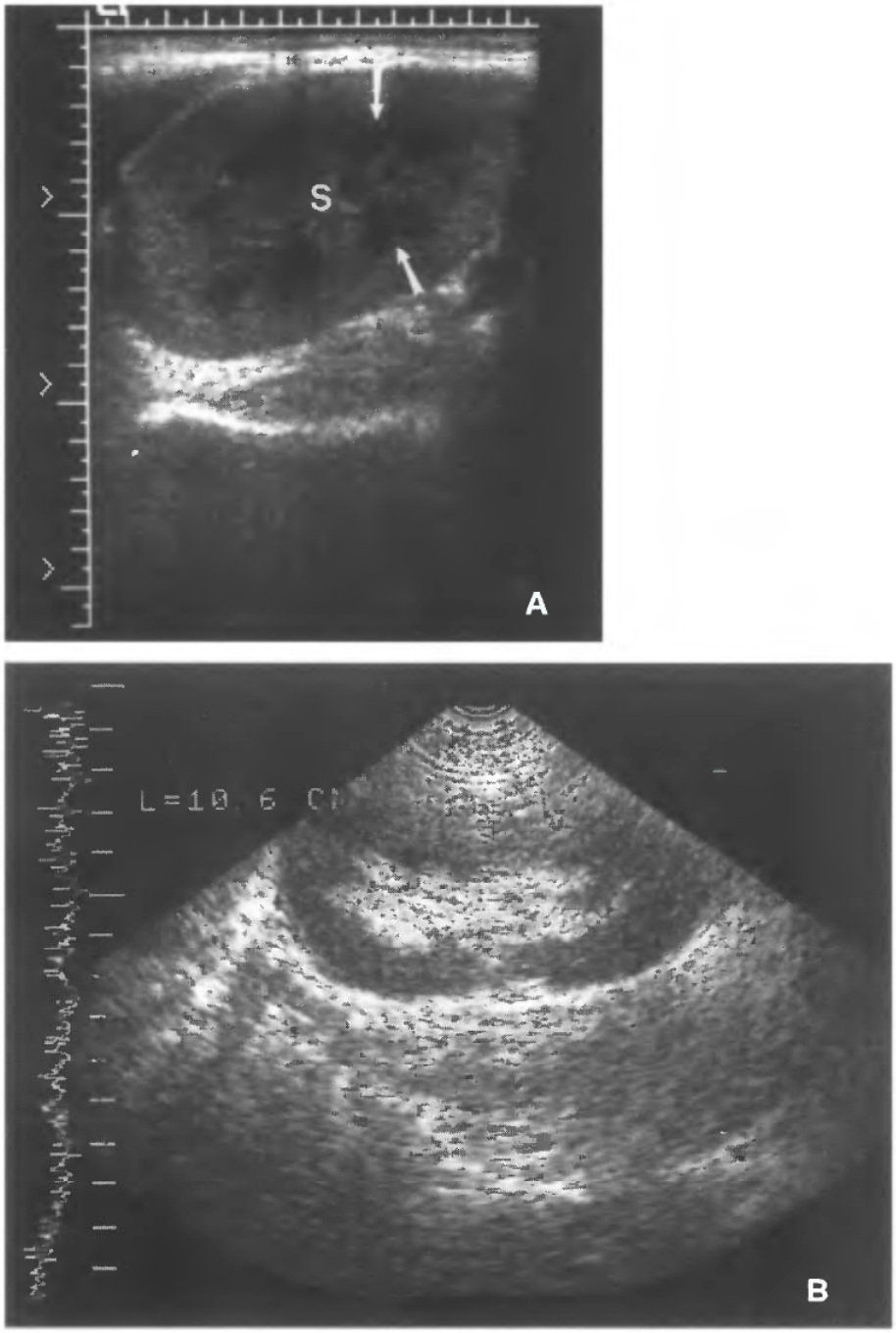

Figure 2 A. Morphological changes associated with moderate acute rejection. The kidney is enlarged and globular in shape. There is complete loss of the central sinus echoes (S) and the pyramids are enlarged (arrows). (From Burdick JF, Racusen LC, Solez K, Williams GM, eds. Kidney Transplant Rejection. 2d ed. New York: Marcel Dekker, 1992:464.) B. The initial sonogram performed 5 months earlier was normal.

Many cases of acute rejection are not associated with sonographically detectable morphological changes in the transplanted kidney, and the absence of sonographic abnormalities does not exclude underlying rejection. In young children, sonography is often inconclusive (12). Whenever there is a strong clinical suspicion of acute rejection, percutaneous biopsy of the allograft is warranted and is usually performed under sonographic guidance, which helps guide the needle toward the area of greatest morphological abnormality.

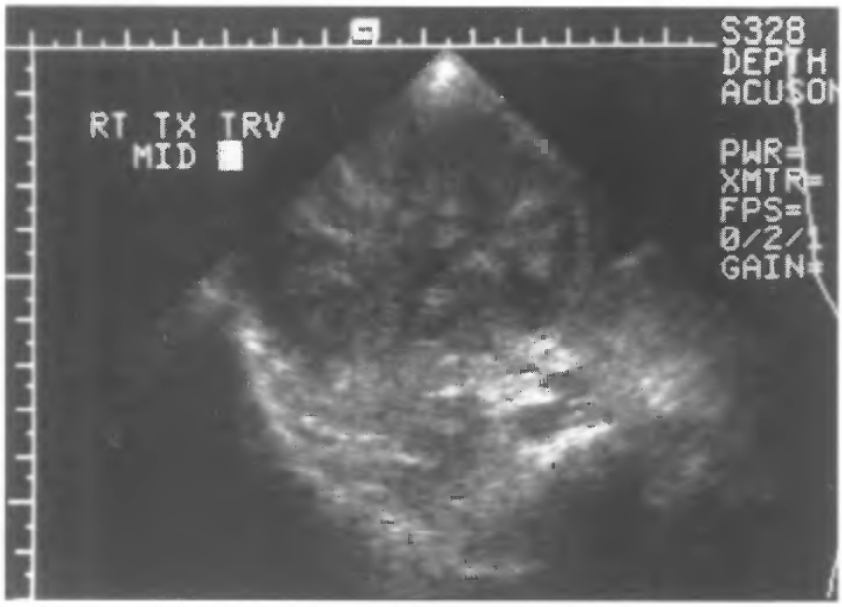

Figure 3 Morphological changes associated with severe acute rejection. There is complete loss of the normal renal architecture, with patchy areas of hyperechogenicity present throughout the cortex. At pathological examination, there was severe interstitial and vascular rejection, with severe vasculitis, cortical hemorrhages, and infarctions. (From Burdick JF, Racusen LC, Solez K, Williams GM, eds. Kidney Transplant Rejection. 2d ed. New York: Marcel Dekker, 1992:465.)

VI. ROLE OF COLOR AND DUPLEX COLOR SONOGRAPHY IN THE DIAGNOSIS OF ACUTE REJECTION

Initial studies evaluating renal transplants with duplex Doppler ultrasound were promising and raised hopes that this modality would be helpful in differentiating acute rejection from other causes of parenchymal dysfunction such as acute tubular necrosis and cyclosporine A toxicity (2,4,22,23). In the vascular form of acute rejection, the small vessels of the transplant are involved in the pathological process, resulting in vasoconstriction, followed by acute necrotizing vasculitis, swelling of the endothelial cells, and fibrointimal proliferation with narrowing of the arterial lumen. The resulting increase in vascular impedance is reflected in the Doppler spectral waveform with loss of the normal diastolic flow and elevation of the resistive and pulsatility indices (Fig. 4) (3,4,22). Rifkin et al. showed (3) that a resistive index value of 0.80 detected nearly 70% of transplants with acute rejection, and values of RI above 0.9 had a 100% positive predictive value for the diagnosis with, however, a sensitivity of only 13%.

Initial enthusiasm for vascular indices measurement in predicting acute rejection has been somewhat dampened in that several recent studies have shown that Doppler sonography results do not necessarily obviate the need for biopsy. A significant number of patients with clinically suspected or pathologically proven acute rejection have an RI in the normal (0.7 or less) or indeterminate (<0.8) range (3,24–26). In addition, finding of an abnormal RI is not specific for acute rejection: many other processes affecting the renal transplant, including severe acute tubular necrosis, severe pyelonephritis, cyclosporine toxicity, acute renal vein thrombosis, obstructive uropathy, and marked compression of the renal parenchyma by a large fluid collection, may affect vascular impedance in the allograft (27,28).

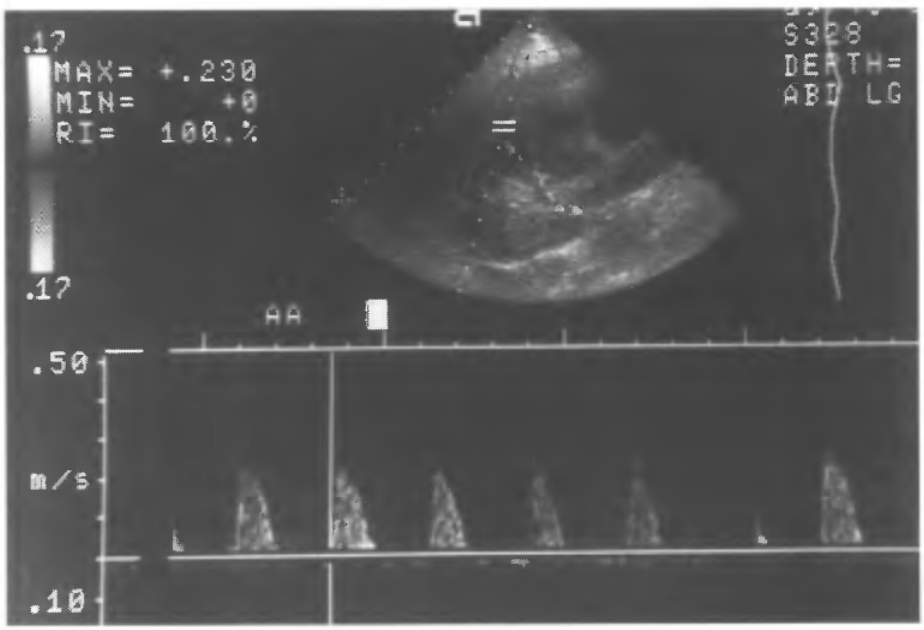

Figure 4 Duplex Doppler changes associated with acute rejection. Doppler spectrum from an arcuate artery shows complete absence of diastolic flow. The resistive index is 1. The transplant was removed several days later and showed severe acute rejection with vasculitis. (From Hamper UM, Sheth S. Ultrasound evaluation of allograft rejection, in: Solez K, Racusen LC, Billingham ME, eds. Solid Organ Transplant Rejection: Mechanisms, Pathology, and Diagnosis. New York: Marcel Dekker, 1996:505.)

However, a highly elevated RI or PI usually indicates poor function of the graft and need for further investigation (29). Even the most severe alteration, reversal of diastolic flow, is not specific for acute rejection but does indicate a poor prognosis (27).

This discrepancy in sensitivity, specificity, and positive and negative predictive value of RI and PI in the diagnosis of acute rejection among various studies can, in part, be explained by other factors: (1) difference in the prevalence of acute rejection and acute tubular necrosis among the patient population (24); and (2) time elapsed between the initial transplant surgery and the time of the Doppler study (29). Doppler indices are of little value in the diagnosis of chronic rejection, and, because transplants older than 1 year are included in some studies (25,30), the sensitivity and specificity of Doppler ultrasound is altered.

The question therefore arises whether to perform duplex ultrasound evaluation of the transplant kidney. I believe that sonography is, despite its limitations, an invaluable diagnostic tool. It is relatively inexpensive, safe, and can be performed at the bedside in extremely sick patients. The clinical differentiation between parenchymal and surgical complications is often problematic, and sonography is quite helpful in recognizing most surgical complications. Sonography is also essential in providing guidance for percutaneous biopsy of the transplant kidney, and in ensuring minimal damage to the organ and maximal safety during the procedure. Finally, whereas isolated measurement of vascular indices lack specificity and sensitivity for the diagnosis of acute rejection, serial measurements may be more valuable. An increase of RI or PI values above baseline study may be the first indicator of potentially serious complications requiring rapid diagnosis and intervention. Doppler parameters often also show improvement after successful therapy of acute rejection (Fig. 5) (6,31).

In summary, despite its limitations, in the appropriate clinical setting, color Doppler

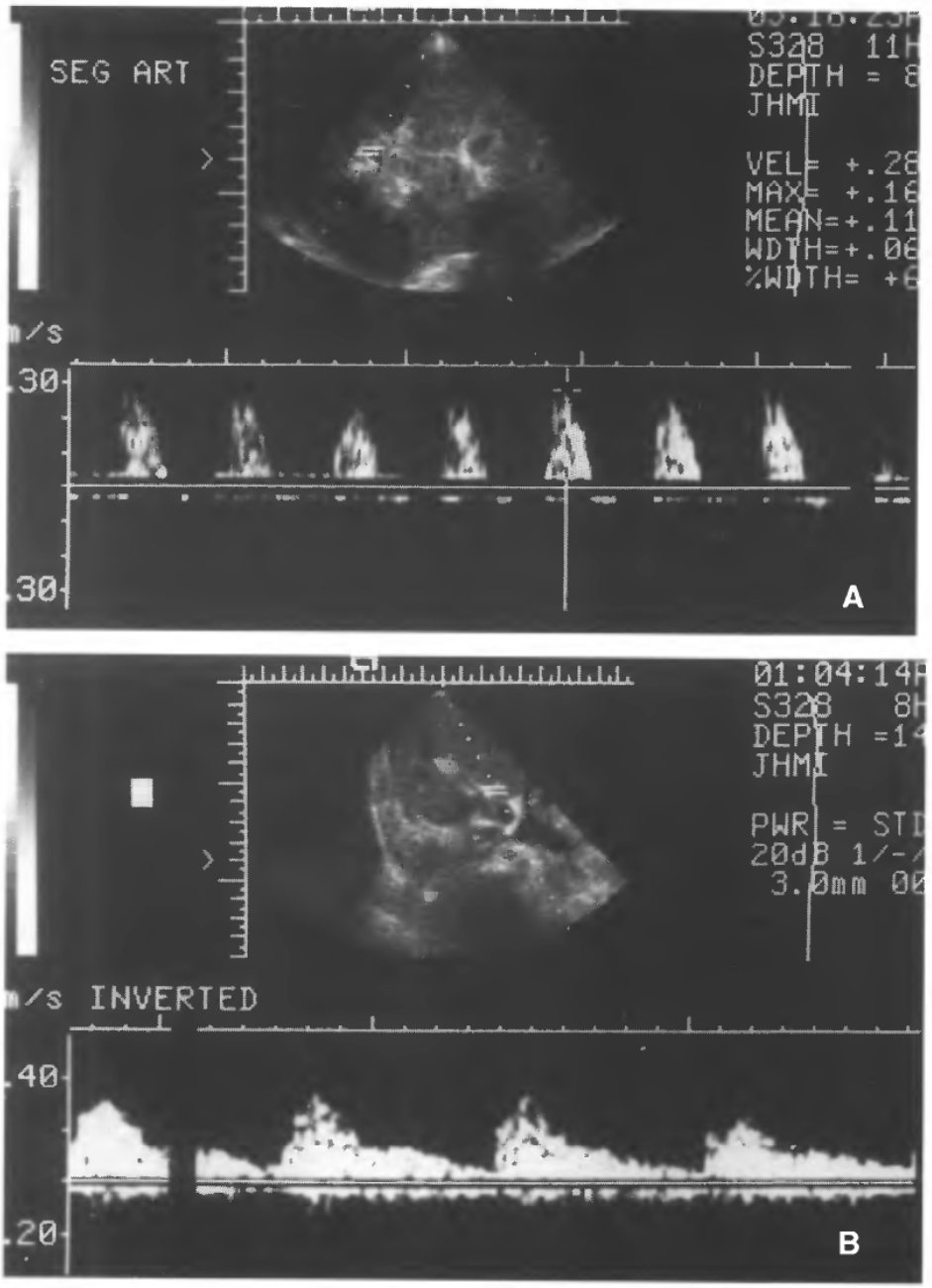

Figure 5 A. Changes in Doppler spectrum after therapy. The Doppler spectrum in the segmental artery shows absence of diastolic flow, suggestive of acute rejection. (From Burdick JF, Racusen LC, Solez K, Williams GM, eds. Kidney Transplant Rejection. 2d ed. New York: Marcel Dekker, 1992:467.) B. The follow-up after corticosteroid therapy shows improvement in the diastolic flow. This finding preceded clinical improvement with therapy.

sonography is invaluable in the management of patients with suspected acute rejection of renal transplant.

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14

History and Prospects for Antilymphocyte Antibody Therapy for Tolerance Induction

FRANCIS L. DELMONICO, DAVID H. SACHS, and A. BENEDICT COSIMI

Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts

I. INTRODUCTION

Since the first experimental evidence by Woodruff and Anderson revealed the immunosuppressive activity of an antiserum raised against lymphocytes (1), antilymphocyte preparations have contributed significantly to the success of organ transplantation. The administration of antilymphocyte antibody sera (ALS), either as a polyclonal preparation (e.g., ATG, ALG) or as a monoclonal antibody (mAb) reactive to human T cells, can delay the onset of acute rejection for weeks following transplantation, enabling the allograft to recover from preservation injury and simplifying the recipient's perioperative management (2,3). More importantly, these ALS preparations have been undisputedly determined to provide the most effective treatment for steroid-resistant rejection episodes, as first reported by Shield et al. after administering ATG (4) and later by Cosimi et al. after administering OKT3 (5).

Because the historical prominence of these ALS preparations would likely be acknowledged by many investigators (6–8), perhaps it is surprising that the role for ALS preparations in future protocols of immunosuppression remains unclarified. Induction ALS therapy following renal transplantation, which gained widespread acceptance over the past decade, has become less widely used. The ALS treatments administered immediately after

transplantation are costly and prolong initial hospital stay. Moreover, they do not appear to influence overall long-term outcome (9), although several high-risk recipient subgroups, including retransplanted or sensitized patients and those with delayed graft function, have been shown to have significantly improved renal allograft survival with induction regimens that include either OKT3 or ALG (7,10). Induction ALS therapy with delayed cyclosporine (CsA) administration following transplantation of nonrenal organs has been similarly associated with better perioperative renal function and delayed cardiac or liver allograft rejection. Long-term survival, however, has not been consistently improved, resulting in no general agreement as to the value of this approach for these recipients (11). Moreover, the recent addition of new pharmacological immunosuppressive agents to therapeutic regimens has likely reduced the incidence of rejection (12), and thus the need for ALS treatment (13).

Although these factors have curtailed the use of ALS therapy in conventional immunosuppressive protocols (and notwithstanding the uncertainty regarding innovative ALS administration for the future), most investigators would also agree that the broad experience with current immunosuppression suggests that new approaches are needed. The complications of indefinite immunosuppression are pervasive, affecting so many aspects of the quality of life intended by successful transplantation (14–16). Despite continuous (intermittently intensified) immunosuppression, attritional allograft loss after the first year following transplantation has been unchanged for the past 3 decades (14). Fig. 1, for example, reveals the renal allograft survival of more than 1000 consecutive cadaver donor recipients at the Massachusetts General Hospital since the inception of the program in 1963.

Upon initial inspection, the data are encouraging, in that they reveal a significant improvement in the rate of early allograft survival (6 months to 1 year), when comparing the first 15 years of service to the survival achieved since 1984. The year 1984 delineates the current era by the addition of CsA to the regimen of maintenance immunosuppression and the routine use of OKT3 for the treatment of steroid-resistant rejection.

However, the rate of attrition in renal allograft survival after the first posttransplant year is exactly the same for all three time periods, as presented in Fig. 1. The significant impact of CsA and OKT3 immunosuppression has therefore been limited to the first year following transplantation, after which the rate of success is no better than it was for recipients of transplants in the 1970s. These observations have been confirmed in numerous

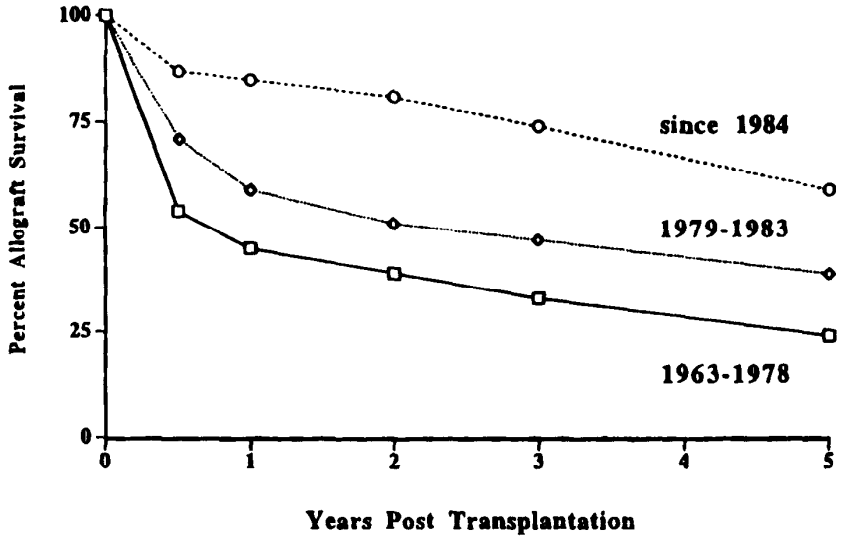

Figure 1 MGH consecutive allograft survival.

long-term survival studies, including the pooled United States nationwide data summarized by Cecka and Terasaki (9). All of the reviews reveal the remarkably constant half-life of renal allografts maintained by conventional immunosuppressive protocols. Thus, ample evidence leads to the conclusion that the fundamental objectives of future therapeutic approaches must be to not only prevent initial allograft rejection but also to induce a state of recipient tolerance that obviates the need for indefinitely administered immunosuppression and avoids the risk of chronic rejection.

The current approach to preventing rejection mainly by long-term administration of combination drug therapy does not predictably lead to recipient tolerance (18). Several reports (summarized in Table 1) document the consequences of unprescribed discontinuation of immunosuppressive therapy (19–21). Withdrawal of immunosuppression, even after years of treatment, usually results in allograft rejection and may lead to patient death. The data in Table 1 also emphasize, however, that a few patients can successfully discontinue all immunosuppressive medications, apparently because donor-specific nonreactivity developed while they received nonselective suppression in the presence of the functioning allograft. Liver allograft recipients may have an immunological advantage for the achievement of this drug-free state; Ramos et al. have reported successful deliberate weaning of all immunosuppression in 16 of 59 (27%) patients studied after 5 or more years of maintenance immunosuppression (20). The significant failure rate, even in this carefully selected patient population, nevertheless emphasizes that this rate of allograft tolerance remains unpredictable, even in the presence of demonstrable donor leukocyte chimerism. Thus, for the vast majority of allograft recipients, current protocols of immunosuppression do not reliably permit drug withdrawal.

Current approaches to adding ALS preparations to the clinical regimen have also been ineffective in altering long-term recipient responsiveness. Thus, despite their success in the prevention (2,3,22) and treatment of rejection (23,24), neither ALS nor mAb therapy has historically accomplished the other significant objective of inducing tolerance.

These historical observations pose an interesting clinical question, which is the focus of this chapter. Why might biological (ALS) preparations be important components of future conditioning protocols designed to develop long-term allograft tolerance, and how will the agents be combined with pharmacological agents that have proved so effective in the short-term control of rejection?

In this review, transplantation tolerance is defined "operationally" as the selective inability of an allograft recipient to respond only to disparate donor antigens, without the necessity of chronic immunosuppressive therapy. Clinically, operational tolerance may be manifested by the complete withdrawal of immunosuppressive drugs from an allograft

Table 1 Outcome After Stopping Immunosuppression

	Allograft Recipient	No. Noncompliant	Result
Iwasaki, 1982 (19)	Renal	33 LRD	19 Rejection
		17 CD	15 Rejection
Schweitzer, 1990 (21)	Heart	3	1 Death
			2 Chronic rejection
	Liver	3	2 Death
	Renal	33 CD; 14 LRD	43 Rejection or death
Ramos, 1995 (20)	Liver	59	15 Rejection

LRD, living related donors; CD, cadaver donors.

recipient, without the subsequent onset of rejection. This clinical phenomenon is distinguished from the classical induction of tolerance experimentally (25) and the subsequent systemic tolerance that can emerge from such in utero manipulation.

The use of the term tolerance does not dictate that particular immunological mechanisms are responsible for the phenomenon and could include both central and peripheral mechanisms. Central tolerance is acquired by exposure to donor antigen in the thymus during T-cell development. In contrast, peripheral tolerance is required to inactivate preexisting T cells that are present in the peripheral circulation at the time of organ grafting, and it has been the basis for most clinical approaches for achieving tolerance in an allograft recipient.

Several potential strategies for using ALS to induce transplantation tolerance have emerged from experimental investigation. These include: (1) the development of anergy mainly by mAb therapies, (2) the clonal deletion of T cells by an ALS preparation followed by thymic education of a new T-cell repertoire in the presence of donor antigen, and (3) the suppression of T cells by an ALS preparation followed by allogeneic lymphohematopoietic cell infusion from the prospective donor. This chapter considers each of these strategies, highlighting the use of ALS preparations (either mAb or ATG) as a unifying element for all of these approaches to tolerance induction.

II. THE DEVELOPMENT OF ANERGY

Anergy occurs when engagement of the T-cell receptor (TCR) is associated with unresponsiveness instead of T-cell activation (26). Experimental models of this approach to tolerance use conditioning regimens that disrupt T-cell signaling necessary for activation of T cells. Current concepts propose that two distinct signals are required to activate a T cell (27). Receiving an antigen-specific signal via the TCR exclusively is downregulatory, preventing full activation and interleukin (IL)-2 production. A second signal, which is not necessarily dependent on the recognition of donor antigen (28,29), also appears to be essential for T-cell activation and T-cell effector function (30). This additional signal may be furnished by cell surface adhesion molecules. Although these adhesion molecules contribute to the binding of the responding T cell to the antigen presenting cell (APC) (31), they also have a costimulatory activity as well, by way of their signaling properties. The second signal receptor-ligand pairs that have received the most investigation are illustrated in Fig. 2. They are CD28–B7 (32), CD2–CD48 (33), CD40–gp39 (30), and CD54–LFA1 (34).

Numerous ALS preparations that have been administered as mAbs and that are reactive with these cell surface costimulatory antigens have been successfully used in rodent

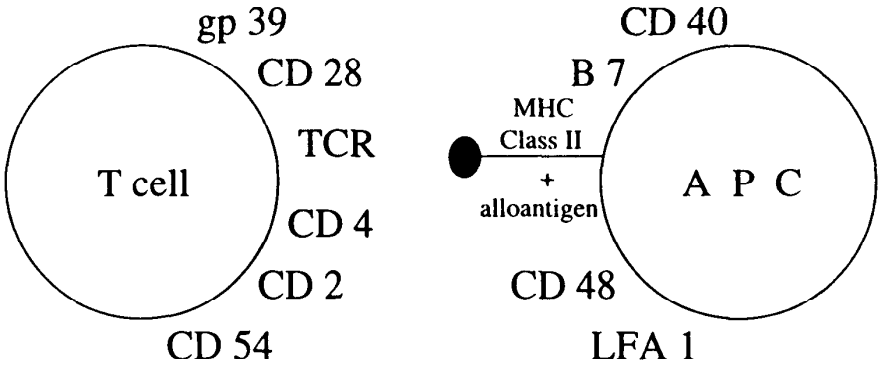

Figure 2 Second signal molecules.

models to produce anergy. These extensive studies using mAb reactive with various T-cell ligands have emphasized the efficacy of targeting specific receptors of the APC (35). By competitively occupying, for example, CD2, B7, CD40, or CD54 antigens, these mAbs alone can block the adhesion of the T cell with the appropriate ligand of the APC. Selective anergy to the simultaneously encountered donor antigen is induced because the second signal molecule is inaccessible to the T cell. As a result of this disruption of the full complement of T-cell signaling, amplifying cytokines of the immune response (e.g., IL-2) are not released (Table 2).

A. CTLA4Ig

Engagement of the CD28 molecule by its natural ligand B7 can be blocked by the soluble protein CTLA4Ig, which is homologous to the CD28 receptor. Lenschow et al. (36) have administered a fusion protein of CTLA4 and human immunoglobulin IgG1 (CTLA4Ig) to murine recipients of human islets (36). Donor-specific tolerance was observed in mice given CTLA4Ig for 14 days after transplantation. The CTLA4Ig-treated recipients of second party human islets had a mean xenograft survival of 4.5 days. In contrast, CTLA4Ig recipients of retransplanted first party donor islets maintained xenograft survival for more than 80 days (the period of analysis).

Lin and colleagues (37) have also used CTLA4Ig to provide long-term acceptance of cardiac allografts in rat recipients additionally treated with donor-specific transfusions. These rodent experiments support the continued investigation of inducing tolerance through the inhibition of the second signal pathway.

B. Anti-CD2

The CD2 is a transmembrane glycoprotein found on T, B, and natural killer (NK) cells that belongs to the immunoglobulin superfamily (38). The CD2 serves both an adhesion and a signal transduction function in these cell types. The ligand of CD2 in humans is LFA3 or CD58 (see Fig. 2). In rodents there is no homologue of LFA3; however, CD48 is the major ligand. Ligation of CD2 with either LFA3 (in humans) or CD48 (in mice) may position the responding T cell and the APC into an optimal distance for TCR-major histocompatibility complex (MHC) interaction (38), and may provide important activation signals. Bromberg et al. have studied the effect of anti-CD2 mAb on the cell surface expression of CD2 and its effect on allograft survival; anti-CD2 mAbs downmodulate CD2 from the cell surface, and this effect is accompanied by T-cell immunosuppression (39). Anti-CD2 mAbs have

Table 2 Mechanisms of Tolerance

	Central	Peripheral (Anergy)
Negative T-cell signaling	–	+
Clonal deletion	+	–
Persistent alloantigen required	–	+
Reeducation of thymus and LN stores	+	+
Chimerism	+	–
Adoptively transferred	–	+
Broken by alloantigen rechallenge	–	+
Pretransplant conditioning	+	–
Posttransplant conditioning	–	+

been shown to suppress cell-mediated immunity of heart allograft recipients (40). Administration of OX34 (7 mg/kg/day i.p.), either for 3 consecutive days immediately before or for 8 consecutive days immediately after transplantation, induced indefinite allograft survival of BN (RT1n) rat hearts transplanted heterotopically to LEW (RT11) recipients.

Squifflet has reported the results of a randomized trial of BTI-322, a rat IgG2b directed against human T and NK cells, as part of an induction therapy in renal allograft recipients (41). All recipients were given CsA, azathioprine, and steroids. The incidence of rejection episodes was reduced in BTI-322–treated patients from 60% in control subjects to 25%.

In vitro observations (J. Hope, 1997, personal communication), suggest that BTI-322 inhibits T-cell proliferation in response to mitogens and allogeneic stimulator cells. Interaction of BTI-322 with responding T cells results in alloantigen-specific anergy. These properties make the study of anti-CD2 mAbs especially attractive for the development of tolerance. In contrast to in vitro responses resulting from OKT3 stimulation, BTI-322 does not induce T-cell proliferation, IL-2, or interferon-δ (IFN) production.

C. Anti-gp39

Activated T cells express a member of the TNF cytokine superfamily, gp39, which serves as a ligand for the APC CD40, thereby enhancing a signaling process between the responding T cell and the APC (30). Larsen and colleagues have treated primary and sensitized murine cardiac recipients with MR1, a hamster mAb specific for murine gp39. A total of 250 μg of MR1 was given at the time of transplantation, and then on days 2 and 4 following transplantation. The time of the mAb treatment was important in that MR1 administration delayed to day 5 after transplantation did not prolong graft survival. Larsen has postulated that inhibition of the CD40–gp39 ligand interaction influences the T effector arm of the immune response.

D. Anti-ICAM-1

The leukocyte function associated molecule LFA-1[CD11a/CD18], a heterodimer of the integrin family, and the intercellular adhesion molecule (ICAM-1) is another receptor ligand pair (see Fig.2) that optimizes the activation of T cells (34). ICAM-1 (CD54) is an integral membrane glycoprotein with five immunoglobulin-like domains (thus a member of the Ig superfamily), which also facilitates leukocyte transendothelial migration (33). Isobe et al. have reported the indefinite survival of cardiac allografts in murine recipients treated with mAb directed against ICAM-1 and LFA-1 (42). Pilot studies of anti-ICAM-1 mAb treatment of cynomolgus (43) and human renal allograft recipients (44) and anti-CD11a mAb treatment of human renal allograft recipients (45) support the approach of disrupting the ICAM adhesion of APC with the responding T-cell LFA-1 in preventing allograft rejection. However, tolerance was not achieved by Isobe unless both mAbs were administered (48).

E. Anti-CD4

Although the mAb blockage of several T-cell adhesion molecules has been successful in producing tolerance, the most extensive study of anergy via mAb therapy has been directed toward the interruption of the CD4 coreceptor activity (46–48). The importance of the CD4 T cell in the development of the immune response is well recognized (49–50). Engagement of the CD4 antigen at the cell surface by the MHC class II antigen of the APC (with the processed alloantigen as shown in Fig. 2) is transduced across the T-cell membrane to the

CD4 cytoplasmic tail, which binds to phosphorylating tyrosine kinases (51). The intracytoplasmic component of CD4 is in proximity to the TCR but it is structurally independent. Anergy may develop if TCR signal transduction occurs without CD4–TCR complex interaction within the cytoplasm (52).

Waldmann and colleagues have shown that tolerance can be produced in murine recipients of MHC-matched heart and skin grafts following the administration of a brief course of anti-CD4 combined with anti-CD8 mAb (53). The mAb effect on the treated T cells has been characterized by Waldmann as "infectious," because tolerance can be adoptively transferred by tolerant CD4 T cells to naive lymphocytes. Other important concepts regarding the mechanism of anti-CD4–mediated tolerance have been elucidated by these investigators, such as the necessity of a sustained display of donor-specific antigen (54). Nevertheless, the significance of these experiments is underscored by the capacity of an ALS (mAb) treatment to consistently induce tolerance in rodent recipients. Because this process does not appear to require a central or thymic redefinition of self-identity, anergy provides an appealing peripheral approach to tolerance induction (53).

These encouraging results from rodent experiments have supported investigation of anti-CD4 administration as a means of inducing anergy in nonhuman primate recipients. However, for historical reference, it should be noted that the investigation of anti-CD4 mAb immunosuppression was initially begun as a prelude to the clinical trial of OKT3 therapy (55). These pilot efforts were designed to evaluate the efficacy of mAb therapy for the prevention and treatment of allograft rejection. The safety and effectiveness of OKT3 could not be evaluated in animal models, because OKT3 does not react with T cells of nonhuman primates other than chimpanzees.

Cynomolgus recipients of renal allografts were first given murine OKT4 mAb in 1980. Subsequent collaborative studies of several anti-CD4 mAbs developed by the Ortho Pharmaceutical Company and the Transplantation Unit of the Massachusetts General Hospital continued for nearly 15 years (56,57). Allograft tolerance was not induced by these anti-CD4 mAb preparations given as the sole immunosuppressive treatment, but mean renal allograft survival was markedly extended from of 8 days to 45.2 ± 6 days (58).

This confirmation of the immunosuppressive effect of selective targeting of CD4-expressing T cells led to phase I clinical trials of murine and humanized anti-CD4 mAbs. Murine OKT4A was administered to 30 human renal allograft recipients, beginning preoperatively (within 3 hours) on the day of transplantation and intended for 12 consecutive days. This study was conducted by the National Institute of Allergy and Infectious Diseases/National Institutes of Health sponsored Cooperative Clinical Trials in Transplantation (59). The standard regimen of CsA, azathioprine, and prednisone was also given so as not to disadvantage the recipient from the excellent results that might otherwise be accomplished with this regimen of immunosuppression (without OKT4A).

In a similar open-label, nonrandomized sequel study to the pilot murine OKT4A trial, 16 renal allograft recipients were given OKTcdr4a (a humanized IgG4 mAb containing the reactive murine OKT4A sequence) (60). Although OKT4A and OKTcdr4a were well tolerated, early episodes of acute rejection were still observed in 11% to 30% of human renal allograft recipients given these mAbs as part of the induction therapy. These mAbs had been administered in the hope of completely averting rejection. The disappointing clinical observations to date have led some investigators to conclude that anti-CD4 mAb induction therapy may never achieve a significant clinical value.

However, the failure of the anti-CD4 mAbs to induce either tolerance in monkeys or completely prevent allograft rejection in humans does not gainsay the successful approach in rodents. It is possible that a dose of anti-CD4 mAb sufficient to provide disruption of the

total T-cell population (comparable to that administered in the rodent models) was not given in these large animal and human trials. It is also possible that the currently available anti-CD4 mAbs are not reactive with all appropriate epitopes of the CD4 antigen, which cosignal T-cell activation with the TCR complex. In either scenario, if the "peripheral approach" is to achieve recipient tolerance through anergy, future clinical trials must address the following paradigm: mAb treatments directed toward costimulatory targets must engage not only the T cells in the peripheral blood circulation but also mature T cells that reside in the thymus, lymph nodes, and spleen. Unless the dosage or efficacy of the mAb is sufficiently increased to disarm T cells in all of these lymphoid stores, the clinical potential of accomplishing recipient tolerance via mAb treatment alone appears unlikely. Thus far, anti-CD4 mAb treatments given only for the first 10 to 14 days after transplantation have not induced allograft tolerance, and these initial discouraging results appear to have diverted investigators to focus more on other approaches.

Nevertheless, the following attractive aspect of the rodent models of mAb-induced anergy suggests that further clinical investigation of this peripheral T-cell manipulation should proceed. Anergy does not require the sustained depletion of T cells (61), nor does it appear to necessitate indefinite T-cell inhibition (62). Given the hazard of opportunistic infection that might result from indefinite and uncontrolled depletion of T cells, mAb therapies that can achieve tolerance without depletion would be more attractive (63).

It is well established that humanized mAb preparations such as Orthoclone cdr-4a do not provoke a human anti-mAb response as is observed with rat or mouse mAb preparations (60). This feature of humanized mAb also provides an opportunity for innovative schedules of mAb treatments, which could be given weekly or monthly if required. Moreover, because indefinite mAb treatment is not a requirement for tolerance in the rodent models, sequential brief periods of mAb treatment following transplantation might also be advantageous. Theoretically, an extended period of mAb T-cell inhibition (negative TCR signaling) may provide the time necessary for alloantigen (displayed on passenger leukocytes or dendritic cells derived from the donor allograft) to gain residence in the thymus and lymphoid stores. Donor-specific alloantigens subsequently encountered by host T cells restored to functional competence as mAb (and later conventional) therapy is withdrawn are not considered as foreign because potentially responding T cells have been rendered anergic. As suggested by Waldmann, a sustained display of alloantigen is necessary for tolerance to persist (54). This theory is supported by experiments of other investigators who have intentionally placed donor antigen in sites of self-recognition such as the thymus.

III. CLONAL DELETION AND THYMIC EDUCATION

Rat islets injected into the thymus of MHC-incompatible recipients are reported to induce a donor-specific nonresponsiveness when recipients are simultaneously given ALS (64). This observation by Naji (65) was significant not only for identifying the thymus as a privileged site for transplantation but also for establishing a novel method of inducing tolerance.

In the use of ALS preparations in the development of tolerance, it is noteworthy that the single dose of ALS was an essential component of the Naji protocol. Animals receiving intrathymic islets without ALS treatment consistently rejected the transplanted allografts. In contrast, tolerance developed in recipients following ALS depletion of host T cells from the peripheral blood circulation. Naji postulated that subsequent repopulation of the T-cell pool required migration of host prothymocytes through the thymus, educating newly developing T cells to be tolerant of foreign antigen.

Bone marrow cells were later given by Naji and colleagues as an alternative source of alloantigen, suggesting that exposure to donor alloantigen was necessary to induce tolerance (65). Operational tolerance was evident in that second islet transplants (syngeneic to the intrathymic islets) placed beneath the capsule of the host kidney were accepted, whereas third party islets were rejected.

The significance of the ALS administration in these seminal experiments has been bolstered by more recent investigations of intrathymic donor antigen injection by Knechtle et al. (66) and other investigators (67), which have corroborated not only this approach but also the ALS requirement. Tolerance was only induced by Knechtle et al. in rat liver transplant recipients who were simultaneously treated with anti-rat lymphocyte serum and intrathymic injection of autologous muscle cells transfected with donor specific Class I MHC antigens. Control animals (no ALS treatment) were not observed to have extended liver allograft survivals beyond 10 to 14 days. The elegant use of transfected donor antigens to recipient cells in the Knechtle experiments revealed the importance of the donor MHC alloantigen (rather than donor islets or bone marrow cells) in the development of the tolerance.

The use of ALS preparations in the development of tolerance by the approach of thymic education is not restricted to antilymphocyte sera. A depleting anti-CD4 mAb preparation has been shown to be equally effective in establishing recipient tolerance in a rodent model following intrathymic injection of donor splenocytes (67). Moreover, Knechtle has administered a murine anti-rhesus CD3 T-cell mAb conjugated to a mutated diphtheria toxin and extended the survival of renal allografts beyond 150 days (68). Animals receiving no immunotoxin had allograft survival of 1 week. Recipients of immunotoxin were also given a single injection of donor cells 7 days before renal transplantation. However, the immunotoxin was apparently so potent that two control animals receiving only a normal saline intrathymic injection also had prolonged allograft survival. In one of these animals, skin grafted 150 days after renal transplantation was accepted without further immunosuppression and without development of a significant increase of anti-donor cytotoxic T cells.

IV. CLONAL SUPPRESSION BY ANTILYMPHOCYTE SERUM AND DONOR HEMATOPOIETIC CELL INFUSION

A. Posttransplant Conditioning

Monaco et al. were the first to observe that "a stable state of tolerance" could be induced in conditioned mice following the infusion of donor allogeneic spleen and lymph node cells (69). A C3H/He × A/Jax F1 hematopoietic inoculum was administered during a period of profound host lymphopenia produced by thymectomy and ALS treatment in A/Jax recipients (Fig. 3). Operational tolerance was confirmed by the indefinite acceptance of C3H/He × A/Jax F1 hybrid skin grafts by the A/Jax recipients. A/Jax recipient chimerism for C3H/He donor cells was inferred by the following additional experiment. A presumed C3H/He × A/Jax spleen cell suspension obtained from the putative chimeric A/Jax recipient was injected into a normal A/Jax mouse. These mice subsequently rejected C3H/He skin grafts in an accelerated fashion, indicating sensitization from the spleen cell suspension of the presumed chimera containing C3H/He splenocytes.

Monaco and Russell later observed, again with precise experimental conditions, that an ALS preparation given as the only immunosuppressive treatment could provide indefinite allograft survival in large animals. Prolonged survival of canine renal allografts was

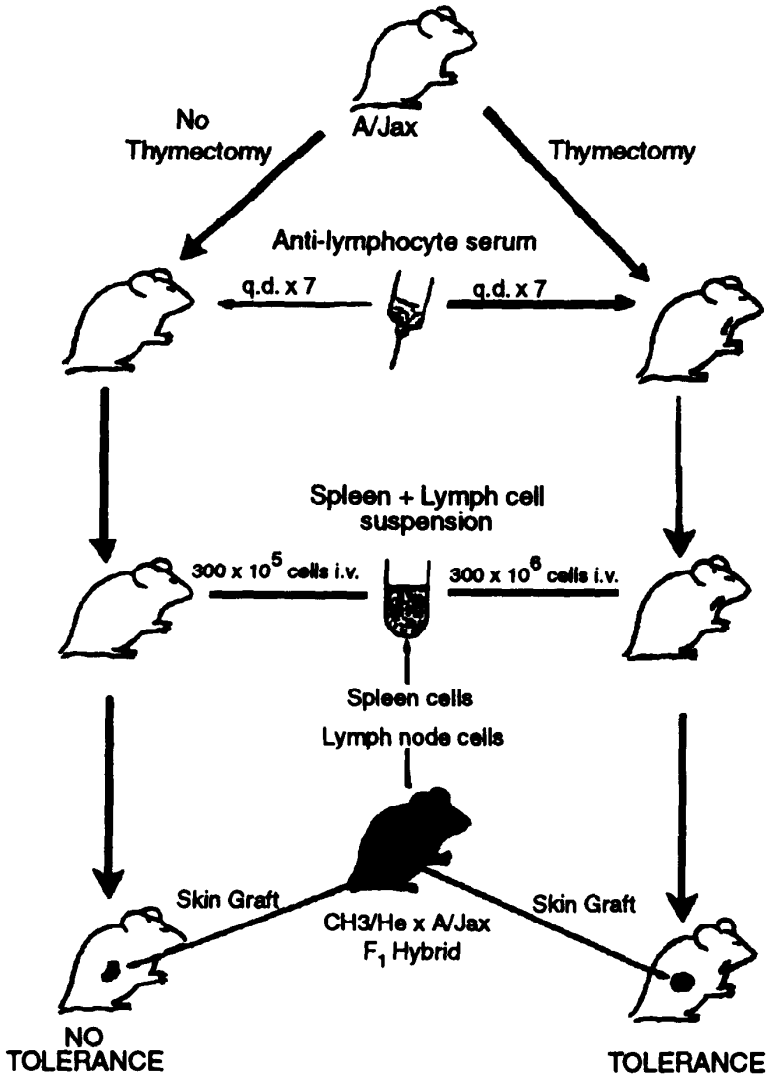

Figure 3 Experimental production of chimeras. (From Ref. 69.)

noted in two recipients following treatment of mongrel dogs with equine anticanine lymphocyte serum (70). However, these animals were not tolerant, in that long-term acceptance of the allograft depended on continued ALS therapy following transplantation. Animals given only pretransplant ALS rejected their allografts within 11 to 56 days after transplantation (71).

Nevertheless, these early observations were significant and they supported an expanded investigation of ALS-induced tolerance in large animals. Subsequent trials revealed that ALS immunosuppression of a canine renal allograft recipient could be enhanced by the simultaneous administration of donor-specific bone marrow (72). This adjunctive measure of bone marrow transplantation was later used by Thomas et al., extending the survival of rhesus monkey skin allografts (73). These initial observations in recipients of skin allografts were subsequently confirmed by the extensive studies performed in renal allograft recipients (74,75). Rhesus monkeys given rabbit antihuman thymocyte globulin for 5 days after transplantation and then allogeneic donor bone marrow on the 12th day, had long-term

renal allograft survival extended from 70 to more than 400 days without the need for additional immunosuppression (74).

The success of the Thomas protocol was especially attractive for clinical application because the ALS and bone marrow could be given *following* renal transplantation and still produce long-term graft survival. No clinical experience was subsequently reported by this group; however, this approach has been evaluated clinically by Monaco et al. (70,75) and later by Barber (76).

Monaco and Russell reported a preliminary experience with three patients who received ATG treatment for 14 days following living donor renal transplantation. The allograft recipients were also given a donor-specific bone marrow infusion on the 21st day after transplantation (75). Two recipients were reported well at 11 and 13 months after transplantation, but immunosuppression was apparently never withdrawn. Thus, operational tolerance was never confirmed. The third allograft was rejected at 3 months after transplantation.

The largest clinical experience with the posttransplant ATG plus donor bone marrow approach to tolerance, propelled by the Thomas experimental model, was reported by Barber et al. (77). Fifty-seven cadaver renal allograft recipients were treated with a 10- to 14-day course of Minnesota ALG, followed 7 days later with an infusion of cryopreserved donor bone marrow (2–3 x 10^8 cells/kg recipient body weight). A group of 54 patients received the contralateral kidney, without the infusion of bone marrow. Recipients of both groups were also given Imuran, CsA, and prednisone.

Although allograft survival in the marrow recipients was excellent (85% at 18 months), episodes of rejection were common, being no different in frequency or severity from those observed in the control group. Barber concluded that the potency of the ALS preparation was less than ideal. Moreover, attempts to withdraw immunosuppression from the marrow-treated patients were not predictably successful despite the detection of chimerism (by polymerase chain reaction [PCR]) of donor DNA sequences in the peripheral blood lymphocytes) in some patients 1 year after treatment (78).

The failure of these clinical efforts to induce tolerance has necessitated a reconsideration of experimental strategy, especially as it pertains to the timing of the ALS and donor antigen presentation. Conditioning a potential recipient with this regimen may be more successfully accomplished if it is performed before transplantation. For example, Morris and colleagues have reported a series of experiments consistently inducing tolerance in adult murine heart allograft recipients by administering the ALS preparation (anti-CD4 depleting mAb) and hematopoietic cell infusion (donor-specific transfusion) at 1 month before transplantation (79).

The consistent success of this pretransplant approach, together with the unsatisfactory clinical experience of the posttransplant protocols previously reviewed, suggests that conditioning protocols implemented after transplantation may require more intense therapy in order to direct recipient responses toward tolerance induction. Thus, we anticipate that some future trials of ALS conditioning will likely be directed toward initiating this therapy at various intervals before transplantation.

B. Pretransplant Conditioning

Pretransplant conditioning strategies introduce an alternative "central" approach to the development of operational tolerance. The central pathway involves a host reeducation of self through a continuous display of allogeneic cells of donor origin, especially in the thymus. In the past, myeloablative irradiation and reconstitution with allogeneic bone marrow

has been used to accomplish this process (80–82). However, there are two major hazards with the use of bone marrow transplantation following recipient myeloablation for the induction of tolerance. If mature T cells are not removed from the donor bone marrow, the recipient could be vulnerable to the effects of graft versus host disease (GVHD). If, on the other hand, mature T cells are completely removed from the bone marrow inoculum before transplantation, the reconstituted recipient is relatively immunoincompetent following successful production of chimerism and transplantation tolerance. Furthermore, there is an unacceptably high incidence of failure of marrow engraftment in lethally conditioned patients receiving HLA-mismatched allogeneic marrow (83,84). These conflicting impediments to the induction of transplant tolerance by this approach are elucidated in the following discussion of the rationale for the development of a mixed chimera that provides for this conditioning regimen.

1. Mixed Versus Full Allogeneic Chimerism

The induction of tolerance can be accomplished either through the creation of a full allogeneic chimera in which the reconstituted lymphohematopoietic elements are of donor origin only (80–82) or through a mixed chimera reconstituted with both allogeneic and self-marrow elements (83,84).

Tolerance to other tissues and organs from the donor of the allogeneic marrow has been demonstrated not only in experimental models of fully allogeneic chimeras but also in those unusual clinical situations in which a recipient of a sibling bone marrow transplant has later received a renal allograft from the same donor (85–87). However, if donor bone marrow infusion is being performed to induce organ allograft tolerance rather than to treat a hematological malignancy, it is neither necessary nor desirable to ablate the host's bone marrow or to establish a fully allogeneic chimera. Immunoincompetence in the fully allogeneic chimera is especially likely when an MHC barrier is crossed.

The probable reason for the immunocompetence of complete chimeras was first suggested by Zinkernagel and colleagues (88,89) and is illustrated in Fig. 4. As diagrammed in this figure of rodents, fully allogeneic chimeras result from lethal irradiation of a recipient and reconstitution with T-cell–depleted allogeneic bone marrow. New T cells that subsequently arise in such recipients are of the allogeneic donor MHC type (D) but are educated in a thymus of host MHC type (H). These new T cells therefore acquire restriction specificities for H + X, in which X is the peptide of a nominal antigen presented by MHC molecules of type H. However, the antigen-presenting cells that present environmental antigens to mature T cells in the periphery are also replaced by the bone marrow transplant and are therefore of MHC type D. The mature T cells thus encounter D + X rather than the H + X to which they are restricted. Some sharing of specificities is undoubtedly responsible for the weak immune responses that occur in such fully allogeneic chimeras, but the majority of MHC-restricted responses are disabled, leading to relative immunoincompetence.

This concept was later validated by the demonstration that mature T cells from fully allogeneic chimeras are capable of responding if appropriate antigen presenting cells of MHC type H are provided (90). As illustrated in Figure 4B, reconstitution of irradiated strain H recipients with a mixture of T-cell–depleted bone marrow cells from host strain (H) plus donor strain (D) leads to production of new mature T cells of both MHC haplotypes. In this case, both mature T-cell populations are restricted through positive selection in the thymus to the recognition of H + X. However, then antigen-presenting cells are present in the periphery of the appropriate strain H type, and immunocompetent interactions can occur.

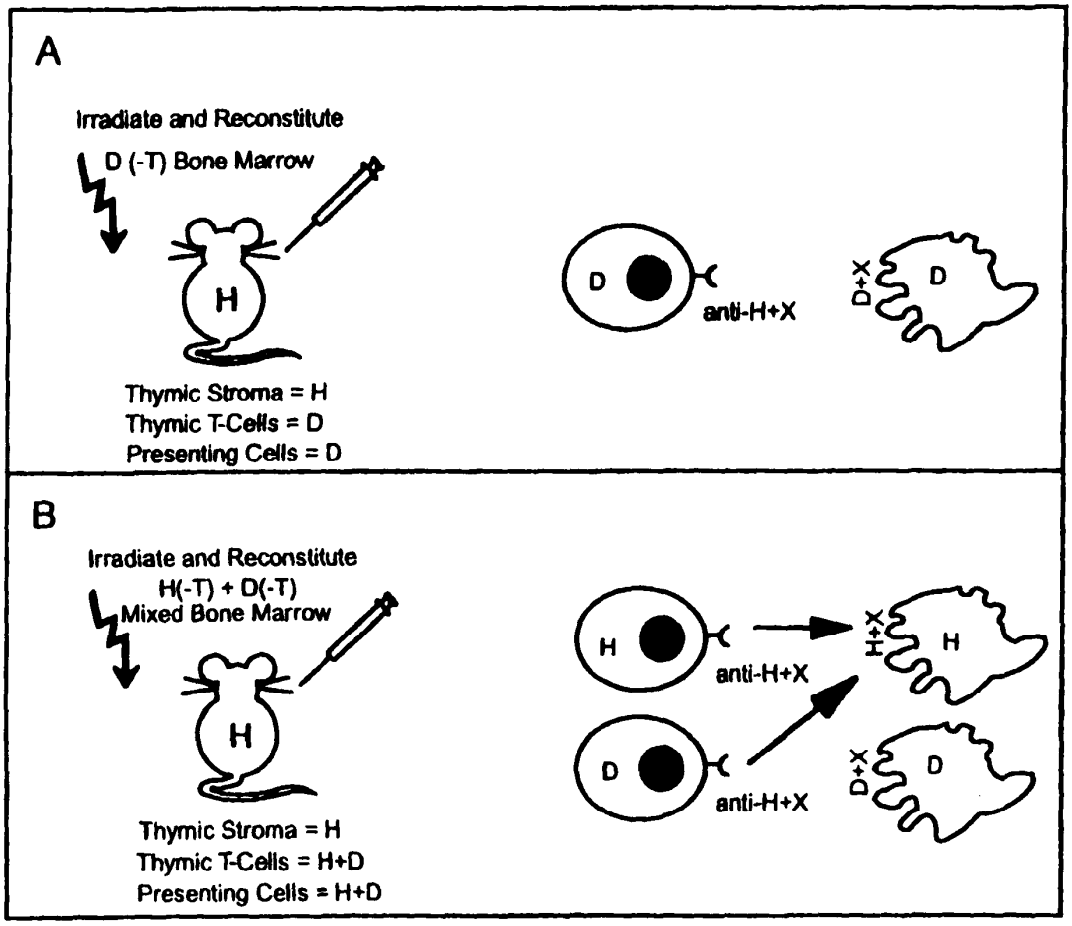

Figure 4 A. Fully allogeneic chimeras. B. Mixed chimeras.

We have referred to animals reconstituted with both allogeneic and self-lymphohematopoietic elements as "mixed" chimeras (83,91). The allogeneic bone marrow derived elements in such animals are responsible for conferring transplantation tolerance, whereas the host-type elements confer immunocompetence.

2. Tolerance Through Mixed Chimerism in Rodents

Our early studies of mixed chimerism involved lethal irradiation of B10 recipient mice followed by reconstitution with mixtures of syngeneic and fully allogeneic bone marrow, both of which were depleted of mature T cells by antibodies and complement before reconstitution (80,83,92). Such mixed chimeras were found to be specifically tolerant to skin grafts from the allogeneic B10.D2 donor strain (80,83). Thus, as illustrated in Fig. 5, the vast majority of mixed chimeras retained B10.D2 skin grafts permanently, yet they were capable of rejecting third party skin grafts (C3H) just as promptly as did normal B10 animals. Thus, induction of mixed chimerism by this procedure resulted in animals that were both immunocompetent and specifically tolerant.

To make this approach to tolerance more acceptable for clinical applications, we have evaluated approaches other than lethal irradiation for adequate recipient T-cell depletion. The most successful regimen relies on the use of ALS preparations to remove the mature T

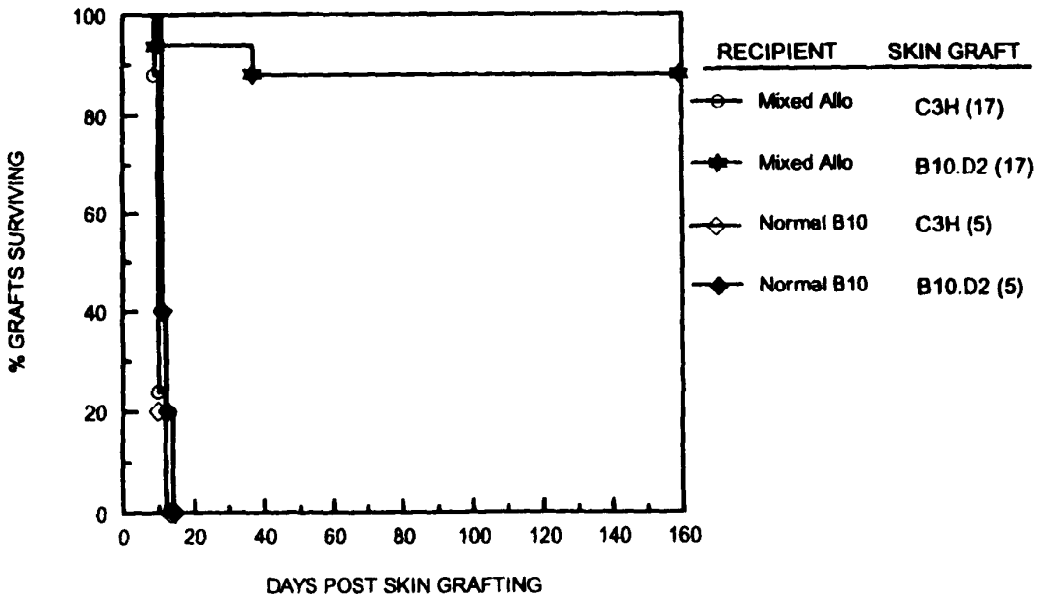

Figure 5 Survival of skin grafts on mixed chimeras.

cells, which are the main impediment to allogeneic engraftment. Cobbold et al. demonstrated that treatment of mice with antibodies to mature T cell subsets (CD4 and CD8) was capable of permitting transient engraftment of MHC-mismatched bone marrow or skin grafts (93). As an extension of this work, we attempted to determine why the engraftment was only transient. When lymphoid compartments of treated animals were examined, depletion of all mature CD4 and CD8 T cells was observed in peripheral blood, lymph nodes, and spleen (94). However, T cells were found in the thymus which were coated with antibody but not depleted. It was concluded that the mechanism by which antibody treatment leads to depletion of mature T cells does not function efficiently within the thymus. A boost of irradiation to the thymus was therefore added to the preparative regimen. Using a protocol consisting of 300 R whole-body irradiation (WBI), 700 R thymic irradiation, and treatment with monoclonal antibodies to CD4 and CD8 (Fig. 6), mixed allogeneic chimerism could be produced in most recipient animals (94).

The pattern of reconstitution of animals prepared by this nonmyeloablative regimen was indistinguishable by flow cytometric analysis from that of animals prepared by lethal irradiation and reconstitution with T cell-depleted host plus donor bone marrow. Like their counterparts prepared by the lethal preparative regimen, the mixed chimeras showed specific tolerance to subsequent skin grafts, retaining B10.D2 skin permanently and rejecting third party skin promptly. In addition, these animals were far more healthy than those produced by the lethal preparative regimen and showed none of the toxic effects of lethal irradiation. Premature graying, for example, was observed only in the small area of the neck where thymic irradiation had been carried out, and the animals gained weight equivalent to untreated cohorts. If no bone marrow was administered, these animals all survived and reconstituted syngeneically, indicating that the preparative regimen was indeed nonlethal.

3. Tolerance Through Mixed Chimerism in Nonhuman Primates

Although a variety of approaches have achieved donor-specific tolerance in rodent recipients, the development of a large animal model in which tolerance could be consistently

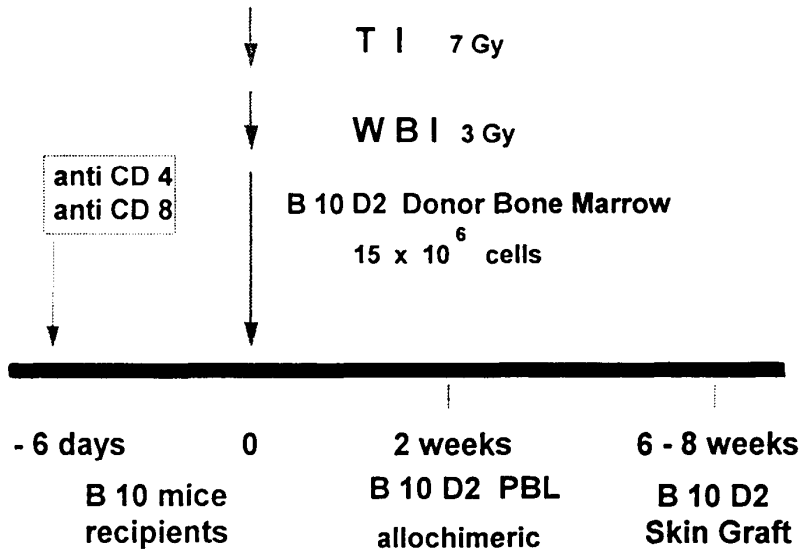

Figure 6 Protocol for mixed allogeneic chimerism. (From Ref. 94.)

derive has proved elusive (75,91). However, the consistent success of this nonmyeloablative regimen in our rodent model has provided a significant impetus to test such a conditioning protocol in nonhuman primate recipients as a prelude to clinical application.

Cynomolgus donor and recipient pairs were selected for compatible blood types, and intentionally mismatched class I and II CyLA antigens. In addition, donor–recipient pairs were selected to ensure that at least one anti-class I mAb could distinguish donor from recipient, so that chimerism could be easily assessed by FACS analysis of donor peripheral blood leukocytes. The nonlethal, nonmyeloablative protocol noted to be successful in the rodent experiments was administered with modifications appropriate for a large animal recipient. Prospective cynomolgus renal allograft recipients were initially treated with 3 Gy of whole body irradiation given as a single dose 6 days before transplantation or as 1.5 Gy fractions on days 6 and 5 (Fig. 7). As in the rodent experiments, additional T-cell depletion by an ALS preparation has proved essential for success. Because no effective mAbs are available, the polyclonal antihuman preparation ATGAM (The Upjohn Company, Kalamazoo, MI) was administered. The timing of the ATG administration was selected with the objective of depleting not only host responsive T cells but also the potential graft versus host capacity of the donor bone marrow T-cell population. Another modification of the rodent protocol was the addition of a recipient splenectomy on the day of the renal transplant. This was initially included in an attempt to reduce the morbidity of the anticipated postirradiation pancytopenia, although some reports indicate that splenectomy itself reduces the incidence of clinical rejection (95). Our continuing studies suggest that splenectomy may, in fact, be an essential component for consistent development of chimerism and allograft tolerance in this model (96).

The donor's bone marrow, ranging from 0.5 to 2.0 x 10^8 bone marrow cells/kg of recipient body weight, was infused after the renal transplant. Because initial studies revealed excellent peripheral blood T-cell depletion but persisting T cells in the recipient lymph nodes following ATG therapy, treatment with CsA was added on day 1 and continued for 4 weeks, after which no further immunosuppression was administered.

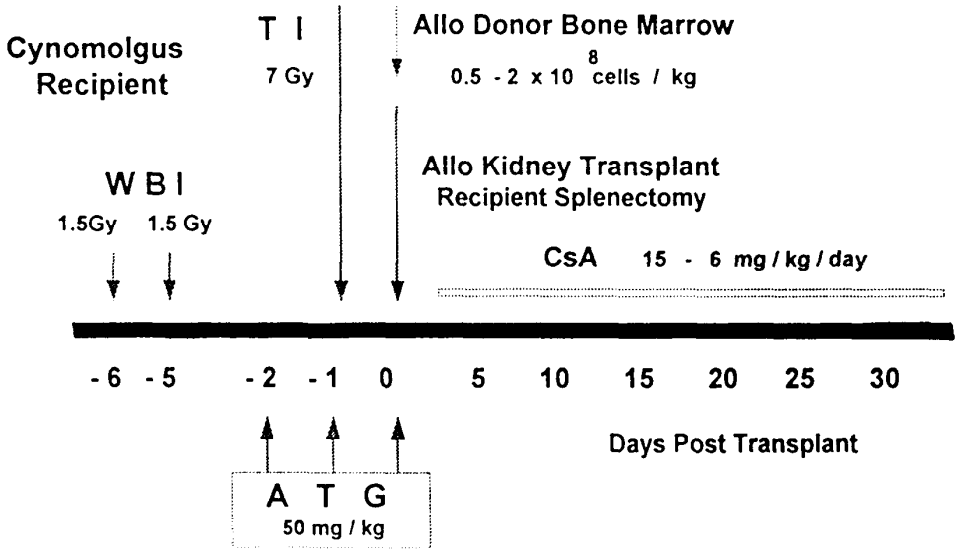

Figure 7 Protocol for donor-specific tolerance. (From Ref. 96.)

Severe pancytopenia was observed between day 7 and 18 in all monkey allograft recipients. Monkeys given the single 3 Gy WBI fraction required transfusion support during this period, whereas those treated with 5 Gy on successive days recovered from the nadir of marrow suppression without transfusions, becoming hematologically normal by day 30. Sequential flow cytometry (FCM) analyses showed clear evidence for multilineage chimerism, initially detected within the first 2 weeks and reaching its maximum around day 20 after transplantation. Thereafter, the levels of detectable chimerism decreased progressively, becoming difficult to distinguish from background staining with isotype control antibodies (i.e., less than 1% to 2%) by FCM.

Nevertheless, in six of nine monkeys receiving the conditioning regimen detailed in Fig. 7, stable tolerance developed to donor antigens, as assessed by mixed lymphocyte reaction (MLR) assays and by sequential monitoring of kidney transplant function for as long as 3 years after discontinuing all immunosuppression. Donor-specific nonresponsiveness was further confirmed in one recipient in which a kidney donor skin transplant placed on the 337th day after renal transplantation survived indefinitely while simultaneously placed third party skin grafts were rejected by day 10. This lack of host response only to original donor skin was demonstrable months after peripheral blood chimerism was undetectable by FCM analysis. It is probable that chimerism as detected by more sophisticated systematic analyses of donor DNA might have been evident either in the peripheral blood, or perhaps more importantly in the thymus or the lymph node stores. Some support for this suggestion is provided by the observation that microscopic sections of lymph nodes and thymus procured from two long-term cynomolgus allograft recipients, electively sacrificed with functioning allografts, revealed donor cells by immunoperoxidase staining. It must be emphasized that in none of these animals was any additional exogenous immunosuppression administered after day 30. We have, therefore, demonstrated that operational tolerance can be relatively regularly induced in primates by this conditioning regimen.

Conceptually, therefore, progeny of donor bone marrow cells (presumably dendritic cells), protected by the conditioning regimen, travel to the thymus and peripheral lymph

node depots, gaining residence at those locations. In the mouse model, recipient reeducation may be accomplished by negative selection (97,98). In the primate, in which a similar process may occur, the development of at least a transient mixed chimerism appears to be essential for the induction of donor nonresponsiveness and transplantation tolerance. Renal allografts were readily rejected in recipients in which multilineage hematopoietic chimerism was not detected.

4. Mixed Versus Microchimerism in Allograft Recipients

Developing mixed chimerism through a conditioning regimen using irradiation, ALS treatment, and bone marrow infusion should be distinguished from the microchimerism unpredictably identified after years of nonspecific immunosuppression.

Starzl has suggested that a state of microchimerism can be induced by the allograft alone, without bone marrow infusion or ALS, because the allograft alone may be able to provide a sufficient number of passenger donor cells (99,100). Retrospectively, Starzl and colleagues initially studied a group of mainly noncompliant patients, whose allografts were not rejected following withdrawal of all immunosuppressive medications (101). They concluded that a stable state of "microchimerism" might evolve many years after the transplantation. Donor cells, expressing histocompatibility antigens or chromosomes identical to those on the "macro" allograft, could be detected (by PCR or gender genetic analysis) in various tissues of these recipients. These cells of donor origin, which have gained a long-lived residence in recipient tissues, are thought to have a negative influence on the recipient's ability to reject the allograft. Burlingham (102) has suggested that circulating donor cells in the microchimeric individual may exert a functional role in suppressing the recipient's capacity to respond. However, a reliable method of intentionally developing the long-lived "microchimerism" observed by Starzl has not yet been developed. Furthermore, several reports emphasize that demonstration of microchimerism does not consistently denote transplantation tolerance for which immunosuppression can be safely withdrawn (103).

To accomplish engraftment of the allogeneic hematopoietic cells so that a mixed chimera can consistently emerge, a prospective, more intensive conditioning regimen appears to be essential. The mixed chimera strategy involves the disruption of the host T cells, which must be temporarily depleted or incapacitated; otherwise, successful engraftment of donor bone marrow will not occur. The ALS treatment is necessary to provide host T-cell suppression, which permits donor bone marrow engraftment, and it may also be necessary to prevent the GVHD capacity of the donor bone marrow T-cell population.

V. CONCLUSION

The goal of tolerance remains the most important consideration for all future protocols of immunosuppression. The usefulness of ALS preparations as a component of new and innovative protocols is emphasized by the requisite inclusion of an ALS preparation in all of the experimental protocols reviewed in this chapter. Rodent protocols for developing tolerance have frequently used either an mAb or ALS treatment. In the only successful large animal strategies for developing tolerance, ATG has also been a necessary component. Thus, our review suggests that the future of tolerance implementation may be dependent on the appropriate administration of an ALS preparation. Given the success that has been accomplished in rodents to large animal models, the prospect for developing tolerance in human allograft recipients appears quite promising.

ACKNOWLEDGMENTS

We gratefully acknowledge the editorial comments of Megan Sykes, M.D., in the preparation of this manuscript.

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15

Mechanisms of Action of Immunosuppressive Agents: Cyclosporine/FK506/Rapamycin

BERNHARD RYFFEL
University of Cape Town, South Africa

I. DRUG DISCOVERY, IMMUNOBIOLOGY OF TRANSPLANT REJECTION, AND IMMUNOLOGICAL INTERVENTION

A. Drug Discovery

The surgical success in vascular anastomoses at the beginning of the century was thwarted by loss of the kidney because of rejection. The immunological basis of events involved in graft rejection was unknown at that time; the notion of histocompatibility antigens and their differences between individuals emerged 60 years later. Successful allotransplantation was performed between identical twins as early as 1950. Irradiation and chemical immunosuppression were first attempted as adjunct therapies in the early 1960s. The milestones of renal transplantation are summarized in Table 1. The discovery of azathioprine and its combined use with glucocorticosteroids marked the beginning of a new era in organ transplantation. Surgeons, encouraged by results with chemical immunosuppression, attempted the transplantation of several other organs, including the liver and heart. Rejection crises were treated with high doses of glucocorticosteroids and/or antilymphocyte serum. An important limitation of the treatment was the toxicity of the immunosuppressants: leukopenia induced by the purine analogue azathioprine and Cushing's syndrome by chronic glucocorticosteroid excess.

Table 1 Milestones of Kidney Transplantation

1902	Experimental kidney transplantation (Ullmann)
1906	Xenotransplantation into humans (Jaboulay)
1954	Kidney transplantation between twins (Murray et al.)
1960	Azathioprine immunosuppression (Calne, Zukoski)
1967	Antilymphocyte globulin (Starzl)
1978	Cyclosporine (Calne)
1983	OKT-3 antibody (Cosimi)
1990	FK506 (Starzl)
1995–2000	Rapamycin, RS 61443 (MPA), brequinar, leflunomide?

The discovery of cyclosporine (1) and its introduction into the clinic in 1978 (1,2) heralded a therapeutic breakthrough that enabled controlled immunosuppression to be used in transplant patients. Cyclosporine proved to be much more selective for the immune system and devoid of adverse effects on the hematopoietic system. However, despite the excellent results achieved, clinicians retain reservations concerning the use of cyclosporine for at least two reasons: renal dysfunction as the major side effect and failure to induce lasting immunological tolerance with cyclosporine (1). The mechanisms of newer immunosuppressants emerging from microbiological research, the macrolide-structured molecules FK506 and rapamycin, as well as immunosuppressive effects of cyclosporine are discussed in this chapter.

It is already evident that each immunosuppressant has its specific profile of adverse effects. The specific toxicities of these immunosuppressive agents are a focus of this chapter. Because side effects are tightly related to pharmacological activity, the mode of action of the immunosuppressants is reviewed and the possible relations to the mode of action are discussed. Understanding of molecular drug action may help to predict drug toxicity; this approach may be necessary for the novel immunosuppressants that are not yet available on the market.

B. Immunobiology of Transplant Rejection

A simplified scheme of cellular events occurring during the interaction between the grafted organ and its host is given in Fig. 1. Schematically, three phases may be distinguished: first, an afferent phase, which includes the recognition of the foreign tissue—that is, the allograft; second, a central phase occurring within the immune system, comprising the differentiation of alloantigen-specific lymphocytic clones into effector cells; third, the final efferent phase, characterized by cellular and humoral effector mechanisms, leading to the ultimate rejection of the allograft (reviewed in Ref. 3). Genetic differences between donor and host are defined by histocompatibility complex molecules expressed on the cell membrane. Donor cells with histocompatibility molecules differing from those of the host are recognized by the host's immune system. Continuous alloantigenic stimulation results in profound activation of helper T (Th) cells with the release of several cytokines leading ultimately to the maturation of effector immune cells (e.g., cytotoxic T cells, activated macrophages, natural killer (NK) cells, and plasma cells). These activated effector cells, acting in concert with several cytokines—for example, interferon (IFN) and interleukins (ILs), which increase the class II expression within the graft, and tumor necrosis factor (TNF), with its direct cytotoxic effect—and specific antibodies attack the graft and cause its rejection. The tremendous progress in the areas of allorecognition, cytokines, and the

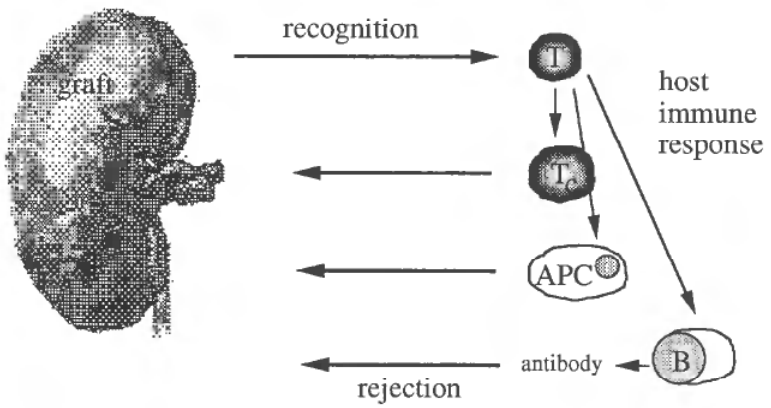

Figure 1 Cellular events leading to graft rejection. Afferent limb: Alloantigen from the graft is presented by dendritic cells (APC). Central part: Recognition of alloantigen by host immune system, activation of antigen presenting cells (APC), T cells, and B cells. Efferent limb: Activated lymphocytes, antibodies and complement, macrophages, and lymphokines destroy the graft.

identification of their specific membrane receptors will allow a better understanding of the molecular events of the graft rejection (4,5).

C. Possible Sites of Immunological Intervention

The interaction of the antigen presenting cell (APC) with the antigen receptor on helper T lymphocyte surface is the central event leading to the activation of the immune system (reviewed in Ref. 3) (Table 2). The activation of the Th cells results in the synthesis and release of several cytokines, affecting the maturation of effector cells with cytotoxic function (Tc, NK) and of B lymphocytes (Fig. 2). In the induction of immune suppression, the first potential site of intervention in the host immune system would be at the level of antigen uptake, followed by processing and final assembly into the host class II molecule of antigen presenting cells. Even though such specificity of suppression is possible in vitro, it may be difficult to achieve in vivo. Agents that inhibit the activation of APCs and their release of cytokines (e.g., IL-1, IL-6, or TNF), however, may have a profound effect on subsequent T-cell activation. The T-cell receptor (TcR), consisting of a heterodimeric antigen-recognition domain and several invariant associated chains (known as the CD3 complex),

Table 2 Potential Sites of Immunosuppression: Macrophage/T-Helper Cell

Antigen presentation	Inhibition of antigen processing, presentation of MHO class II molecule
	Bioengineered xenotransplants
Signal recognition	Blocking antibodies against T-cell receptor, accessory molecules of T-cell activation, specific peptides
Signal transduction	Inhibition of specific kinases or phosphatases: fyn-, lck-kinase, CD45 phosphatase, calcineurin. PLCγ, MAP-kinase, G-proteins
Gene transcription	Inhibition of cytokine or receptor
	Modulation of regulatory gene (myc, myb, rb, p53, cyclins)
Cytokine action	Neutralizing antibodies against cytokine or cytokine receptor
	Soluble receptors
Inhibition of cell cycle	Antimetabolites

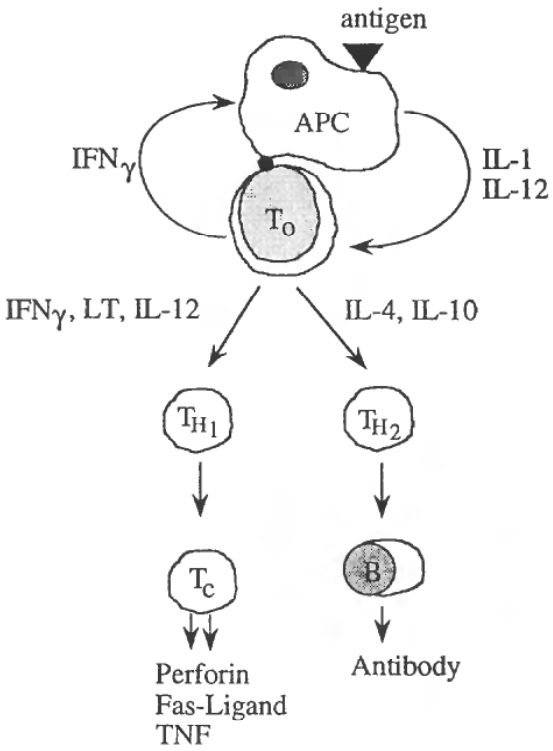

Figure 2 Central role of T-helper lymphocyte, cellular interactions. Antigen is processed by antigen-presenting cells (APC) and presented within the major histocompatibility molecules to the T-cell (T_0) receptor. Activated APC, release IL-1, IL-12, and TNF, whereas activated T-helper cells (TH) synthesize several interleukins, which have multiple regulatory effects, shifting the immune response to a TH1-type response with the differentiation of cytotoxic T lymphocytes (Tc) or to a TH2 response, resulting in B-cell differentiation and antibody-secreting plasma cells.

is another potential site of immunointervention, which would theoretically prevent recognition of the antigenic signal (6). Antibodies directed against TcR or CD3 may block the access of the processed antigen presented by the class II molecules. Instead of antibodies, irrelevant peptides may be used, which compete for the class II site, thereby preventing correct recognition by the TcR. The interaction of TcR with the specific antigenic peptide presented by class II molecules provides the specificity of the reaction but is alone insufficient for T-cell activation. Other membrane molecules, such as CD4/8 and several accessory molecules such as CD28/CTLA4, FAS-L, and CD40-L on T lymphocytes, are necessary to increase the affinity of the cellular interaction. Thus, prevention of these additional contact sites, predominantly by antibodies, is another potential avenue for inhibition of T-cell activation.

Upon recognition by the TcR, the antigenic signal is transduced through complex biochemical pathways, which include the activation of several enzymes—such as phospholipase C_γ, kinases (e.g., fyn, lck, and MAP kinase), and phosphatases, including CD45 and calcineurin. A key feature of this transduction is the release of inositol-3-phosphate, thus mobilizing intracellular calcium, which, with diacylglycerol, activates protein kinase C. Recent reports concerning cyclosporine A (CsA) and FK506 have concluded that the phosphatase calcineurin plays an important role through dephosphorylation of the nuclear factor NFAT. Upon dephosphorylation, NFAT is translocated into the nucleus, where it binds to the IL-2 promotor, resulting in IL-2 gene transcription. However, other factors

(e.g., NFKB and AP-1) were shown to be necessary for IL-2 gene activation. The control of IL-2 gene activation/transcription is an active area of research.

The genetic program induced by antigen stimulation is complex and includes the active transcription of at least 70 genes. The inhibition of gene expression of molecules such as cytokine receptors, interferons, and TNF must also be considered for the induction of immunosuppression.

Pharmacological modulation of antigenic processing within the APC is also a potential avenue to immunosuppression that remains unexploited.

II. OVERVIEW OF IMMUNOSUPPRESSIVE AGENTS

This chapter provides a short overview of several presently used immunosuppressants; additional agents are summarized briefly (Table 3):

A. Azathioprine

Azathioprine is an imidazole derivative of 6-mercaptopurine, a purine analogue with antimetabolite properties, inhibiting primary immune responses. Because azathioprine immunosuppression is mediated by inhibition of cell proliferation, it is not surprising that other systems, especially the hemopoietic system, are also adversely affected. Leukopenia is the main dose-limiting side effect (7–10). Azathioprine is often combined with steroids and CsA as maintenance immunosuppressive therapy, but it has no effect in antirejection therapy.

Table 3 Overview of Immunosuppressant Agents: Chemical and Biological

Drug	Mode of Action	Side Effects
Azathioprine	Antimetabolite, inhibition of purine biosynthesis	Myelotoxicity
Cyclosporine	IL-2 gene transcription	Nephrotoxicity
Glucocorticosteroids	Inhibition of IL-1 synthesis	Cushing's syndrome
FK506	IL-2 gene transcription	Nephrotoxicity/neurotoxicity
Rapamycin	IL-2 signal transduction, inhibition of S6 kinase	?myelotoxicity
Leflunomide	IL-2 signal transduction	?myelotoxicity
Deoxyspergualin (DSG)	IL-2 signal transduction?	?myelotoxicity
Mycophenolate mofetil (MPA)	Inhibition of de novo purine synthesis	?myelotoxicity
Brequinar (BQR)	Inhibition of de novo purine synthesis	?myelotoxicity
OKT3	Activation and sequestration of T lymphocytes	Fever
Antibodies	MHC class II IL-2R ICAM-1, FLA-1, CD4, B7, CTLA4 IFNγ, TNF	?

B. Antilymphocyte Antibodies

Polyclonal antibodies raised against human lymphocytes in rabbit or horse are in clinical use for the treatment of transplant rejection. These antisera bind to circulating lymphocytes, causing a sequestration in the spleen and resulting in subsequent lympholysis. Monoclonal antibodies (anti-CD3) have the advantage of being a homogeneous product, in contrast to the antisera. Although monoclonal antibodies act similarly to the polyclonal antibodies, an important difference is the mitogenic activity of anti-CD3 monoclonal antibodies on T cells, which leads to their activation, resulting in fever caused by the release of cytokines (11–14).

C. Glucocorticosteroids

Steroids inhibit T-lymphocyte activation by blocking the release of IL-1 from macrophages and subsequent IL-2 synthesis. Furthermore, steroids cause the lysis of lymphocytes. The immunosuppressive properties of steroids can be used clinically for maintenance and antirejection therapy.

Steroids have pleiotropic effects on other systems in the body (e.g., inhibition of inflammation and interference with several metabolic pathways, causing hyperglycemia). The adverse metabolic effects of chronic therapy, manifesting as Cushing's syndrome, are usually dose-limiting (15,16).

D. Cyclosporine A

Cyclosporine A is a fungal peptide that inhibits IL-2 gene transcription. The immunosuppressive property of CsA is successfully used clinically for maintenance and antirejection therapy. Recent investigations reveal that CsA confers immunosuppression by inhibiting the calcineurin phosphatase. It is assumed that deficient dephosphorylation of NFAT prevents its nuclear translocation and IL-2 gene transcription (1).

E. FK506, Rapamycin

These molecules are two novel and potent immunosuppressants with macrolide structure. Whereas FK506 has a similar mode of action to CsA (i.e., inhibition of IL-2 gene transcription by affecting calcineurin phosphatase), rapamycin is the prototype of a novel class of immunosuppressants. Rapamycin has no effect on IL-2 gene expression but potently blocks IL-2 signal transduction by inhibition of the S6 kinase (2,17,18).

III. CYCLOSPORINE—MOLECULAR MECHANISM OF ACTION, RELATION TO TOXICITY

Cyclosporine A is a member of the cyclic undecapeptides (Fig. 3), which have immunosuppressive properties (1). It has been successfully used to prevent allograft rejection and to treat several autoimmune diseases (2). Molecular studies on its mode of action have revealed that CsA prevents T-lymphocyte activation at the level of cytokine gene transcription (reviewed in Refs. 3,17,18). Recent investigations with macrolide immunosuppressants showed that FK506 has the same effect on cytokine gene transcription as CsA, thereby inhibiting T-lymphocyte activation (19–22). These findings provoked investigations at the molecular level designed to identify possible pathways common for both immunosuppressants. Such studies may provide important insights into the control of cytokine gene activation.

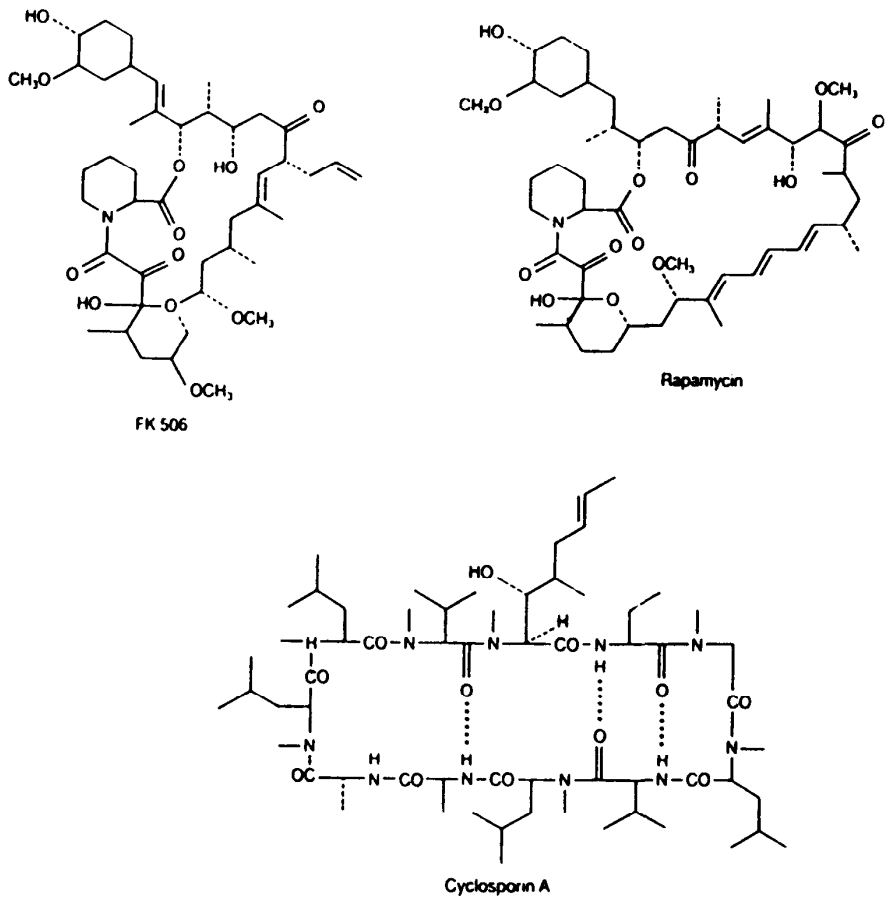

Figure 3 Comparison of the structures of cyclosporine, FK506, and rapamycin.

A. Cellular Uptake of CsA, Evidence of Intracellular Receptors

Specific, saturable, and reversible binding was shown for murine and human mononuclear blood leukocytes using a ^{3}H-CsA derivative (23,24). Investigations over a broader concentration range in erythrocytes and in several nucleated cell types revealed two components of cell binding (3): a saturable cytosolic binding at low CsA concentrations and a nonsaturable, nonspecific partitioning into the membrane at higher CsA concentrations. These findings, together with evidence for CsA accumulation within the cell, suggest the existence of an intracellular binding protein (25).

The discovery of cyclophilin A (CPH-A), an 18-kD protein that specifically binds CsA, was a seminal contribution to the understanding of CsA-mediated events (26), opening many avenues of further research. The amino acid sequence of CPH-A was apparently not related to any known protein (27); however, it was later established that CPH-A was homologous to a prolyl-peptidyl cis-trans-isomerase, also known as rotamase (28,29). Active cyclosporines were shown to bind to CPH-A (30) and inhibit its rotamase activity.

The specificity of cyclosporine–CPH-A binding was investigated by hydrophobic interactions using an LH-20 column (27), competitive solid phase enzyme-linked immunosorbent assay (31,32), and photoaffinity labeling (33). Binding to CPH-A correlated with the immunosuppressive activity of cyclosporine analogues. Amino acids 1, 2, 10, and 11 of the CsA molecule were found to be essential for cyclophilin binding. Subtle changes in these residues reduced both the affinity for CPH-A and in vitro immunosuppressive activity (32,34).

The synthesis of a photoaffinity-labeled cyclosporine analogue allowed the identification of several additional CsA binding proteins (33,35,36). In the T-cell line Jurkat, labeled proteins of 21, 25, 40, and 60 kD were identified (37). The labeled proteins at 21 and 25 kD were identical with CPH-A and CPH-B (Fig. 4). CPH-B is a second CsA-binding protein, which has an endoplasmic reticulum retention signal (38–40). Two new members of this family were also identified (Table 4): CPH-C, which reportedly has a restricted

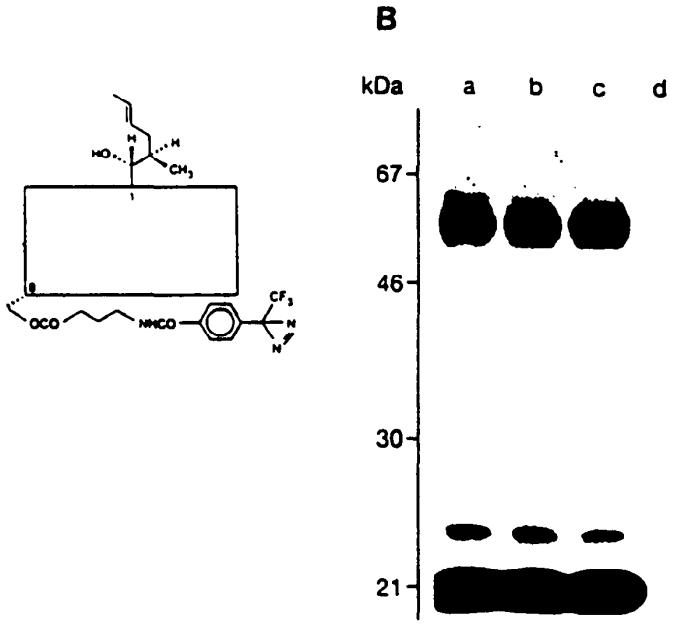

Figure 4 A: In vivo detection of CsA receptor proteins. B: Structure of photoaffinity-labeled derivative. The human T-cell line Jurkat is incubated with titrated photoaffinity label–probe. After UV cross-linking, the labeled cellular proteins are separated by SDS-PAGE and the proteins detected by fluoroautoradiography (a). The specificity of binding is defined by competition with 10× molar excess CsA (d) or by lack of competition by inactive CsH (b) or FK506 (c).

Table 4 Properties of CsA and FK506 Binding Proteins (Immunophilins)

Name	Molecular Weight (kDa)	(%)	Homology	Location[a]	Rotamase Activity
Cyclophilins					
CPH-A	18		—	c	+
CPH-B	22		64	m	+
CPH-C	23		?	?	+
CPH-D	22		72	m	+
CPH-40	40		?	c	+
CPH-45	45		?		
FKBPs					
FKBP12	12			c	+
FKBP13	13		60	m	+
FKBP25	25		40	n	+
FKBP59	59			n?	+

[a]Cellular location: membrane (m), cytosol (c), nuclear (n).

tissue distribution (41), and CPH-D (42). These latter proteins are less abundant than CPH-A and have a molecular mass of approximately 22 kD; thus, they were not distinguishable from CPH-B by photoaffinity labeling. Kieffer et al. (43) purified a 40-kD protein (CPH-40) by affinity chromatography; the partial sequence analysis of this protein showed homology with CPH-A. CPH-40 antiserum did not cross-react with CPH-A in immunoblot analysis. Another 45-kD CsA-binding protein, which is phosphorylated, was reported but not further characterized (44). The roles of several CsA target proteins have not been resolved.

B. Cyclophilin Distribution and Function

All the members of the CPH family have rotamase (prolyl-peptidyl cis-trans-isomerase) activity, which is inhibited by CsA (42,45). CPH-A exhibits the highest specific activity and is most sensitive to CsA inhibition. Macrolide-derived immunosuppressants, however, do not affect this CPH rotamase activity. All CPHs are highly abundant in both lymphoid and nonlymphoid tissues. The subcellular localization of CPH proteins has been investigated by biochemical cell fractionation studies and immunoelectron microscopy. Cyclophilin A and B are found in both cytosol and nucleus, and demonstrate no specific association with organellar structures (46–48). CPH-B and D possess a membrane localization signal and are found in the endoplasmic reticulum–membrane fraction.

The relative abundance and high conservation of cyclophilins suggest, however, an important role in normal cell function (26,46,49,50). The search for an endogenous ligand of CPH has been hitherto unsuccessful. The initial uptake and intracellular concentration of active cyclosporines at cytosolic and/or nuclear sites (25), followed by inhibition of rotamase activity are two potential roles for CPH-A in CsA-mediated immunosuppression. However, the role of rotamase inhibition has been questioned, because the IL-2 gene transcription is fully inhibited at just 1% occupancy of CPH-A.

C. The CsA–Cyclophilin Binding Site

The CsA–cyclophilin complex has been investigated by x-ray and nuclear magnetic resonance (NMR) techniques (51–55). CPH-A exhibits a β barrel shape with a radius of 17 Å. The main structural elements are two perpendicular four-stranded β sheets and two well-defined α helices (Fig. 5A). Most of the hydrophobic side chains are packed in a hydrophobic core. Other hydrophobic residues occur in the contact region between the two helices, the β sheets, and in the CsA binding site. Replacement of the two cysteines (Cys 62 and 115) by alanine affected neither CsA binding nor rotamase activity. The sole tryptophane residue (Trp 121) is, however, necessary for binding (56). The macrolide-binding protein, FKBP, has no significant homology with CPH-A. Common three-dimensional surface structures that could preexist or be induced by CsA or FK506 on their respective immunophilins are areas of intense research.

D. Calcineurin as a Target of the CsA–Cyclophilin Complex

As shown in Fig. 5, the photolabile cyclosporine derivative labeled not only cyclophilin but also a 60-kD protein in Jurkat T cells, which is most likely calcineurin. It was recently demonstrated that the phosphatase calcineurin forms a complex with the drug and immunophilin (41,57). The complex was only formed in the presence of the CsA and cyclophilin together with calmodulin and calcium.

Calcineurin consists of catalytic (A) and regulatory subunits (B). Calcineurin B has a molecular weight of 19 kD and high homology to calmodulin. Calcineurin A, the 61-kD

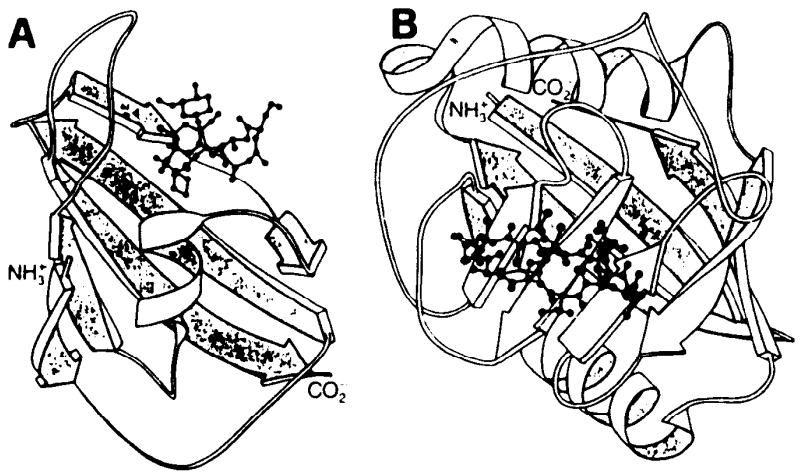

Figure 5 A: Three-dimensional structure of the complex of human FKBP. B: With FK506 and human CPH-A with CsA. The data were obtained by x-ray and NMR analyses. (From Refs. 251 and 69.)

subunit, contains the catalytic domain of the serine-threonine phosphatase. Calcineurin occurs ubiquitously in the body and is a highly conserved protein. Two isozymes of calcineurin A (type I and II) occur as the result of alternative splicing events (58). The C terminus contains an inhibitory domain and an adjacent calmodulin binding domain, which are rapidly removed by limited proteolysis. The central part of the protein, being resistant to proteolysis, harbors the catalytic domains and is identical for the two isozymes of calcineurin. This region shows extensive similarities to the catalytic subunits of protein phosphatases 1 and 2B, which define a distinct family of protein phosphatases. The 40 amino acid N-terminal fragment, which is specific for calcineurin, contains 11 successive prolines, possibly important for the binding to CPH-A/B or to FKBP.

E. Role of Calcineurin in IL-2 Gene Transcription

Calcineurin is abundant in lymphoid cells (59). Fruman et al. (60) demonstrated calcineurin phosphatase activity in lysates of Jurkat T cells. CsA inhibited cellular calcineurin activity at drug concentrations that inhibit IL-2 synthesis in activated T cells. These findings taken together suggest that calcineurin plays a role in T-cell activation. Another approach chosen to investigate the role of calcineurin T-cell activation was the cotransfection of an IL-2 promotor-linked reporter-gene construct together with murine calcineurin A into Jurkat cells. As expected; the overexpression of calcineurin caused relative resistance to the immunosuppressants, necessitating higher concentrations to achieve the same immunosuppressive effect (61,62). These results implicate calcineurin as a component of the T-cell receptor signal transduction pathway. The present understanding of the molecular events leading to the inhibition of IL-2 gene transcription is depicted in Fig. 6.

F. Molecular Mechanisms Leading to CsA Nephrotoxicity

The mechanisms leading to nephrotoxicity are being examined. At immunosuppressive doses, CsA causes a reduction of the glomerular filtration rate (GFR) and slight arterial hypertension (63,64). The present knowledge of the pathogenesis of this renal dysfunction

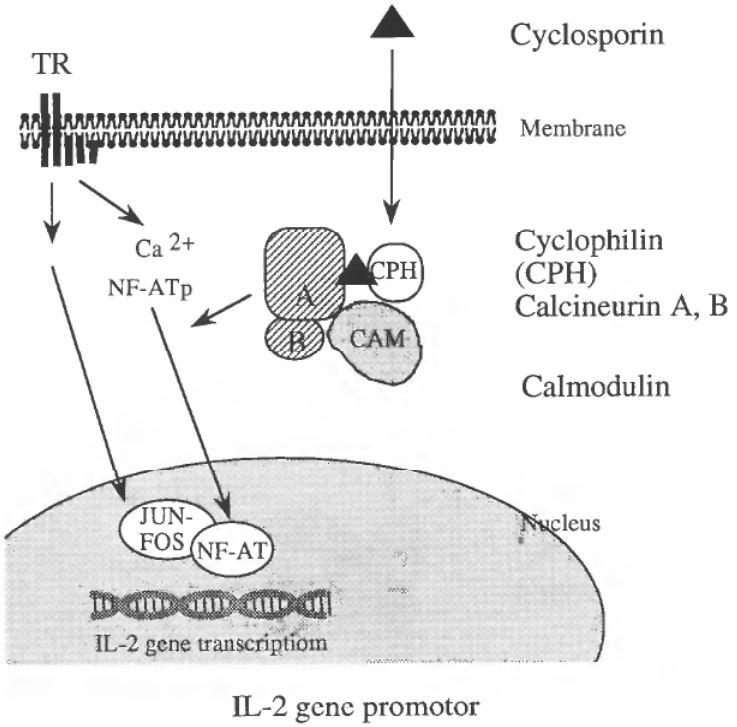

Figure 6 Schematic representation of the drug–immunophilin complex, binding and inhibition of the calcineurin phosphatase, thus inhibiting the translocation of the cytosolic subunit of the nuclear factor of T-cell activation (NF-ATc).

is summarized in Fig. 7: CsA may affect renal mesangial cells directly, resulting in reduction of the GFR. In addition, endothelial cells and smooth muscle cells, especially of the afferent arterioles, may respond directly to CsA and release further vasoactive mediators. At higher concentrations, the tubular epithelial cells themselves may develop degenerative changes.

The abundance of CPH-A, B, and calcineurin, at least as determined by Western blot analysis, does not differ between drug-sensitive (lymphocytes and kidney) and drug-resistant organs (50,65,66). Friedman and Weissman (41) claimed that CYP-C occurs only in the immune system and the kidney. CPH-C could, therefore, explain the relative tissue-specificity of CsA action and perhaps account for the specificity of organ toxicity, that is, nephrotoxicity. We could not confirm this observation (unpublished). Attempts have been made by several groups to correlate the ability of several CsA derivatives to bind cyclophilin with their immunosuppressive and toxic activity in vivo (22). However, presently available in vitro data do not confirm such a relationship. The formation of drug–immunophilin complexes with calcineurin is very likely in renal target cells, because abundant calcineurin is present in the kidney (65,66). The CsA–cyclophilin complex associates with renal calcineurin and inhibits its phosphatase activity in vitro. Higher-molecular-weight complexes consisting of calcineurin and cyclophilin in the presence but not in the absence of CsA were found (Fig. 8). Thus, receptor-bound CsA inhibits renal calcineurin in vitro.

The next question concerns the identification of the substrate of renal calcineurin–immunophilin complex. Possible candidate substrates include the effector peptides, leading to toxicity, or factors upstream, leading to effector molecules of toxicity. Presently, candidate peptides considered as important for the development of nephrotoxicity are endothelin, renin, tissue factor, and TGF-β.

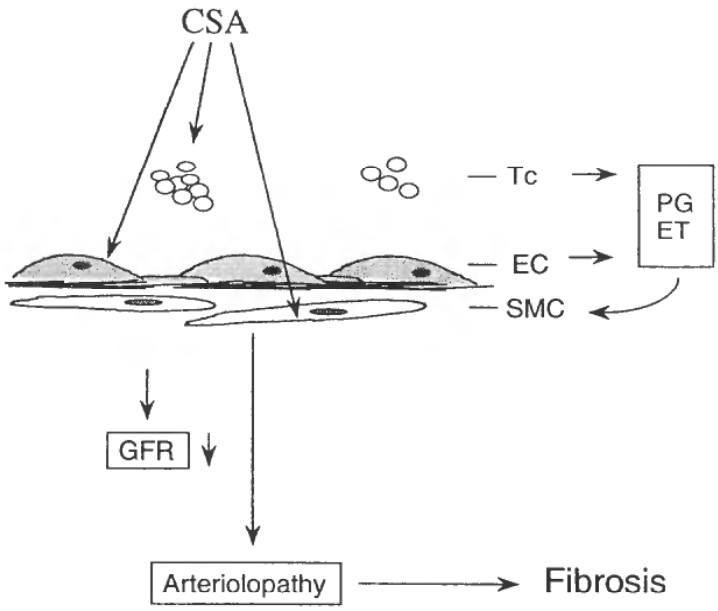

Figure 7 The cellular targets of cyclosporine comprise endothelial cells (EC), smooth muscle cells (SMC), and thrombocytes (Tc). CsA may cause adherence of platelets to the endothelium and synthesis and/or release of endothelin (ET) and prostaglandin (PG), which may be followed by endothelial and smooth muscle cell damage and regeneration. The direct or indirect contractile effect of CsA on the smooth muscle cell causes a reduction of glomerular filtration rate (GFR); the progressive arteriolar damage (arteriolopathy) results in focal interstitial fibrosis.

Despite initial enthusiastic reports, FK506 was shown to cause a similar type of nephrotoxicity to CsA in addition to neurotoxicity in controlled clinical trials. The related macrolide rapamycin with its different mode of immunosuppression-induction is devoid of nephrotoxic side effects (Ryffel, unpublished). One possible explanation is that rapamycin neither binds to calcineurin nor inhibits calcineurin-phosphatase activation. These findings suggest that the immunophilin drug–calcineurin complex may indeed be involved in the development of the nephrotoxicity of both CsA and FK506.

IV. FK506 AND RAPAMYCIN—MACROLIDES INTERRUPTING DISTINCT PATHWAYS

Macrolide immunosuppressants are a structurally distinct family of immunosuppressants, composed of FK506 and rapamycin (see Fig. 4). Whereas FK506 inhibits cytokine gene transcription in a manner identical to that of CsA, rapamycin has a completely different mode of action (67–69).

A. Cellular Receptors

Both FK506 and rapamycin bind to a 12-kD cytosolic protein, FKBP12 (70–76). FKBP12 has no homology to any known protein but has rotamase activity comparable to that of the CPHs. The rotamase activity of FKBP12 is inhibited by FK506 and rapamycin but is not affected by CsA. Because both FK506 and rapamycin have similar inhibitory effects on

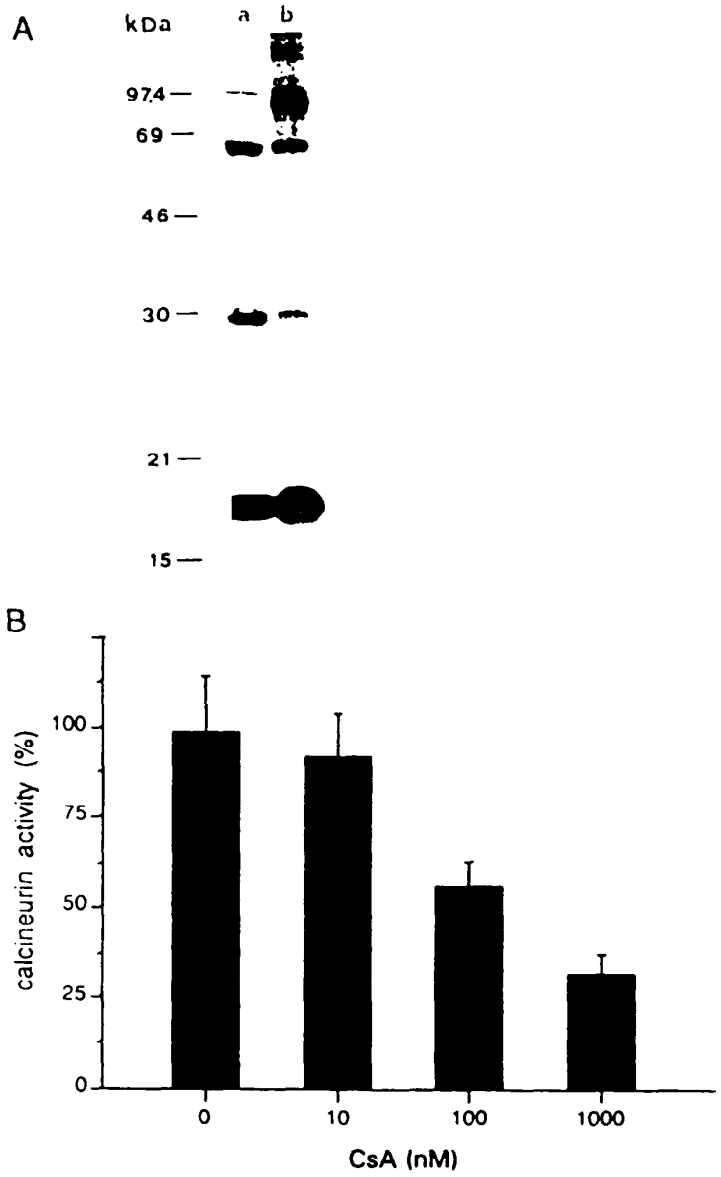

Figure 8 Evidence for immunophilin–drug–calcineurin complex and inhibition of calcineurin activity in rat kidney. A: Ternary complex of calcineurin with cyclophilin and CsA in rat kidney. Renal tissue lysate was incubated in the absence (a) or presence of 1nM CsA (b) with the chemical cross-linker DST. The proteins were separated on a 7.5% SDS PAGE and transferred on nitrocellulose, and calcineurin complexes were detected with anticalcineurin A/B antiserum. B: Inhibition of calcineurin phosphatase activity by CsA. Renal tissue lysate was incubated with several concentrations of CsA, and calcineurin activity was determined using a radiolabeled synthetic peptide.

FKBP12, inhibition of the rotamase fails to explain the different actions of the two drugs. Additional members of the family with binding specificity for the macrolide immunosuppressants were sought (Table 4). Indeed, several other FKBPs are able to bind FK or rapamycin (77–84a).

The three-dimensional structure was investigated by NMR and x-ray crystallography (Fig. 5B). The main structural element is a five-stranded antiparallel β sheet, which wraps around a short helix without any similarity to CPH-A. FK506 binds in a shallow cavity

between the α helix and the β sheet, half of the ligand being buried in the receptor protein. The binding site is composed of conserved aromatic residues (for review, see Ref. 18).

B. Calcineurin as a Common Target of Drug–Immunophilin Complex

Since it was shown that the rotamase activity was also inhibitable by nonimmunosuppressant macrolides and that the rotamases have no absolute substrate specificity, it has been suggested that the FKBPs and immunophilins in general may have a "dominant" function; binding of the drug to the cognate immunophilin may thus result in a gain of function (37). Proline binding by immunophilins might be an important property for the association with common target proteins. The demonstration that the phosphatase calcineurin forms a complex not only with CsA–cyclophilin but also with FK506–FKBP was an exciting observation, possibly explaining the identical mode of action (41,57). The complex forms only in the presence of the drug, its cognate immunophilin, calmodulin, and calcium. Rapamycin bound to FKBP does not form a complex with calcineurin. The immunosuppressants CsA and FK506 inhibit calcineurin phosphatase activity in the presence of their specific immunophilins and calcium (66,85).

C. FK506 Blocks IL-2 Gene Transcription by Inhibiting the Calcineurin Phosphatase

Fruman et al. (60) demonstrated that calcineurin phosphatase activity in lysates from Jurkat T cells was inhibited by CsA and FK506, with rapamycin having no effect. Cotransfection of an IL-2 promotor-linked reporter-gene construct together with murine calcineurin A into Jurkat cells caused relative resistance to the immunosuppressants CsA and FK506 (61,62). These results implicate calcineurin as a component of the T-cell receptor signal transduction pathway. A likely substrate of calcineurin is the nuclear factor of activated T cells (NFAT), a cytoplasmic phosphoprotein. It is hypothesized that, upon T-cell activation, NFAT is dephosphorylated and translocated into the nucleus, where it binds to the IL-2 promotor region. Thus, it may be assumed that inhibition of the calcineurin phosphatase prevents this dephosphorylation and subsequent IL-2 gene transcription (see Fig. 6).

D. Rapamycin Inhibits Signal Transduction Through S6 Kinase Activation

It is well established that rapamycin has no effect on calcineurin activity and does not inhibit IL-2 gene transcription. The discovery that rapamycin completely and rapidly inhibits IL-2–induced phosphorylation and activation of p70 S6 kinase was a major breakthrough in the understanding of the differing modes of action of the closely related molecules. The blockade of biochemical events proximal to p70 S6 kinase activation by rapamycin implicates this signaling pathway in the regulation of T-cell entry into the S phase. In addition, Chung et al. (80) showed that rapamycin blocked phosphorylation and activation of p70 S6 kinase in a variety of animal cells of nonlymphoid origin. These studies demonstrate that a growth factor–induced signaling event, not merely restricted to T cells, may be impinged upon by rapamycin through the induction of a blockade of entry into the S phase (68,69).

Rapamycin, which binds to the same immunophilin as FK506, FKBP12, neither associates nor inhibits calcineurin phosphatase. Importantly, rapamycin has no effect on IL-2 gene transcription. An interesting observation was the recent discovery of FKBP25 (see Table 4), a rapamycin-specific receptor. It may be speculated that FKBP25 targets rapamycin to the rapamycin-sensitive S6 kinase. In contrast to CsA and FK506, rapamycin

inhibits the activation process at a later stage (e.g., the IL-2 receptor-induced entry into S phase and subsequent T-cell proliferation) (81–83). Kuo et al. (67) presented evidence that IL-2 selectively induces the phosphorylation and activation of the p70 S6 kinase.

V. AZATHIOPRINE, STEROIDS, AND ANTILYMPHOCYTE ANTIBODIES, STILL USEFUL FOR MAINTENANCE IMMUNOSUPPRESSION AND REJECTION THERAPY

Azathioprine, an imidazole derivative of 6-mercaptopurine, was the first immunosuppressant that allowed successful renal transplantation to be performed. Azathioprine was synthesized by Hitching and Elion and was found to be more portent than 6-mercaptopurine (84). Both compounds are purine analogues and inhibit cell replication in general. The importance and relative specificity of the inhibited pathway of purine metabolism for lymphocytes is clearly demonstrated by the profound immune incompetence associated with adenosine deaminase deficiency. Azathioprine proved to be a potent immunosuppressant and is useful in preventing allograft rejection, but it has no therapeutic effect in acute rejection episodes. Azathioprine is usually combined with steroids for effective immunosuppression. In this combination, 1-year graft survival is on the order of 50%. The main limitation of azathioprine immunosuppression, referable to its general inhibitory effect on cell replication, is myelosuppression (7–10,84b,85).

Glucocorticosteroids, which are normally produced by the adrenal cortex, have a broad spectrum of activity; they possess metabolic, antiinflammatory, and immunosuppressive activities. Their antiinflammatory and immunosuppressive properties are used to therapeutic advantage in several clinical conditions, including autoimmune diseases, allergic reactions, and allograft rejection. The effect of steroids on the immune system is fairly well established. Steroids inhibit the release of the macrophage-derived IL-1, thereby blocking the IL-1–dependent activation of T lymphocytes and synthesis of IL-2, the central T-cell growth factor. Steroids also result in the lysis of T lymphocytes, an effect that is more profound in murine than in human lymphocytes (15,16,86).

Steroids used at low doses are useful as adjunct immunosuppressants and are of prime importance in rejection therapy. For rejection therapy, high-dose intravenous bolus injections of methylprednisolone (up to 1 g) are given, sometimes in conjunction with antilymphocyte serum or anti-CD3 antibody.

Adverse effects of steroids, especially of long-term therapy, are well known and include osteoporosis, diabetes, gastric ulcer, infections, and water and electrolyte retention, described as Cushing's syndrome.

Antilymphocyte antibodies, polyclonal antibodies raised against human lymphocytes in rabbit or horse, are in clinical use for the treatment of transplant rejection. These antisera bind to circulating lymphocytes, causing a sequestration in the spleen and resulting in subsequent lympholysis. Monoclonal antibodies (anti-CD3) have the advantage of being a homogeneous product, in contrast to the antisera. Although acting similarly to the polyclonal antibodies, an important difference is the mitogenic activity of anti-CD3 monoclonal antibodies on T cells, which leads to their activation, resulting in fever caused by the release of cytokines (11–14).

Thus, the main side effect is the so-called cytokine release syndrome, which usually is manifest by fever; more severe signs such as rash or hypersensitivity reaction and allergic shock are very rare, because the patients have a maintenance immunosuppressive therapy. A common fear with combinations of immunosuppressants is the higher risk of lym-

phoma development, which has been reported with OKT3 but which is a common concern for any drug combination.

VI. RISKS OF IMMUNOSUPPRESSION AND DRUG TOXICITY

Any form of immunosuppression bears the risk of infections and tumor development. The incidence of infection depends on the dose and the specificity of the immunosuppressant. Viral infections are the most frequent complication. With respect to tumor development, one may distinguish between direct genetic damage and indirect or epigenetic mechanisms (87,88). Although alkylating agents predominantly make up the first group, any type of immunosuppressant increases the risk of tumor development resulting from a weakening of immune surveillance. The tumor types found in immunosuppressed patients include lymphomas, skin tumors, and occasionally brain tumors (88). The risk of these complications may be reduced by minimal dose immunosuppressive therapies.

Specific toxicity associated with a given immunosuppressant is as follows:

1. Fever and malaise after OKT3 therapy (known as cytokine-release syndrome) commonly occur and are due to the systemic release of endogenous cytokines, including TNF, IL-1, and interferons. Therapy with antibodies against other cellular targets (e.g., IL-2 receptor antibody, adhesion molecules) likely will have some systemic adverse reactions, as outlined for OKT3 (89).

2. The bone marrow is a common target of antiproliferative drugs. Bone marrow depression after azathioprine and several of the novel immunosuppressants, which have emerged from the area of tumor therapy and have general antiproliferative properties, are frequent and will be dose limiting.

3. Glucocorticosteroids used at high dose for the prevention of acute rejection are also a common component of maintenance immunosuppressive regimens in combination with CsA or azathioprine. However, the long-term administration of steroids is followed by the well-known Cushing's syndrome.

4. Reduction of glomerular filtration rates occur commonly with CsA and FK506 immunosuppression. Dose limiting of such a therapy is, however, renal arteriolar disease, resulting in interstitial fibrosis and occasionally in renal failure. This is a well-recognized complication and can be avoided by switching to an alternative immunosuppressive regimen.

5. Gastrointestinal erosions, bleeding, and ulcers are found with steroids and all the antiproliferative type of immunosuppressants.

6. Chronic rejection is one of the major threats to long-term heart and kidney transplant function. Chronic graft rejection is characterized by progressive narrowing of the graft arteries and arterioles, interstitial cellular infiltration and fibrosis, glomerular changes, and tubular atrophy. The obliteration of the vascular lumen is due to infiltration of the intima by mononuclear cells, proliferation of vascular smooth muscle cells and fibroblasts of the media, and migration into the intima, with increased deposition of extracellular matrix proteins. Graft failure caused by chronic rejection is an important cause of graft loss within 5 to 10 years after transplantation. The pathophysiology of chronic rejection is complex and involves a number of mechanisms, including endothelial cell damage, monocyte-macrophage activation, proliferation of smooth muscle cells and fibroblasts resulting from the release of several cytokines and oxygen radicals, and release of vasoactive peptides, including endothelin and nitric oxygen, as reviewed in Fellström and Larsson (90) and Häyry et al. (91). Because the pathology is characterized by invasion of immunoreactive cells within the vessel wall, a key to the prevention of chronic vascular rejection might be

the optimization of the immunosuppressive treatment. Presently there is no established means of preventing chronic vascular rejection and the proposed experimental strategies, such as interruption of prostaglandin metabolism, somatostatin inhibitors, and others, have to be tested in patients (90).

VII. EFFECTIVE IMMUNOSUPPRESSION WITHOUT RISK—THE FUTURE?

What is possible to achieve in the near future? Two lines of research will probably lead to the desired goal of long-term graft acceptance and maybe to tolerance: first, further developments in drug discovery and, second, genetic modifications of the graft or host.

For drug discovery, the long list of novel immunosuppressants with only limited adverse effects is a source of hope. Most of the discoveries have been made by an ever-increasing and more sophisticated screening of natural products. Mechanistic studies with the novel experimental immunosuppressants might provide new insights into pathways of gene activation. The identification of pivotal proteins such as regulatory phophatases and/or kinases may also represent the key for a rational drug design.

The induction of transplant tolerance is a goal that is presently achievable only in rodent models. Some of the novel agents have promise in the prevention of xenograft rejection, and it is possible that these agents may help to unravel novel pathways.

With the advent of novel immunosuppressive agents and technical improvements in transplant surgery, xenotransplantation is being considered in light of the shortage of human kidneys so that more patients might benefit from organ replacement therapy. The rejection of discordant xenografts is very different from classic cell-mediated allograft rejection. It appears to involve the binding of naturally occurring host antibodies to xenograft antigens, followed by the activation of complement (92,93).

Genetic modifications of the graft or the host will be an area of intense research. These procedures must include the identification of the relevant target proteins for graft acceptance, identification of stable and safe vectors for gene transfer, and establishment of an ethical basis for such procedures.

Xenotransplantation of healthy organs with tolerance induction is the ultimate goal of medical intervention in the immune system (94). Impressive advances in the area of tolerance have been achieved, generating hope for clinical application of this goal.

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16

Cyclosporine: Clinical Use in Kidney Transplantation

BERTRAM L. KASISKE

University of Minnesota School of Medicine and Hennepin County Medical Center, Minneapolis, Minnesota

I. INTRODUCTION

Cyclosporine A (CsA), first discovered in 1972, is a product of the soil fungus *Tolypocladium inflatum.* It is a lipophilic, cyclical polypeptide containing 11 amino acids, one of which is unique to CsA. Cyclosporine A has a molecular weight of 1203 kD. Borel et al. first described the potent immunosuppressive effects of CsA in 1976 (1). The first phase I clinical trial was carried out by Calne and coworkers in seven renal transplant recipients (2). The results of studies from these investigators, which demonstrated both efficacy and toxicity, eventually led to large-scale clinical trials in Canada (3) and Europe (4). These trials convincingly demonstrated the efficacy of CsA in preventing acute renal allograft rejection and in improving short-term graft survival. The advent of CsA also brought heart and combined heart-lung transplantation (5), as well as liver transplantation (6), into the clinical mainstream.

Cyclosporine A appears to be most effective in preventing the initiation of allograft rejection. Indeed, in rat transplant models, CsA is relatively ineffective if given after the immune response has been induced (7). CsA inhibits the proliferative response of lymphocytes stimulated with both mitogens and antigens, and it inhibits the generation of cytotoxic T cells. However, the chief mode of action seems to be centered on T-helper lymphocytes,

where CsA prevents the production of interleukin 2 (IL-2). The effect of CsA on B cells is less clear. CsA does not appear to interfere with T-suppressor cells.

There is no convincing evidence that there is a receptor for CsA on cell membranes. However, once in cells, CsA binds to calmodulin and cyclophilin. It was once thought that the binding of CsA to cyclophilin, a peptidyl-prolyl *cis-trans* isomerase, was directly responsible for preventing the activation of the IL-2 gene. However, it is currently thought that the CsA–cyclophilin complex binds to calcineurin, thereby preventing calcineurin from activating the nuclear transcription factor of activated T cells (NFAT). This inhibition of NFAT prevents the normal transcription of the IL-2 gene and, to a lesser extent, genes for other cytokines important in cell proliferation. For further discussion of the mechanisms of CsA action see Chap. 15.

II. MONITORING CSA THERAPY

Although extremely variable, the bioavailability of CsA is about 30%. Therefore, an oral dose approximately three times higher than an intravenous dose, on average, achieves a comparable blood level. However, in some individuals, as little as 10% of the oral dose may be absorbed. A number of factors may affect CsA bioavailability. Malabsorption, diabetic gastroparesis, biliary diversion, and food may all affect absorption. In addition, bioavailability tends to increase in the first few weeks after transplantation (8).

Cyclosporine A is lipophilic and binds to lipoproteins and red blood cells. Not surprisingly, increases in both plasma lipids and hematocrit are associated with higher whole blood CsA levels (9). Whether changes in lipid levels affect delivery of CsA to cells and, therefore, the pharmacodynamic activity of CsA, is unclear. It is possible that lower lipid levels could lead to higher free CsA levels and more cellular uptake. Indeed, in liver transplant recipients very low lipid levels were associated with systemic CsA toxicity (10). In heart transplant recipients, the lipid-lowering agent pravastatin decreased the severity of rejection, possibly by a direct effect of 3-hydroxy-3-methylglutaryl enzyme CoA (HMG-CoA) inhibition, as postulated by the investigators (11). However, it is also possible that lowering lipids increased free levels and cellular uptake of CsA in these cardiac transplant recipients. Alternatively, CsA may be taken up by cells via lipoprotein receptors. If this is the case, then the amount of CsA delivered to cells could be a function of both lipoprotein receptor expression and plasma lipoprotein levels. In short, although whole blood CsA and lipid levels vary in parallel, the effects of these changes on CsA efficacy and toxicity are unknown.

Cyclosporine A is metabolized in the liver by the cytochrome P-450 system. Cytochrome P-450 is a highly inducible enzyme system that metabolizes a wide variety of drugs and toxins. This fact explains many of the alterations in CsA levels caused by drug interactions (Table 1). There are several metabolites of CsA, some of which may have immunosuppressive effects.

Cyclosporine A can be measured by several different techniques. Data from Europe suggest that most centers there use semiautomated immunoassay techniques (12) and that few use the gold standard, high-performance liquid chromatography (HPLC). Among the immunoassays used worldwide are the INCSTAR CYCLO-Trac SP (Incstar, Minneapolis, MN) radioimmunoassay (RIA), the Abbott Tdx (Abbott Diagnostics, Chicago, IL) monoclonal antibody fluorescence polarization immunoassay (FPIA), and the Syva enzyme multiplied immunoassay technique (EMIT). Some centers also use a polyclonal antibody with the Abbott Tdx FPIA. To a greater or lesser extent, antibodies may measure metabolites along with the parent compound, leading to levels that tend to be higher (10%–30%) than

Table 1 Common Drug Interactions with Cyclosporine A

Drug	Mechanism	Effect on CsA Blood Levels
Antibiotics		
Erythromycin	P-450[a] inhibition	↑↑
Rifampicin	P-450 induction	↓
Isoniazide	P-450 induction	↓
Antifungal agents		
Ketoconazole	P-450 inhibition	↑↑↑
Fluconazole	P-450 inhibition	↑↑
Itraconazole	P-450 inhibition	↑↑
Anticonvulsants		
Barbiturates	P-450 induction	↓↓↓
Phenytoin	P-450 induction	↓
Carbamazepine	P-450 induction	↓
Benzodiazepines	—	↔
Valproic acid	—	↔
Calcium channel blockers		
Nifedipine	—	↔
Amplodipine	—	↔
Felodipine	—	↔
Isradipine	—	↔
Nicardipine	P-450 inhibition	↑
Verapamil	P-450 inhibition	↑
Diltiazem	P-450 inhibition	↑↑
Methylprednisolone	P-450 inhibition	↑
Danazol	P-450 inhibition	↑
Lipid-lowering agents	↓ Plasma binding	↓
Metoclopramide	↑ Absorption	↑

[a]Cytochrome P-450 enzymes.

those determined by HPLC (12,13). However, metabolites may also be immunosuppressive to some extent, and there are no good clinical data suggesting that any assay is superior to any other when it comes to predicting clinical events.

The need to monitor CsA blood levels is compelling, given the fact that CsA has a very narrow therapeutic window (i.e., the difference between a therapeutic and a toxic dose is relatively small). However, how to best monitor CsA therapy has been an ongoing controversy. Some researchers have argued that routine blood levels are so unreliable that monitoring CsA therapy is a waste of time and money. Others have advocated the use of detailed pharmacokinetic studies in every patient. Standard practice probably lies between these two approaches.

A number of studies have shown that low CsA blood levels correlate with the occurrence of subsequent acute rejection episodes in renal allograft recipients and that high CsA levels are associated with CsA nephrotoxicity (generally defined in these studies as a high serum creatinine) (14,15). Although statistically significant associations between CsA blood levels and clinical events have been reported by many investigators, the reliability of blood CsA levels for predicting outcomes in an individual patient appears to be quite low.

In general, when random trough whole blood CsA levels are greater than 250 to 300 ng/mL, the chances are good that an increase in serum creatinine is due to CsA nephrotoxicity (15). When trough levels are less than 100 ng/mL, it is more likely that an increase in serum creatinine is due to acute rejection (15). These are only crude correlations and most CsA blood levels fall in the middle range, where the predictability of clinical events is too low to be reliable.

It probably is true that pharmacokinetic studies correlate more closely with clinical events than do random trough whole blood levels (15). However, even with pharmacokinetic studies, the predictability of clinical events is not high. Moreover, obtaining detailed pharmacokinetic studies is cumbersome for both patients and physicians. Although there have been few comprehensive surveys (13), it appears that relatively few centers routinely obtain pharmacokinetic studies to monitor CsA therapy. The ideal way to monitor CsA therapy is with an assay of the biological effects of CsA. So far, however, attempts to measure the pharmacodynamic activity of CsA have met with only limited success and results are not available for routine clinical practice (16,17).

Most transplant centers obtain periodic trough CsA levels and adjust the dose of CsA if levels fall outside of a fairly wide range (13). Levels may also be obtained when there is an increase in serum creatinine. If, for example, there is a rise in serum creatinine, the level of CsA is found to be high, and the serum creatinine returns to baseline when the dose of CsA is reduced, then the patient can be followed closely with a presumptive diagnosis of CsA toxicity. Whenever there is any doubt, a biopsy is usually obtained.

The dosing interval for CsA is usually every 12 hours (13). However, some patients may have a short terminal elimination half-life that makes the use of a shorter dosing interval appropriate. Others may occasionally have a long half-life, suggesting that the dosing interval could be once daily. There are few studies examining the relationship between dosing interval and clinical outcomes; however, it may be reasonable to obtain a pharmacokinetic study in a patient who seems to have persistently high or low trough levels and to adjust the CsA dosing interval when the half-life is unusually short or long.

III. CLINICAL USE OF CSA EARLY AFTER RENAL TRANSPLANTATION

A. CsA Toxicity Early After Renal Transplantation

The clinical utility of CsA lies in its ability to prevent acute allograft rejection. However, the use of CsA is associated with both systemic and renal toxicity in the early posttransplantation period. Acute systemic toxic manifestations of CsA include hypertension, electrolyte abnormalities, acid-base disturbances, tremor, and other less common neurological abnormalities (Table 2). The most common and problematic manifestation of CsA toxicity in the early post-transplant period is decreased allograft function.

B. The Pathogenesis of Acute CsA Nephrotoxicity

The pathogenesis of acute renal dysfunction induced by CsA is still not completely understood (18). Certainly renal vasoconstriction seems to play a prominent role. A number of studies in humans and animal models have suggested possible mechanisms for the CsA-induced renal vasoconstriction. Increased sympathetic nervous system activity has been suggested as one mechanism leading to CsA-induced renal vasoconstriction (19,20). However, this is unlikely to be the only explanation, because renal dysfunction is common early

Table 2 Cyclosporine Toxicity

Adverse Effect	Frequency
Delayed graft function	↑↑↑
Acute reversible decrease in renal function	↑↑↑
Thrombotic microangiopathy	↑
Tubular/electrolyte abnormalities	
Hyperkalemia	↑↑
Hypomagnesemia	↑↑
Metabolic acidosis	↑↑
CsA-associated arteriopathy	↑
Chronic renal interstitial fibrosis	↑↑↑
Cardiovascular disease risk factors	
Increased total cholesterol	↑↑↑
Increased low density lipoprotein cholesterol	↑↑↑
Increased triglycerides	↑↑
Decreased high density lipoprotein	↑
Hyperglycemia/diabetes	↑↑
Hypertension	↑↑↑↑
Hyperuricemia/gout	↑↑
Hypercoagulability	↑
Hepatotoxicity	↑
Seizures/central nervous system toxicity	↑
Tremor	↑↑↑↑
Hypertrichosis	↑↑
Gingival hypertrophy	↑↑

after renal transplantation when the kidney is still largely denervated. The renin angiotensin system could play a role; however, evidence indicates that the renin angiotensin system is either unchanged (21,22) or suppressed (23,24) in patients given CsA. Clinical data regarding the role of inhibition of vasodilatory and vasoconstricting prostaglandins in the renal vascular effects of CsA have been contradictory (18). For example, urinary levels of thromboxane have been reported to be normal in patients given CsA (25). However, discontinuation of CsA led to lower urine thromboxane levels in patients with idiopathic uveitis (26). In vitro data suggest that CsA may have direct vasoconstrictor effects in arteries and arterioles (18). These effects could be mediated by changes in endothelium-dependent relaxing factor (nitric oxide), endothelin, or direct and indirect effects on intracellular calcium levels.

In addition to the reversible vasoconstrictive effects of CsA seen early after transplantation, CsA may also have direct, acute, toxic effects on renal tubules and vasculature. Histologically, high doses of CsA can cause vacuolization and other nonspecific changes of renal tubular cells and a spectrum of arteriolar lesions ranging from medial hyalinization to an acute syndrome indistinguishable from hemolytic uremic syndrome (HUS) or thrombotic microangiopathic purpura (TTP). However, the pathogenesis of these lesions is not well defined. In the renal tubule, CsA could have direct toxic effects, for example, by causing lysomal release of intracellular toxins or by suppressing mitochondrial respiration.

With regard to the arteriolar lesions, CsA can cause platelet aggregation that could theoretically play an important role in endovascular microthrombus formation and fibrin deposition.

C. Delayed Graft Function

A number of uncontrolled studies have shown that delayed renal allograft function seems to be more problematic when therapy with CsA is initiated immediately after transplantation. However, there is a large amount of center-specific variability in the incidence of delayed graft function and in the apparent effects of CsA on its incidence and severity. In a Canadian multicenter trial (n = 209) the incidence of delayed graft function was 39% in the standard group and 52% with CsA ($p = 0.08$) (3). The number of days on dialysis was 3.7 ± 7.7 compared to 7.8 ± 13.0, respectively ($p = 0.01$) (3). In a randomized controlled trial in Minneapolis (n = 230), the incidence of delayed graft function was 30% with conventional immunosuppression compared to 31% with CsA (p = not significant) (27). However, the number of dialysis treatments required was increased from 7.4 ± 3.1 in the conventional immunosuppression group to 15.8 ± 18.1 with CsA ($p < 0.05$). Michael and coworkers conducted a clinical trial in which they randomly allocated patients with delayed graft function at 12 to 24 hours after transplantation to Minnesota ALG and prednisone (n = 19) or CsA (10 mg/kg/day) and prednisone (n = 26) (28). They also found that CsA significantly prolonged delayed graft function from a mean of 9.7 days in the control group to 13.7 days in the CsA group ($p < 0.05$). Thus, it is clear that CsA can exacerbate delayed graft function. However, many other factors, such as the duration of cold ischemia time, influence the severity of delayed graft function, and the impact of CsA on delayed graft function is, therefore, center specific.

Despite the fact that CsA appears to prolong delayed graft function, short-term graft survival has nevertheless improved with CsA. Thus, whether the increased duration of delayed graft function seen with CsA adversely affects graft survival is unclear. In general, delayed graft function has been found to be associated with subsequent graft failure (29,30). The possible negative impact of CsA on graft survival caused by an exacerbation of delayed graft function presents a major clinical conundrum, because the immunosuppressive effects of CsA are most efficacious if CsA is initiated before, or soon after, transplantation. A common strategy has been to delay the initiation of CsA until renal allograft function is established and to use monoclonal or polyclonal antibody "induction" therapy in the early post–transplant period. Indeed, in a survey of U.S. transplantation program directors, polyclonal or monoclonal induction therapy was favored by 54% and 51%, respectively, of kidney, 54% and 55% of heart, and 48% and 53% of heart-lung programs (31). Induction therapy was favored by 76% and 72% of pancreas transplant program directors, whereas only 37% and 35% of liver transplant program directors favored induction therapy (31).

Recent data have suggested that prolongation of delayed graft function seen with the early initiation of CsA may be ameliorated with the concomitant use of a calcium channel blocker. Indeed, some preliminary data suggest that the use of a calcium channel blocker may make it possible to give CsA before or immediately after transplantation, thereby obviating the need for antibody induction therapy. Two uncontrolled trials in renal transplant recipients seemed to confirm the lack of adverse effects from replacing antibody induction therapy with the immediate institution of CsA (32,33). We recently reported preliminary results of a randomized, controlled clinical trial substantiating the advantages of this approach in renal transplant recipients (34). Likewise, an uncontrolled trial in heart trans-

plant recipients also seemed to indicate the feasibility of replacing antibody induction with the immediate use of CsA combined with a calcium channel blocker (35). Although, the optimal way to use CsA in the very early post-transplant period requires further study, the use of calcium antagonists may allow clinicians to replace antibody induction with the immediate initiation of CsA.

D. Acute Reversible Renal Dysfunction

Acute CsA toxicity was first described in bone marrow transplant patients, and was associated with very high doses and blood levels of CsA (36). Acute renal dysfunction in heart transplant recipients is common in the first few days after transplantation. It is characterized by oliguria and low urinary sodium excretion, and is most severe in patients with pretransplant renal dysfunction (37). Acute renal dysfunction has also been seen with intravenous administration of CsA following liver transplantation (38). Acute nephrotoxicity is less severe early after renal transplantation, probably because lower doses of CsA are generally used in renal transplant recipients. Acute reversible nephrotoxicity is most often manifest as a mild increase in serum creatinine during the first few weeks after renal transplantation. It is infrequently associated with oliguria.

The mild, reversible renal dysfunction attributable to CsA is usually not a major clinical problem per se. However, it is problematic because it must be distinguished from acute renal allograft rejection. Efforts have been made to distinguish acute reversible CsA renal toxicity from acute rejection using clinical criteria (39,40). Both are most frequent in the first few weeks after transplantation. The increase in serum creatinine is often slower and of a lesser degree (25% or less) in CsA toxicity than in acute rejection. Fever and oliguria are more often seen in association with acute rejection than in CsA toxicity, and trough CsA levels tend to be lower in acute rejection compared to CsA toxicity. Unfortunately, no combination of clinical or laboratory findings distinguish acute rejection from CsA toxicity with certainty. If the CsA blood level is high, then mild, gradual increases in serum creatinine can be managed by reducing the dose of CsA and closely monitoring the patient to be certain that creatinine levels decline. Otherwise, an allograft biopsy should generally be obtained to rule out acute rejection.

E. Acute CsA Arteriopathy and Thrombotic Microangiopathy

Severe arterial and arteriolar lesions are associated with allograft failure in a high proportion of cases (41). Occasionally, a full-blown clinical syndrome develops with histopathological findings similar to HUS or TTP (42). In these cases, there is usually a rapid and marked decline in renal function, evidence of platelet consumption, and a microangiopathic, hemolytic anemia. Treatment is empirical. In addition to discontinuing the CsA, a number of therapies have been tried, including anticoagulation, additional immunosuppression, and plasmapheresis or exchange. The outcome is often poor. It has been suggested that patients whose underlying renal disease was HUS or TTP may be at higher risk for development of a recurrence if CsA is used after renal transplantation.

F. Systemic CsA Toxicity Early After Transplantation

Probably the most common systemic complication of CsA in the early posttransplantation period is hypertension. In one report, hypertension occurred in 70% during the first month (43). Although the pathogenesis of hypertension is multifactorial, CsA probably plays a major role. Tremor is another common manifestation of nonrenal, CsA toxicity in the early posttransplantation period (see Table 2). Indeed, tremor occurred in 20% of renal transplant

recipients given CsA during the first 3 months after transplantation (43). Hyperuricemia was also common in the early posttransplantation period (31%–38%), but gout was rare (<0.5%) (43). Although the incidence of elevated transaminase levels was high during the first 3 months after transplantation (20%–49%), the role of CsA in causing these transaminase elevations is unclear (43). The incidence of diabetes was also high early after transplantation. During the first 2 months, 7% of patients were given diagnoses of diabetes, and 2% to 4% required insulin (43).

G. Dosing Early After Transplantation

Early studies used relatively high starting doses of CsA. Initial oral doses of 14 to 20 mg/kg/day were common in renal transplant recipients (3,27,44). Higher doses were found to be associated with a higher incidence of toxicity and subsequent studies in renal transplant recipients have shown that lower initial doses of CsA can be used (e.g., 8–10 mg/kg/day) at least in low-risk patients (45–49). The optimal starting dose for CsA is probably dependent on the risk of acute rejection and on what other immunosuppressive drugs are used for rejection prophylaxis in the early posttransplantation period.

Once therapy with CsA has been initiated, most centers adjust the dose of CsA according to a predetermined, therapeutic range based on trough whole blood CsA concentrations (13). The target ranges are generally higher in the early posttransplantation period, compared to those used in the late, maintenance period (Table 3). Most centers maintain these higher target levels for the first 3 to 6 months after transplantation. The therapeutic ranges used tend to be higher in heart and liver recipients compared with kidney recipients (see Table 3). Therapeutic ranges are higher in nonkidney organ recipients because the consequences of rejection (usually death) are much greater. Moreover, with similar immunosuppression protocols, therapeutic target trough ranges for CsA measured

Table 3 Therapeutic Ranges for Cyclosporine A Stratified According to Transplanted Organ, Immunosuppressive Regimen, Induction/Maintenance Therapy, and Immunoassay Technique

Method	Kidney[a] Triple Therapy	Heart[a] Triple Therapy	Liver[a] Triple Therapy	Liver[a] Double Therapy
Induction[b]				
HPLC	150–225 (5)	250–325 (1)	225–300 (4)	
mFPIA	250–375 (6)	300–400 (3)	250–313 (8)	300–375 (2)
m^{125}I-RIA	160–200 (5)	250–325 (2)	250–300 (3)	
EMIT	125–200 (2)	275–375 (2)		125–200 (2)
Maintenance				
HPLC	100–150 (5)	125–175 (2)	100–150 (6)	100–150 (1)
mFPIA	100–250 (8)	150–250 (5)	135–200 (8)	150–250 (3)
m^{125}I-RIA	75–150 (5)	90–160 (2)	150–238 (4)	
EMIT	75–150 (2)	150–250 (3)		75–150 (2)

[a]The ranges are the median values (μg/L) for the minimum and maximum trough cyclosporine concentrations (whole blood). The number of contributing centers is given in parentheses.

[b]In some centers, antilymphocyte antibodies were also included as part of induction therapy. *Abbreviations:* HPLC = high-performance liquid chromatography; mFPIA = monoclonal antibody fluorescence polarization immunoassay; m^{125}I-RIA = monoclonal antibody INCSTAR radioimmunoassay; EMIT = enzyme-multiplied immunoassay technique.

Source: Ref. 13.

by the monoclonal antibody Abbott TDx FPIA are approximately 28% higher than those measured with HPLC (13). It was surprising that target trough levels were similar in centers using an RIA and in those using HPLC, because levels measured with RIA tend to be approximately 22% to 30% higher than levels measured with HPLC.

IV. CLINICAL USE OF CSA LATE AFTER TRANSPLANTATION

How CsA should be used in the late posttransplantation period (i.e., beyond the first 6 to 12 months after transplantation) is unclear. Indeed, whether CsA needs to be continued indefinitely in all renal allograft recipients is controversial. Worldwide, it appears that most transplant recipients continue to receive CsA in the late posttransplantation period. Data from the European Dialysis and Transplant Registry show that among 12,953 first cadaver kidney transplant recipients who initially received triple-drug therapy and had a functioning allograft for at least 1 year, all but 830 (6.4%) still received CsA at 1 year (50). In a U.S. survey, the number of program directors who used only prednisone and azathioprine for maintenance immunosuppression was as follows: 20.2% for kidney, 4.9% for heart, 0.0% for heart-lung, 0.0% for liver, and 8.0% for pancreas transplant recipients (31). There are reasons to want to minimize the use of CsA in the late posttransplantation period. These include systemic toxicity, nephrotoxicity, and cost. If CsA is continued indefinitely, the optimal dose of CsA in the late posttransplantation period is poorly defined. Similarly, few data suggest how CsA therapy should be monitored in the late posttransplantation period. All of these uncertainties are compounded by the introduction of new immunosuppressive agents that can be used with, or in place of, CsA in the late posttransplantation period. Benefits of using CsA in the late posttransplantation period must be weighed against the potential for chronic nephrotoxicity.

A. The Pathogenesis of Chronic CsA Toxicity

Whether the pathogenesis of chronic CsA nephrotoxicity is related directly or indirectly to the pathogenesis of acute, reversible renal toxicity is unclear. It is possible that chronic renal vasoconstriction could lead to ischemia and thereby cause glomerular and interstitial fibrosis. However, it is also possible that CsA could have more direct effects on cells, cytokines, and growth factors that lead to fibrosis. Studies in rats have shown that CsA can increase renal interstitial osteopontin (a macrophage adhesion protein) and osteopontin mRNA, macrophages, transforming growth factor β (TGF-β) mRNA, and interstitial fibrosis (51,52). Experiments using cell cultures have also shown that CsA can induce procollagen, collagen, TGF-β, and TGF-β mRNA (53–56). Because TGF-β is generally believed to arrest cell proliferation and to stimulate matrix production, this cytokine could play a key role in the tubular atrophy and interstitial fibrosis associated with chronic CsA nephrotoxicity. However, it is likely that fibrosis is caused by an imbalance of multiple cytokines, growth factors, matrix proteins, and collagenase. Thus, it is difficult to extrapolate the results of cell culture experiments, which measure only a few of these factors, to the pathogenesis of fibrosis in vivo.

B. Histopathology of Chronic CsA Toxicity

There are few, if any, histological changes that are diagnostic of chronic CsA nephrotoxicity. Glomerulosclerosis, tubular atrophy, and interstitial fibrosis are typical of both chronic

rejection and chronic CsA toxicity (57,58). The most distinctive renal lesion associated with CsA is a vasculopathy that is characterized by protein (hyalin) deposits replacing necrotic smooth muscle cells in arterioles, the so-called CsA-associated arteriopathy (59). These lesions may be seen early after transplantation, but are more common in biopsies obtained after the first 3 months after transplantation (59). The frequency of the lesions has declined over the years in renal transplant recipients, possibly reflecting a reduction in CsA dose (59). Cyclosporine A–associated arteriopathy appears to be reversible when the dose is discontinued or reduced (60).

C. CsA Nephrotoxicity Late After Transplantation

Whether CsA causes clinically relevant nephrotoxicity in the late posttransplantation period continues to be debated in a void of sound clinical data. The most compelling data supporting the notion that CsA can cause clinically relevant, chronic, nephrotoxicity has been reported for heart transplant recipients (61–63). Indeed, a number of heart transplant recipients are on dialysis. Although there are other reasons for renal failure to develop in heart transplant patients, CsA may certainly play a major role. Heart transplant recipients have few alternatives but to continue CsA therapy.

There are no controlled studies in renal transplant recipients examining the long-term effects of CsA on renal allograft histopathology. It is impossible to distinguish CsA nephrotoxicity from chronic rejection in the absence of histopathological studies, and it is certainly plausible that clinically silent rejection and CsA nephrotoxicity may both affect long-term allograft function. A number of studies have purported to demonstrate stable renal function in renal allograft recipients chronically receiving CsA treatment. In a recent analysis of 1663 patients treated with CsA at six different centers, for example, there was little change in serum creatinine over a median follow-up of 36 months (64). However, studies that have examined the effects of CsA on serum creatinine suffer not only from the uncertainty created by the absence of histopathological studies, but also from the insensitivity of measurement of serum creatinine to detect chronic allograft dysfunction. In short, there are, as yet, no conclusive scientific data to firmly establish the presence or absence of CsA nephrotoxicity late after renal transplantation.

A recent prospective study demonstrated histological evidence of chronic CsA nephrotoxicity in the native kidneys of pancreas transplant recipients (65). Most worrisome was the evidence of progression between 2 and 5 years after transplantation. A large number of trials have examined renal structure and function in patients with autoimmune diseases treated with CsA (66–68). These trials have confirmed the presence of chronic histopathological changes consistent with CsA toxicity, even when doses of CsA were less than 5 mg/kg/day.

D. CsA Systemic Toxicity Late After Transplantation

Systemic toxicities include those caused by immunosuppression in general (e.g., infections and malignancies) and those that are unique to CsA. Although there are no data from large, well-controlled trials, it does not appear that the long-term use of CsA confers a unique risk for infections and malignancies. Certainly, immunosuppression therapy of any kind increases the risk of life-threatening infections and malignancies. However, there does not appear to be anything unique about CsA that increases that risk in comparison to comparable immunosuppression with other agents. Thus, as for other immunosuppressive agents, the use of CsA should be no more than that which is necessary to maintain long-term graft

survival without incurring undue risk of potentially life-threatening infections and malignancies.

Cardiovascular disease is one of the leading causes of death in the late posttransplantation period. There are no controlled clinical trials of sufficient size or duration to prove that CsA increases the incidence of cardiovascular disease events in the late posttransplantation period. However, the long-term use of CsA appears to adversely affect a number of cardiovascular disease risk factors, including lipoproteins (69,70), blood pressure (71,72), and glucose intolerance (see Table 2) (73–75) Cyclosporine A may also adversely affect some theoretical or putative cardiovascular disease risk factors, including serum uric acid levels (76,77), coagulation parameters (78,79), and vascular endothelial cell function (80–82).

Cyclosporine A has other potential long-term systemic toxic effects, including hypertrichosis and gingival hyperplasia. Less well documented is the potential for long-term neurotoxicity. The incidence and severity of peripheral neuropathy and central nervous system toxicity in the late posttransplantation period, including tremor, are unclear.

Certainly, the expense of CsA continues to be a problem for many patients in the late posttransplantation period. This is not only a problem in third world countries but also in the United States where Medicare coverage for immunosuppression does not currently extend beyond the third posttransplantation year. Clearly, cost has been a major reason for minimizing the use of CsA in the late posttransplantation period (83).

E. Discontinuing CsA Late After Transplantation

Whereas there are a number of compelling reasons for discontinuing CsA in the late posttransplantation period, there may also be equally plausible reasons for continuing CsA indefinitely. The most compelling reason is prolongation of patient and graft survival. Clearly, CsA has decreased the incidence of graft failure in the early posttransplantation period. However, the reduction in acute rejection and improvement in allograft survival are fully manifest in the first few months after transplantation. In contrast, there are no convincing data that the rate of graft failure in the late posttransplantation period has been reduced by the use of CsA.

Perhaps the strongest argument for continuing CsA indefinitely has been the occurrence of acute rejection episodes within a few weeks or months after discontinuing CsA therapy. Indeed, an "immunological addiction" to CsA appears to develop, whereby withdrawal of CsA often leads to acute rejection episodes in the late posttransplantation period, at a time when the incidence of acute rejection episodes would otherwise be quite low. This phenomenon was first noted in a number of uncontrolled studies that reported a very high incidence of acute rejection in association with CsA withdrawal. These reports led many in the transplant field to conclude that it was folly to discontinue CsA electively. However, some researchers have said that the timing and rate of CsA withdrawal are important determinants of postwithdrawal acute rejection and that the rate of acute rejection could be rendered acceptably low if CsA withdrawal were carried out under the right circumstances.

Whether CsA should be electively withdrawn has been addressed in several randomized controlled trials. A recent metaanalysis of these trials showed that the incidence of acute rejection was indeed increased after CsA withdrawal (Figure 1) (84). However, during the relatively short follow-up in these trials, there was no increase in graft failure (Figure 2). Not included in the metaanalysis was a more recent randomized controlled trial with 5 years follow-up that confirmed these results (85).

The seemingly paradoxical observation that CsA withdrawal leads to an increase in

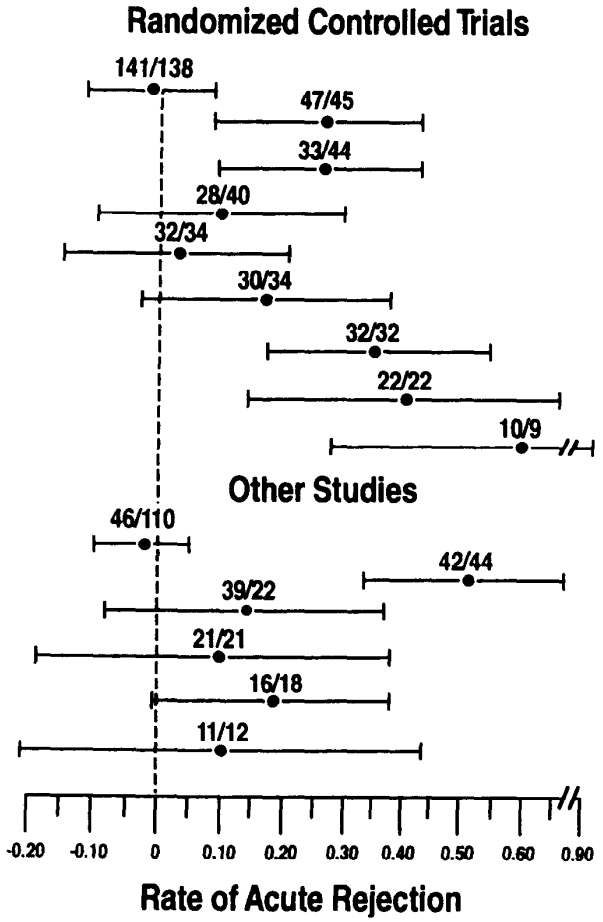

Figure 1 Results of randomized controlled trials examining the effects of CsA withdrawal on the incidence of acute rejection. Shown are the means and 95% confidence intervals for the individual and pooled studies. Failure of the 95% confidence interval to cross zero indicates a statistically significant difference at $p < 0.05$. (From Ref. 84. Copyright 1993, American Medical Association.)

acute rejection episodes without an increase in graft failure could be due to a number of factors. Although longer follow-up or larger trials could have shown increased graft failure associated with CsA withdrawal, this seems unlikely in the absence of any trend in the combined trials. It also seems improbable that the results of the randomized trials were influenced by publication bias, although this is difficult to exclude. Similarly, given the number of trials and the consistency of results, it seems improbable that patients in the randomized controlled trials were somehow different than patients in other centers. Alternatively, the result may simply indicate that acute rejection episodes after CsA withdrawal rarely lead to graft failure or that CsA itself may lead to graft failure as often as graft failure results from CsA withdrawal.

Differences in graft survival with or without long-term CsA therapy use have been reported in large registries (86). However, retrospective analyses of registry data should be interpreted with caution, and any inferences from such analyses should either be avoided altogether or confirmed in controlled clinical trials. There are several reasons why CsA may have been discontinued in the patients reported in the registry, including decreased allograft function. These unreported reasons for withdrawing CsA could well explain the observed results without having to implicate CsA withdrawal per se as a cause of graft failure. In any

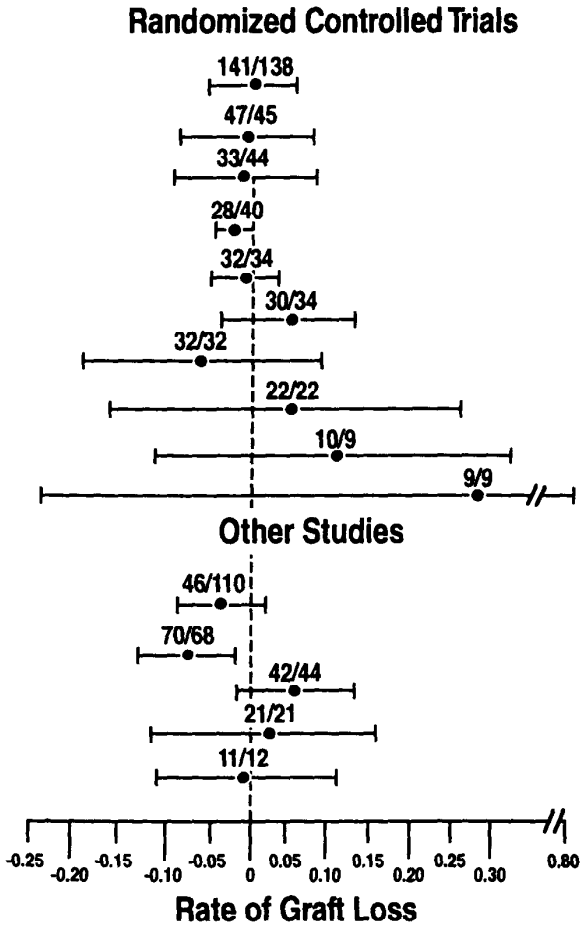

Figure 2 Results of randomized controlled trials examining the effects of CsA withdrawal on the incidence of graft failure. Shown are the means and 95% confidence intervals for the individual and pooled studies. Failure of the 95% confidence interval to cross zero indicates a statistically significant difference at $p < 0.05$. (From Ref. 84. Copyright 1993, American Medical Association.)

case, it is usually unwise to base prospective therapeutic decisions on data from retrospective analysis of registry data.

It is attractive to think that it may be possible to reduce the risk of acute rejection after CsA withdrawal by carefully selecting patients. At Hennepin County Medical Center, we attempted to define risk factors for an increased incidence of acute rejection following CsA withdrawal among 192 patients (87). An early or rapid compared to late or slow taper, major histocompatibility (DR) mismatches, and younger age were each independently associated with a greater likelihood for acute rejection following CsA withdrawal. Although the theory has never rigorously been tested, there is a feeling among other investigators that CsA withdrawal should only be attempted in stable patients, that is, patients who have had no recent acute rejection episodes. Blacks have also been identified as more likely to have acute rejection following CsA withdrawal (83).

All of the trials examining the effects of CsA withdrawal have been carried out using patients given azathioprine and prednisone. There have not yet been any randomized controlled trials reporting the effects of CsA withdrawal in patients treated with mycophenolate mofetil. Mycophenolate mofetil has been shown to reduce the incidence of acute rejection episodes in the early posttransplantation period (88,89). It is reasonable to expect that

the incidence of acute rejection after CsA withdrawal may also be less in patients maintained on mycophenolate mofetil. However, this must be demonstrated in randomized controlled trials.

F. The Dose of CsA Late After Transplantation

Several retrospective analyses have examined the relationship between CsA dose and outcome in the late posttransplantation period. In general, most of these studies have shown an inverse correlation between dose and the incidence of acute rejection or graft failure (90–92). However, it is plausible that doses were reduced because patients were not doing well rather than vice versa. Thus, it is only possible to conclude that higher doses give a better outcome in prospective randomized controlled trials. Such trials are essential before drawing conclusions, because higher doses may also cause more toxicity. Indeed, in a study of risk factors for chronic CsA nephrotoxicity in nontransplant patients with autoimmune disease, the risk of biopsy-confirmed nephrotoxicity was less if the dose of CsA did not exceed 5 mg/kg/day (67). In the end, which immunosuppressive agents should be used at what doses should be tested using clinical trials that take into account the likelihood that not all patients have the same risk for rejection.

V. NEW CSA PREPARATIONS

Despite efforts to develop a less toxic analogue of CsA, for example, cyclosporine G (93,94), CsA remains the mainstay of immunosuppression therapy in transplantation. Recently, a microemulsion form of CsA was developed to enhance bioavailability and improve its pharmacokinetic profile. Clinical trials in renal transplant recipients have indicated that the microemulsion formulation is absorbed more rapidly and more completely than CsA (95–98). Indeed, after a milligram per milligram conversion from CsA to the microemulsion formulation, the steady-state peak concentration increased 39% to 73%, and the area under the curve increased 15% to 44% (95–99). Trough concentrations were similar before and after conversion. However, measurement of whole blood trough concentrations correlated more closely to the area under the curve after conversion to the microemulsion formulation (95–98). Despite higher blood levels after a milligram per milligram conversion from CsA to the microemulsion formulation, toxicity did not appear to be increased (95–98). In particular, renal function was similar before and after a milligram per milligram conversion. Both the intrapatient and interpatient variability in whole blood levels appear to be less with the microemulsion formulation compared to CsA (14,99). Similar pharmacokinetic data have been reported from clinical trials in liver transplant recipients, as reviewed recently by Noble and Markham (100).

In both kidney and liver recipients, the incidence of acute rejection appears to be similar with CsA and the new microemulsion formulation (100). Whether there are advantages to using the microemulsion formulation beyond the more predictable blood levels compared to those seen with CsA is unclear. It is also unclear whether more predictable levels of CsA will translate into better outcomes after transplantation, compared to those achieved with equivalent doses of CsA. However, there currently appears to be compelling reasons to use the microemulsion formulation in place of CsA, with all other things being equal, for example, cost (101). Thus, it is probably reasonable to begin new patients on the microemulsion form of CsA. However, it is less certain whether to convert patients who are receiving standard CsA and are clinically stable. If conversion is undertaken, a milligram

per milligram dose conversion, with close monitoring of CsA levels, seems to be the most reasonable strategy.

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Tacrolimus and Mycophenolate Mofetil as Primary Immunosuppression for Renal Allograft Recipients

GAETANO CIANCIO, GEORGE W. BURKE, DAVID ROTH, KEITH ZUCKER, VIOLET ESQUENAZI, ANNE ROSEN, and JOSHUA MILLER

University of Miami School of Medicine, Miami, Florida

I. INTRODUCTION

Immunosuppression, although necessary to enable the graft to escape the consequences of immune surveillance, carries risks for the patient. There is an associated increase in neoplasms, opportunistic infections, and end-organ toxicity. In addition, even with excellent patient compliance, rejection (acute and chronic) remains a major limitation that contributes to the loss or decrease in the function of the allograft.

New drugs have recently been added to the decade-long clinical dominance of cyclosporine (CyA). This agent, the cornerstone of immunosuppressive agents to suppress allograft rejection, was introduced by Borel et al. (1) and Calne et al. (2) in the mid-1970s. In 1989, Starzl et al. (3), after 2 years of animal experimentation, introduced tacrolimus (originally designated as FK506 by the Fujisawa Pharmaceutical Company of Japan) as a potent immunosuppressant for liver transplants. Also, in recent years, a variety of novel purine and pyrimidine biosynthesis inhibitors have been tested for transplantation therapy. The leading agent to replace azathioprine, used in immunosuppressive regimens for a generation, is the newly approved, semisynthetic morpholinoethyl ester of mycophenolic acid known as mycophenolate mofetil (MMF). It was pioneered by Allison (4) and Sollinger (5) and their coworkers. This agent was developed by the Syntex corporation, now a component of Roche Pharmaceuticals.

II. TACROLIMUS (FK506)

Tacrolimus is a macrolide antibiotic, isolated from *Streptomyces tsukubaensis,* which binds to an intracellular lymphocyte protein (FKBP-12). A complex of tacrolimus–FKBP-12, calcium, calmodulin, and calcineurin is then formed, and the phosphatase activity of calcineurin is inhibited, eventually preventing the generation of nuclear factor of activated T cells (NF-AT), a nuclear protein thought to initiate cytokine gene transcription. Consequently, a chain of events starting with interleukin (IL)-2 and interferon-γ production, IL-3 and IL-4, granulocyte-macrophage colony stimulating factor production, and IL-2 receptor expression inhibition, resulting in inhibition of mixed lymphocyte reactions, and cytotoxic T-cell generation in vitro (6,7). Tacrolimus appears to be 10 to 100 times more potent than cyclosporine for inhibition of IL-2 synthesis in in vitro testing (8).

Although the oral bioavailability of tacrolimus is somewhat variable, unlike cyclosporine, the enteric absorption of tacrolimus is not dependent on solubilization by bile salts. However, similar to cyclosporine, tacrolimus is primarily metabolized in the liver through the cytochrome P-450 system. Less than 1% of a dose is excreted in the urine. Dosing adjustments may not be required in patients with renal failure or who are undergoing dialysis, but it is required in patients with hepatic dysfunction.

Adverse side effects include headache, nausea, vomiting, diarrhea, pruritus, tremor, abdominal pain, diabetes mellitus, and impairment of renal function. These are generally reversible with dose reduction.

Starzl first reported on the salvage of liver allografts in 7 of 10 patients who had failed conventional immunosuppression for rejection (3). Because tacrolimus is so much more potent than cyclosporine (CyA), there has been a growing expectation of beneficial immunosuppressive effects of tacrolimus in solid organ transplantation.

Tacrolimus was approved for use in liver transplants by the Food and Drug Administration (FDA) in 1994, and it has exhibited efficacy in the transplantation of the kidney (9–12) (approved by the FDA in 1997), small bowel (13), simultaneous pancreas/kidney (14,15), heart (16), and lung (17), and in the therapy of graft versus host disease (GVHD) in bone marrow transplantation (18).

A. Tacrolimus as "Rescue" Therapy

Acute rejection is common following renal transplantation, occurring more frequently during the first 6 months postoperatively. High-dose steroid and/or monoclonal or polyclonal antilymphocyte antibody therapy have been used to reverse graft rejection. However, in some cases, rejection persists and progresses despite this therapy. Before the introduction of tacrolimus, persistent rejection led to loss of the graft. Recent studies with tacrolimus suggest that it may suppress or reverse ongoing refractory rejection, even if antilymphocyte preparations have failed (19–21).

Our first experience with tacrolimus was as "rescue" therapy for renal allografts with ongoing rejection failing to respond to CyA. This work was done in conjunction with the University of Pittsburgh, where the concept had been introduced. Between November 1, 1992, and November 30, 1993, one hundred and two consecutive kidney transplantations were performed. Protocol acceptance for transplantation required at least one DR compatibility with the donor. The Waters pulsatile preservation apparatus has been routinely used in all cadaver (CAD) transplants since 1978 at this center. Biopsy-proven rejection episodes requiring OKT3 treatment occurred in 29 patients (at least one-third were diagnosed as

acute vascular rejection). This was followed by recurrent rejection in 21 patients. Of these, 20 were converted to tacrolimus therapy. Stabilization occurred in 19 of the 20 patients (serum creatinine returning to baseline levels seen after OKT3 treatment in all but one patient who stabilized at a higher level). The only exception was a patient with progressive rejection over 6 months confirmed by repeat biopsy and subsequent kidney loss despite tacrolimus conversion. In this group of 102 patients, the actuarial patient and graft survival rates at 1 year in the CAD group were 99% and 95%, respectively (9).

Woodle et al. (22) reported a multicenter trial evaluating the efficacy and safety of tacrolimus in the treatment of refractory renal allograft rejection. Of 73 patients included in the study, 59 (81%) had previously received at least one course of antilymphocyte antibody as rejection therapy. Therapeutic responses included improvement in 78% of patients, stabilization in 11%, and progressive deterioration in 11%. Actuarial patient and graft survival rates were 93% and 75%, respectively. Posttransplant lymphoproliferative disorder was diagnosed in a single patient. The most commonly observed adverse events were neurological and gastrointestinal. Tacrolimus therapy was discontinued in 18% of patients because of continuing rejection (11% for progressive, unrelenting rejection and 7% for recurrent rejection) and in 8% of patients because of adverse events. Conclusions were that tacrolimus provides effective reversal of refractory rejection, good long-term renal allograft function, low incidence of recurrent rejection, and an acceptable safety profile in renal allograft recipients with refractory rejection.

B. Tacrolimus as Primary Therapy

1. The Experience of the U. S. Multicenter FK506 Kidney Transplant Group

In 1996, the Multicenter FK506 Kidney Transplant Group reported the results of phase II open-label concentration-ranging trial of tacrolimus versus cyclosporine. One hundred and twenty patients were randomly assigned to a cyclosporine-based or one of the three tacrolimus-based regimens designed to achieve low (5–14 ng/mL), medium (15–25 ng/mL), or high (26–40 ng/mL) trough whole blood levels. Initial doses of tacrolimus were 0.2, 0.3, or 0.4 mg/kg/day, respectively. Ninety-two patients completed a 1-year follow-up. Patient survival rate was 98% for tacrolimus and 92% for cyclosporine, and the graft survival rate was 93% and 89% in the tacrolimus and cyclosporine groups, respectively. The incidence of acute rejection was 14% in the tacrolimus group and 32% in the cyclosporine group ($p = 0.048$) by day 42 after transplantation. However, the incidence of rejection episodes requiring treatment at 1 year was similar in both groups (33% for tacrolimus and 32% for cyclosporine). Nephrotoxicity occurred with a similar frequency in both groups, but the incidence of neurotoxic adverse events, primarily headache, insomnia, paresthesia, and tremor, occurred more frequently and required dosage or treatment change more often with tacrolimus than with cyclosporine during the 1-year follow-up. Hyperglycemia requiring insulin therapy occurred more frequently in the tacrolimus group than in the cyclosporine group. A total of 17 (25.4%) tacrolimus-treated patients required insulin during the first year of therapy; 12 of those patients were insulin-dependent at 1 year.

The target range of whole blood levels that optimizes efficacy and minimizes toxicity seems to be 5 to 15 ng/dL. The corresponding recommended initial dose of tacrolimus for kidney transplant recipients is 0.2 mg/kg/day. These results indicate that the efficacy and safety were comparable to that for cyclosporine for primary immunosuppression in patients undergoing cadaveric kidney transplantation. In this study, correlations between tacrolimus

dosage, rejection, adverse effects, and infections were not reported. The main objective was to compare tacrolimus versus cyclosporine with regard to toxicity and patient and graft survival at 1-year follow-up (23).

In 1997, the results of a phase III multicenter trial were published, reporting a randomized open-label comparison of tacrolimus versus cyclosporine-based immunosuppression in patients receiving cadaveric kidney transplants. A total of 412 patients were randomized to tacrolimus (n = 205) or cyclosporine (n = 207). One-year patient survival rates were equivalent, that is, 95.6% for tacrolimus and 96.6% for cyclosporine ($p = 0.576$). Corresponding 1-year graft survival rates were 91.2% and 87.9% ($p = 0.289$) for tacrolimus and cyclosporine, respectively. There was a significant reduction in the incidence of biopsy-confirmed acute rejection in the tacrolimus group (30.7%) compared with the cyclosporine group (46.4%, $p = 0.001$), which was confirmed by blinded review. There was also a reduction in the use of antilymphocyte therapy for rejection (10.7% versus 25.1%, respectively; $p < 0.001$). Impaired renal function, gastrointestinal disorders, and neurological complications were commonly reported in both treatment groups, but tremor and paresthesia were more frequent in the tacrolimus group. The incidence of posttransplant diabetes mellitus was 19.9% in the tacrolimus group and 4% in the cyclosporine group ($p < 0.001$). In the tacrolimus group, this was subsequently shown to be reversible in many patients with successful reduction in tacrolimus and steroid dosage. It was concluded that tacrolimus was more effective than cyclosporine in preventing acute rejection in cadaver renal allograft recipients and that it significantly reduced the use of antilymphocyte antibody preparations. Tacrolimus was associated with a higher incidence of neurological events, which were rarely treatment limiting, with posttransplant diabetes mellitus, which was frequently reversible, and with a lower incidence of hyperlipidemia (24).

2. The University of Miami Experience

Tacrolimus has been used as maintenance immunosuppression in first cadaver kidney transplantation in our program since the entry phase of a randomized multicenter phase III trial was completed in August 1994, in which it was compared with cyclosporine (24). Polyclonal or monoclonal antilymphocyte antibody induction was routinely used, together with maintenance methylprednisolone and (less frequently) low-dose azathioprine. Since September 1995, mycophenolate mofetil has replaced azathioprine. In this protocol, tacrolimus is instituted when renal function has been demonstrated to be adequate (serum creatinine decreasing to less than 4 mg/dL). The tacrolimus dose starts at 0.1 to 0.2 mg/kg/day and is adjusted to maintain trough level of 10 to 20 ng/mL.

From this clinical protocol, a group of 100 consecutive tacrolimus-treated patients were compared with a historical control group of 100 consecutive cyclosporine-treated patients. The two groups did not differ in age, gender, original disease, organ source, cold ischemia time, or panel reactive antibody (PRA) at time of transplant. However, although the protocol of accepting a minimum of 1 D-related match was not changed between the groups, the tacrolimus group had a greater ABDR mismatch (3.6 ± 1.3 versus 2.7 ± 1.4, $p = 0.0001$) and were more likely to have received OKT3 induction than anti-thymocyte globulin (ATGAM) ($p < 0.05$). Maintenance azathioprine was given to 39% of the FK506 patients versus 60% of the CYA group ($p = 0.0001$). No MMF was used in this earlier protocol. The tacrolimus trough levels were 13.5 ± 5, 13.4 ± 5.1, 13 ± 4.3, and 13.2 ± 4.3 at 1, 3, 6, and 12 months, respectively. There was no difference in the number of antihypertensive medications or the incidence of posttransplant diabetes mellitus (PTDM). Despite significantly more CyA patients receiving lipid-lowering agents (27% versus 10%, $p = 0.0008$),

Table 1 Results of FK506-Treatment Compared with CyA-Treatment

	FK506 (n = 100)	CyA (n = 100)	*p*
Rejection (%)	22	37	0.03
BP meds (#)	1.6 ± 0.9	1.6 ± 0.8	NS
PTDM (%)	22	14	NS
Cholesterol*	202 ± 53	245 ± 54	0.001

BP meds = blood pressure medications; PTDM = posttransplant diabetes mellitus; * = posttransplant level; NS = not statistically significant.

the serum cholesterol remained higher in the CyA group ($p = 0.001$). The tacrolimus group had fewer biopsy-proven acute rejection episodes than the CyA group ($p = 0.03$) (Table 1). The mean serum creatinine concentration at 1 year did not differ (1.5 versus 1.6 mg/dL) between groups. Actuarial 1-year patient and graft survival rates for the tacrolimus and CyA groups were greater than 92% (not statistically different). No patients discontinued tacrolimus; however, 13 CyA patients were converted to tacrolimus as part of a rescue protocol for refractory rejection. There were two deaths in each group attributable to sepsis. The incidence of serious cytomegalovirus (CMV) infection was no different between groups because ganciclovir prophylaxis was used, but the detection by shell vial assay or polymerase chain reaction (PCR) of CMV in the peripheral blood of asymptomatic patients never treated for rejection was higher in the FK506 group (10). We concluded that tacrolimus was an effective agent when used as primary therapy for kidney transplantation, resulting in this series in a similar degree of diabetes mellitus and hypertension, with less acute rejection and hyperlipidemia compared with a historical CyA-treated group (10,25).

III. MYCOPHENOLATE MOFETIL

Mycophenolate mofetil (MMF) is a semisynthetic derivative of mycophenolic acid, which is isolated from *Penicillin glaucum*. Mycophenolic acid causes a noncompetitive reversible inhibition of inosine monophosphate dehydrogenase. The resultant interference with the de novo pathway of guanine nucleotide synthesis and DNA replication is highly specific for T and B lymphocytes, which are dependent upon this pathway for purine synthesis because there is a lack of a salvage pathway (26). Following oral administration, mycophenolate mofetil is metabolized to mycophenolic acid, the biologically active component. Elimination of mycophenolic acid occurs primarily through renal and hepatic glucuronidation, with a significant reentry through an enterohepatic pathway and biliary excretion.

In a preliminary trial of mycophenolate mofetil in cadaveric renal transplantation, patient and graft survival at 18 months were 100% and 95%, respectively (27). In 75 kidney transplant recipients with biopsy-proven refractory rejection, successful rescue was obtained in 52 (69%) recipients (28).

Adverse events reported for mycophenolate mofetil in the trial were primarily gastrointestinal (nausea, diarrhea, or constipation), and these could be managed with dose reduction. Other events that warranted dose reduction included leukopenia, thrombocytopenia, anemia, and neutropenia. No reports of nephrotoxicity or diabetogenicity were reported.

Mycophenolate mofetil has been approved by the FDA for the prevention of rejection following renal transplantation. The approval was based on the results of three large, multicenter, prospective, double-blind trials that involved 55 transplant institutes on three continents, the largest series of multicenter trials ever performed in transplantation (29).

A. The U.S. Renal Transplant Mycophenolate Mofetil Study Group

Mycophenolate mofetil is probably one of the first immunosuppressive drugs to undergo a thorough, rigorous, and critical evaluation in clinical transplantation.

The phase III U.S. multicenter trial was a prospective, double-blind study conducted in 14 centers and enrolling 499 patients. All patients received induction therapy with antithymocyte globulin (ATG), followed by cyclosporine and prednisone. They were divided into three equal groups, with the patients in one group receiving standard therapy with azathioprine and the patients in the other two groups receiving mycophenolate mofetil at a dose of 2 or 3 g/day. Biopsy-proven acute rejection episodes or treatment failure occurred in 47.6% of patients in the azathioprine group compared with 31.1% ($p = 0.0015$) and 31.3% ($p = 0.0021$) of patients in the MMF 2 g and 3 g treatment groups, respectively. The MMF-treated groups also demonstrated a longer delay until the first biopsy-proven rejection or treatment failure, lower requirement for antilymphocyte therapy to treat breakthrough rejection (azathioprine, 44.5%; MMF 2g, 24.8%; and MMF 3g, 21.1%, respectively), and fewer full courses of antirejection therapy compared with the azathioprine control group (azathioprine, 20.1%; MMF 2g, 10.3%; MMF 3g, 5.4%, respectively). At 6 months (and 1 year) after transplantation, however, graft and patient survival rates were similar in all treatment groups. There was no significant difference between the groups with respect to adverse events, except for increased gastrointestinal disturbances (especially diarrhea), leukopenia, and rare tissue-invasive cytomegalovirus infections in the MMF-treated groups (30).

B. Tacrolimus and Mycophenolate Mofetil for Renal Allograft Recipients as Primary Immunosuppression

Tacrolimus and mycophenolate mofetil (MMF) have each been demonstrated to be effective immunosuppressants when combined with other agents in kidney transplantation. In retrospective analysis, a cohort of patients who received the combination of tacrolimus, MMF, and steroids (FK/MMF/Med, n = 72) was compared with prior experience with tacrolimus and steroids (FK/Med, n = 98) as maintenance immunosuppression for kidney recipients. Patients who had primary nonfunction (1 patient in the FK/Med group) or death (2 patients in the FK/MMF/Med group) during the initial admission, or patients enrolled in other pharmacological studies were excluded. Patients who were given allogeneic bone marrow (BM) (16 FK/MMF/Med patients and 19 FK/Med patients), however, were included (31). Most patients received antibody induction (Table 2); tacrolimus was instituted when the serum creatinine (Cr) concentration was less than 4 mg/dL. Mycophenolate mofetil was usually begun at 2 g/day by day 2 postoperatively. There were no significant differences in gender, race, ABDR mismatch, PRA, or original disease. FK/MMF/Med patients were older, more likely to have received a cadaveric graft, and had shorter follow-up. The mean daily dose of FK was significantly lower in the FK/MMF/Med group as were FK levels at months 2 to 6 after transplantation (Table 3).

The incidence of biopsy-proven acute rejection (AR) within the 6 months was signif-

Table 2 Recipient Immunosuppression

Induction Immunosuppression
Kidney Transplantation
OKT3 Start on day 2 for about 10 days at 5 mg/day
FK506 0.1 mg/kg twice a day by mouth when creatinine is less than 4 mg/dL
Steroids 500 mg/day × 3, followed by Medrol 1 mg/kg
Decreasing over 2 weeks to 0.5 mg/kg
Decreasing over 4 weeeks to 0.33 mg/kg
Decreasing over 3 months to 0.25 mg/kg
Mycophenolate mofetil 1 g twice a day
Maintenance Immunosuppression
Steroids 4 mg/day for 3 months
FK 506 aiming at maintaining level of 5–15 ng/mL (whole blood) for the first 12 months after transplantation.
Mycophenolate mofetil 1 g twice a day
Treatment of Rejection Episodes
Biopsy-proven rejection episodes = OKT3 and/or Solu-Medrol

Table 3 FK506, Mycophenolate Mofetil, and Steroid Treatment versus FK506 and Steroid Treatment

	FK (mg/kg/d)	AR (%)	PTDM (%)	PS (%)	GS (%)
FK/MMF/Med (n = 72)	0.12	7	18	100	100
P value	0.001	0.002	NS	0.12	0.06
FK/Med (n = 98)	0.19	22	22	96	94

FK = tacrolimus; MMF = mycophenolate mofetil; Med = Medrol, AR = acute rejection, PTDM = posttransplant diabetes mellitus, PS = patient survival, GS = graft survival.

icantly lower in the FK/MM/Med group versus the FK/Med group (with no difference between BM and no BM recipients). The incidence of posttransplant diabetes mellitus, monthly serum CR measurements, cholesterol levels, and numbers of blood pressure medications did not differ between groups. Actuarial patient survival at 1 year was 98% for the FK/MMF/Med group and 96% for the FK/Med group. Graft survival at 1 year did not differ, although the result approached statistical significance (97% versus 94% for the FK/MMF/Med and FK/Med groups, respectively). The mean starting dose of MMF was 1897 ± 288 mg/day and was 1161 ± 471 mg/day at most recent follow-up. Mycophenolate mofetil was discontinued in 22 patients, mostly because of gastrointestinal intolerance and/or leukopenia. Serious adverse events occurred in four FK/Med patients (one Kaposi's sarcoma, two posttransplant lymphoproliferative disorders, one fungal infection) and in

three patients in the FK/MMF/Med group (one tuberculosis, two fungal infections). Asymptomatic CMV viremia was more common in the FK/MMF/Med patients, although there were no differences in tissue-invasive disease (32).

IV. CONCLUSION

We have observed therapeutic efficacy of tacrolimus in both (1) induction (i.e., prevention of acute rejection) and (2) treatment for relapsing or resistant rejection when used as rescue therapy for renal allografts. There has been much progress since the early studies with tacrolimus, when it was used as an alternative immunosuppressive agent that was potent enough to reverse OKT3-refractory or recurrent rejection in patients treated with cyclosporine-based therapy without resorting to the use of excessive doses of steroids and additional antilymphocyte preparations (9,19–22).

The onset of CMV disease has been shown to be critical during the first 3 postoperative months and presumably is aggravated as a consequence of the intense immunosuppression required during this period. Tacrolimus-treated patients in our experience had predominantly asymptomatic CMV infection typified by shell vial assay or PCR positivity in peripheral blood buffy coat samples; this occurred to a somewhat greater extent than in cyclosporine-treated patients (10). However, because of a reduced rate of early graft rejection and, as a result, less overall immunosuppressive therapy, and because of the routine use of prophylactic ganciclovir therapy, the incidence of clinical CMV infection may well have been commensurately reduced. Per therapeutic protocol during the past 4 years, CMV-antibody–negative patients receiving a kidney from CMV-antibody–positive donors are treated with preemptive intravenous ganciclovir, 5 mg/kg twice a day for 14 days, followed by ganciclovir, 1 g by mouth three times a day for 3 months. All other recipients receive intravenous ganciclovir followed by acyclovir, 800 mg by mouth four times a day for 3 months. Doses are modulated according to renal function. When steroid-resistant rejection develops, requiring antilymphocyte therapy, the ganciclovir is reinstituted during rejection therapy.

We have observed five (2.1%) cases of Epstein-Barr virus (EBV)-positive posttransplant lymphoproliferative disease (PTLD) since the shift from cyclosporine to tacrolimus for maintenance immunosuppression and for rescue therapy (33). This is still within the 2% to 4% incidence of PTLD that has been reported in kidney transplant patients using maintenance cyclosporine therapy (34), but it is more than we have seen previously at this center.

One of the drawbacks of tacrolimus is an increased incidence of glucose intolerance (higher incidence of diabetes mellitus and new-onset insulin requirement) (23,24). However, there is no detailed encompassing report as yet about the risk factors for new-onset diabetes mellitus under tacrolimus. Tanabe et al. (35) reported the correlation between the oral glucose tolerance test (OGTT) and posttransplant insulin requirement. A higher incidence rate of diabetes mellitus was seen in the patients who had abnormal OGTT patterns compared with the patients with normal OGTT patterns. Pirsh et al. (24) reported that covariate analyses of race, steroid use, and trough levels of tacrolimus were significant predictors for the development of diabetes mellitus. The risk for blacks or Hispanic patients was 3.3 times that of whites for development of diabetes mellitus. Seventy-five percent of the patients in whom diabetes mellitus developed had maximum total daily steroid doses of 25 mg/day or greater within a 30-day period before the onset of diabetes mellitus.

In conclusion, it appears that there is an overall early benefit to giving cadaver kidney transplant recipients maintenance tacrolimus therapy in terms of morbidity and finan-

cial cost, which may translate into an eventual advantage in graft survival by reducing rates of acute rejection.

Mycophenolate mofetil is well tolerated with an acceptable safety profile. The gastrointestinal side effects that we observed were managed by dose reduction, and they necessitated drug discontinuation in very few cases. Mycophenolate mofetil appears to be a well-tolerated immunosuppressant that is efficacious in preventing renal allograft rejection and improving graft survival when administered with cyclosporine and steroids (30), perhaps even more so with tacrolimus and steroids (32). If prevention of rejection leads to improved long-term survival, the superiority of tacrolimus and mycophenolate mofetil used together will become apparent as treated patients are monitored through long-term follow-up.

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18

Newer Immunosuppressive Agents: Mycophenolate Mofetil, Sirolimus, 15-Deoxyspergualin, Brequinar, and Combination Therapy

FERDA SENEL and BARRY D. KAHAN

University of Texas Medical School at Houston, Houston, Texas

I. INTRODUCTION

The development of immunosuppressive therapy to dampen host alloimmune responses to foreign antigens has been a critical factor in the success of organ transplantation. The present matrix of agents includes corticosteroids, azathioprine (Aza; Imuran, Burroughs-Wellcome), cyclosporine (CsA; Sandimmune, Sandoz, Switzerland), tacrolimus (TRL; FK506; Prograf, Fujisawa, Japan), and antilymphocyte preparations (OKT3 and antihymocyte globulin [ATG]). These agents are used in a panoply of combination regimens in which minimal doses of the individual agents are used to reduce their toxic side effects, which are responsible for the appreciable patient morbidity associated with transplantation. Thus, a niche exists for new, more potent, more specifically directed, less toxic, and truly synergistic immunosuppressive drug combinations.

The modern era of chemical immunosuppression began when the antiproliferative drug 6-mercaptopurine (6-MP) and its nitroimidazole derivative Aza, competitive inhibitors of de novo purine synthesis, were developed in the early 1960s. Despite the "slippery slope" of Aza-corticosteroid immunosuppression, this regimen was the baseline treatment for almost 2 decades (1–6).

After its introduction, CsA, a lymphokine synthesis inhibitor that inhibits T cells

selectively, quickly became the cornerstone of immunosuppressive regimens despite the array of drug-induced toxic side effects. Although TRL was found to inhibit lymphokine generation more potently than CsA, its therapeutic window is at least as narrow as that of CsA. Therefore, new drugs are being explored for their promise to broaden the therapeutic window of CsA, namely sirolimus (SRL, rapamycin; Rapamune, Wyeth-Ayerst), which blocks cytokine signal transduction; 15-deoxyspergualin (15-DS, Behringwerke, Germany), which inhibits lymphocyte differentiation pathways; and brequinar sodium (BQR; DuP785, Dupont-Merck) and mycophenolate mofetil (MM; RS-61443, CellCept, Syntex Laboratories), which display selective inhibition of nucleoside synthesis, namely pyrimidine and purine generation, respectively. Although these individual agents offer alternative therapeutic approaches, the greatest boon to the transplant enterprise will be the discovery of a synergistic regimen that allows CsA dose reduction and practically eliminates acute or chronic allograft rejection.

II. MYCOPHENOLATE MOFETIL

Mycophenolate mofetil (MM), a semisynthetic analogue of mycophenolic acid (MPA), which is derived from the fungus *Penicillium glaucum* (7,8), is converted by the liver into MPA and metabolized to its glucuronide, which is then secreted into bile and converted by intestinal glucuronidase into active MPA, thereby undergoing enterohepatic cycling. The high drug concentrations in the bowel may explain the gastrointestinal toxicity of MM (9).

A. Mechanism of Action

Mycophenolic acid inhibits the S phase of the cell cycle (10) by noncompetitively inhibiting inosine monophosphate dehydrogenase (IMPDH), particularly isoenzyme type II, which converts inosine monophosphate to guanosine monophosphate in the de novo purine biosynthesis pathway (Fig. 1), thereby depleting guanosine nucleosides (8). Types I and II isoenzymes of IMPDH, which each consist of 514 amino acids, display an 84% homology

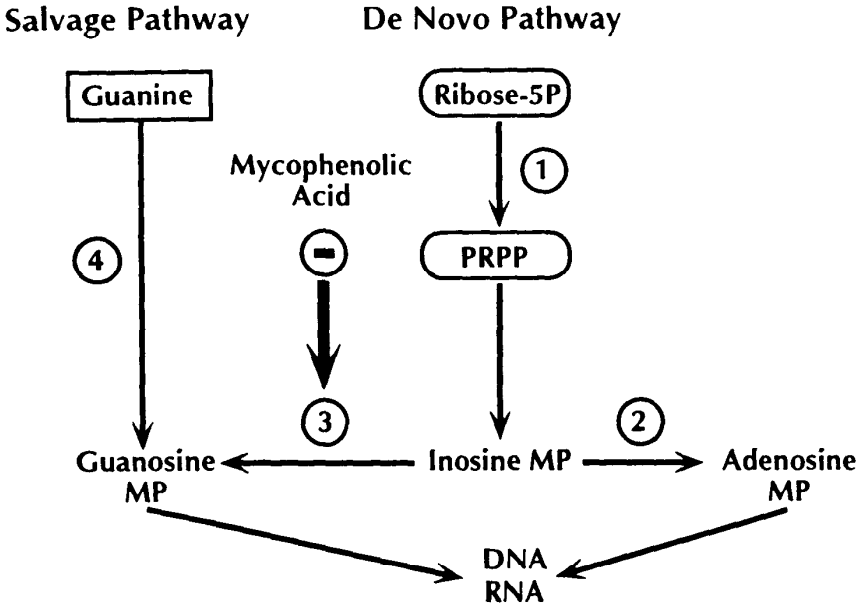

Figure 1 Purine biosynthesis and mechanism of action of mycophenolic acid PRPP:5-phosphoribosyl-1-pyrophosphate. 1, PRPP synthetase; 2, adenosine deaminase; 3, inosine monophosphate dehydrogenase; and 4, HGPRTase.

(11). Type I is the predominant isoenzyme in resting human leukocytes; type II is constitutively expressed in leukemic and ovarian neoplastic cells and is upregulated after stimulation of lymphocytes with phytohemagglutinin (PHA) or Epstein-Barr virus (EBV) (12). The type II isoform is five times more sensitive to MPA than the type I isoform (13). Two observations suggest that the de novo but not the salvage purine synthesis pathway is crucial for the mitogen-driven proliferative responses of human T and B lymphocytes. Children with inherited adenosine deaminase deficiency, that is, a reduced level of the enzyme that converts inosine monophosphate to adenosine monophosphate, have decreased numbers and functions of T and B lymphocytes, but normal neutrophil, erythrocyte, and platelet counts (14). Also, children with Lesch-Nyhan syndrome, due to a lack of hypoxanthine-guanine phosphoribosyltransferase (HGPRTase), have essentially normal numbers and functions of T and B lymphocytes (15).

In addition, MPA decreases the intralymphocyte concentrations of guanosine triphosphate (GTP) and deoxyguanosine triphosphate (dGTP) (16) and thereby may reduce the transfer of mannose or fucose to glycoproteins (17), such as the intercellular adhesion molecule (ICAM) and the very late antigen (VLA)-4 (18). Although addition of 1 μmol/L MPA appeared to reduce the incorporation of 3H-mannose into immunoprecipitated VLA-4 on exposure of PHA-activated human peripheral blood lymphocytes (PBLs), flow cytometry failed to reveal decreased VLA-1 surface expression (19). Thus, the extent or significance of the effect of MPA on glycoproteins is unclear.

In vitro addition of 30 to 100 nM MPA halves (IC_{50}) the proliferative responses of T cells stimulated by PHA, interleukin (IL)-2, or primary mixed lymphocyte reaction (MLR) (20). At a concentration of 100 nM, MPA almost completely inhibits B-cell proliferative responses to pokeweed mitogen (PWM) and staphylococcus protein A (21). Oral administration of MPA (30 mg/kg/day) to rodents inhibits antibody production in response to sheep erythrocytes (SRBC) or xenogeneic cells (22).

B. Pharmacokinetics

The MPA is metabolized principally by glucuronyl transferase to its inactive phenolic glucuronide (9). High-performance liquid chromatography (HPLC) methods have been used to estimate MM and MPA concentrations in plasma, where they are almost exclusively distributed, without evidence of temperature or concentration dependence. Most of the drug in plasma associates with nonalbumin proteins; less than 10% of the agent is associated with lipoproteins (23). The unbound fraction of plasma MPA ranges from 7.2% to 16.5%. Pharmacokinetic studies have demonstrated that the prodrug MM is rapidly absorbed after oral administration and hydrolyzed to free MPA, the active drug. Between day 1 and day 20, the maximum concentration (C_{max}) of MPA and the area under the concentration-time kinetic curve (AUC) show a dose-proportionate increase (Table 1), suggesting increased oral absorption, enterohepatic recycling, or drug accumulation during the early posttransplant period. Furthermore, there is substantial interpatient variation in pharmacokinetic properties (24). Although the ingestion of food alters the pharmacokinetic profile, reducing the C_{max} by 40%, it does not change the AUC value. In contrast, antacids not only reduce the C_{max} by 33% but also lower the AUC by 17%. Thus MM should be administered on an empty stomach.

C. Animal Transplant Models

Morris et al. (25) found that MM at doses of 40 to 100 mg/kg/day prolonged the survival of neonatal Balb/c(H-2^d) heart pinnal implants in C3H/km(H-2^k) mice and, at doses of 10 to

Table 1 Pharmacokinetic Parameters of Mycophenolic Acid in Renal Transplant Recipients Following Oral Administration

	Day 1			Day 20		
Dose	t_{max} (h)	C_{max} (μg/mL)	AUC (μg/h/mL)	t_{max} (h)	C_{max} (μg/mL)	AUC (μg/h/mL)
1 g qd	6.7 (±2.3)	1.6 (±0.9)	10.4 (±8.1)	0.7 (±0.3)	9.1 (±2.1)	24.1 (±8.6)
1 g bid	6.1 (±4.8)	2.6 (±2.5)	24.6 (±11.2)	1.6 (±1.3)	8.2 (±3.6)	65.4 (±10.9)
1.5 g bid	9.6 (±3.6)	5.1 (±6.2)	39.0 (±27.8)	1.1 (±0.6)	13.0 (±8.4)	73.2 (±10.3)

40 mg/kg/day, prolonged the survival of heterotopic Brown Norway (BN) ($RT1^n$) vascularized cardiac allografts in Lewis (LEW) ($RT1^l$) rats to more than 200 days. Upon rechallenge, donor (BN) but not third party strain (ACI; $RT1^a$) atrial transplants placed beneath the renal capsule were accepted (26). The coronary arteries of four BN heart grafts from hosts treated for 100 days showed no evidence of graft coronary disease in one case, mild intimal proliferation in two grafts, and moderate intimal thickening in one case. A subsequent study by Steele et al. (27) showed that MM (40 mg/kg/day × 14 days followed by continuous therapy with 30 mg/kg/day) significantly ($p = 0.011$) reduced the degree of intimal proliferation of ACI aortic allografts in LEW recipients at 3 months. In contrast, Schmid et al. (28) failed to document an effect of 100-day treatment with CsA and/or MM to prevent transplant vasculopathy in heterotopic F344 rat heart grafts in LEW hosts; however, treatment with CsA and/or MM appeared to reduce the degree of glomerulosclerosis and arterial obliteration in renal allografts. In this renal allograft model, LEW hosts engrafted with F344 kidney allografts, Azuma et al. (29) found that administration of MM (15 mg/kg/day), beginning at either day 1 or at 8 weeks, mitigated the chronic rejection process that appears in untreated rats after 12 weeks, including progressive proteinuria, prominent cellular infiltrates, particularly macrophages in the glomeruli and perivascular areas, and upregulated expression of intracellular adhesion molecule-1 (ICAM-1), cytokines, tumor necrosis factor (TNF)-α, tumor growth factor (TGF)-β, and platelet-derived growth factor (PDGF). In mongrel dogs, treatment with MM (40 mg/kg/day) prolonged kidney allograft survival from 8.1 (±1.2) to 36.0 (±9.6) days (7) (Table 2). The dogs experienced dose-related gastric toxicity without an apparent increased susceptibility to infection. Another study in which reduced (10 mg/kg) MM doses were administered in conjuction with CsA (5 mg/kg) and prednisone (0.1 mg/kg) documented that renal allograft rejection episodes in mongrel dogs were sensitive in 14 of 16 dogs (87.5%) to a course of increased doses of MM (80 mg/kg twice a day for 3 consecutive days) (7).

D. Clinical Trials

1. Phase I

A pilot study in 25 psoriatic patients showed that the severity of the disease was ameliorated in 68% of patients by 12 weeks after the initiation of therapy; in 15 patients, the disease completely cleared. The major side effect was gastrointestinal toxicity; including nausea, diarrhea, abdominal cramping, decreased appetite, abdominal distention, and weakness (30). Lynch et al. (31) reported three neoplasms, including one recurrence, and two primary neoplasms at 38 and 46 weeks among a cohort of 38 psoriatic patients given MPA. A third study of 85 psoriatic patients treated for up to 13 years (mean dose = 7368 mg/day) revealed gas-

Table 2 Mycophenolate Mofetil in Animal Experiments

Species (Organ)		Dose (Route)	Duration (d)	n	Mean Survival Time (d)	*p* Value
Mouse (heart)		Control	NA	105	10	—
		40–100 mg/kg (p.o.)	0–13	5–10/group	12–14	<0.01
Rat (heart)	BN→LEW	Control	—	5	7	—
		10–40 mg/kg (p.o.)	0–50	4–10/group	10 to >210	<0.01
Rat (atrial tissue)	BN→LEW[a]	40 mg/kg (p.o.)	0–50	3	>100	—
	ACI→LEW[b]	40 mg/kg (p.o.)	0–50	3	<14	<0.001
Canine (kidney)		Control		5	8.1	—
		40 mg/kg (p.o.)		6	36	<0.05
		20 mg/kg plus CsA (5 mg/kg) plus prednisone (0.1 mg/kg)		16	122.4	<0.002

[a]Atrial tissue of BN donor transplanted into LEW recipient that had previously received BN heart.
[b]Atrial tissue of ACI donor transplanted into LEW recipient that had previously received BN heart.

tric toxicity among 72.4% of patients, the percentage of which decreased to 13% to 27% after several years (32). Additionally, herpes zoster infections developed in 11.6% of patients and neoplasms in six patients (32). An open label study of MM treatment for patients afflicted with severe rheumatoid arthritis (33) reported clinical improvement in two-thirds of patients given 1 to 1.5 g MM twice daily.

Sollinger et al. (9,24) conducted an open label ascending dose trial, administering MM doses of 100 to 3500 mg/day de novo in combination with CsA and prednisone to 48 recipients of primary cadaveric kidney transplants. Significantly fewer patients experienced rejection episodes at doses above 2000 mg/day. The gastrointestinal side effects included ileus, gastritis, nausea, and vomiting. One patient (1000 mg/kg dose) experienced hemorrhagic gastritis that required drug discontinuation. There was no evidence of dose-dependent infectious complications, myelosuppression, or hepatotoxicity.

2. Phase II

Several open label studies suggest that MM displays immunosuppressive properties. Among 14 pediatric renal transplant patients given MM in combination with CsA and steriods for immunoprophylaxis, fewer rejection episodes occurred in patients receiving 23 mg/kg twice a day than among those receiving 15 mg/kg twice a day (34).

Some phase II open label studies suggest that MM may be used to "rescue" grafts from apparently unremitting rejection. Among 20 patients experiencing "refractory" allograft rejection, MM (2000–3500 mg/day) reversed the process in 4 of 5 patients who received living-related donor (LRD) grafts and in 7 of 15 cadaveric (CAD) graft recipients (24). A second study by Laskow et al. (35) showed that MM reversed rejection episodes in 18 of 27 renal allograft recipients given MM within 28 days after steroid and OKT3 therapy failed. A similar study in liver transplantation demonstrated 21 of 23 rejection reversals, but at the price of a high infection rate and one patient death caused by cytomegalovirus (CMV). An open label multicenter pilot trial claimed successful rescue in 52 of 75 (69%) patients who were deemed "refractory" based on biopsy-proven persistence of rejection despite at least one course of antilymphocyte globulin (ALG)/OKT3 (36). The overall impression was that MM rescue tends to be more successful when the creatinine level at the time of entry is less than 4 mg/dL (79% versus 52%). In addition to an overall infection rate of 40%, MM was discontinued in 19 patients because of treatment failure and in 11 cases for recurrent disease, lymphoma, pancreatitis, ureteral leak, cytomegalovirus colitis, gastrointestinal toxicity, and cancer in a native kidney. However, because of the open label nature of the study and the lack of a comparator treatment, the study was not deemed sufficiently powerful to permit a labeling indication.

3. Phase III

Recently, the findings of two pivotal trials have been reported. The European, double-blind, placebo-controlled multicenter study of 491 patients (37) examined the benefit of adding 2 (n = 165) or 3 g (n = 160) of MM versus placebo to a CsA/prednisone regimen. Patients given 2 (30.3%) or 3 g (38.8%) MM displayed significantly fewer biopsy-proved rejection episodes during the first 6 months or withdrew early from the trial (for any reason) compared with those in the placebo group (56%). MM reduced the incidence of acute rejection episodes by 60%, but did not improve 1-year graft survival rates (Table 3).

The U. S. double-blind, randomized multicenter study (38) compared the efficacy of MM with that of Aza when combined with CsA/prednisone for prophylaxis of acute allograft rejection in 495 primary renal transplant recipients after induction of immunosuppression with 5 to 14 days of ATG treatment. Patients given MM doses of either 1 (n = 167)

Table 3 European Mycophenolate Mofetil Study Group: Biopsy-Proved Rejection Episodes, Treatment Failure, Graft and Patient Survival Rates

Condition Entry	Placebo	MM (2 g)	MM (3 g)	*p* Value
Total treatment failures	93 (56.8%)	50 (30.3%)	62 (38.8%)	<0.001
Biopsy-proved rejection	77 (46.4%)	28 (17%)	22 (13.8%)	NS
Graft loss/death/other treatment failure	16 (9.6%)	22 (13.3%)	40 (25%)	NS
All graft loss or death	17 (10.2%)	11 (6.7%)	14 (8.8%)	NS
All graft losses	15 (9%)	7 (4.3%)	10 (6.3%)	NS
Deaths with functioning kidney	2 (1.2%)	4 (2.4%)	4 (2.4%)	NS

NS = not significant.

or 1.5 g (n = 166) twice daily showed a significantly lower incidence of biopsy-proved rejection episodes or treatment failure than patients treated with Aza (1–2 mg/kg/day, n = 166). Both the incidence of and the time to the first biopsy-proved rejection episode were significantly lower among patients receiving MM treatment. The incidence of moderate or severe rejection episodes (diagnosed by biopsy) was lower in the MM-treated groups, which required less OKT3 therapy. However the incidence of diarrhea, esophagitis, gastritis, gastrointestinal bleeding, leukopenia, and CMV infections were higher among patients receiving MM. The most frequent adverse events were anemia, hypertension, and diarrhea; the most common events leading to discontinuation of MM were leukopenia and sepsis. The 6-month patient and graft survival rates were comparable in all groups (Table 4). Thus, the benefit of MM is at least partially offset by the increased rate of infections, particularly CMV infection. A comparison of the efficacy of MM showed a more marked dose dependency in the reduction of acute rejection episodes among African-Americans versus non-African Americans. These findings suggest that African Americans may benefit from higher MM doses (Table 5) (39).

In aggregate, these experimental and clinical results suggest that MM is a promising

Table 4 Mycophenolate Mofetil Study Group

	Aza	MM (2 g)	MM (3 g)
Patients	164	165	166
Biopsy-proved rejection or treatment failure[a]	79 (47.6%)	52 (31.1%)	52 (31.3%)
First biopsy-proved rejection	63 (38%)	33 (19.8%)	29 (17.5%)
Grade 1	29 (17.5%)	18 (10.8%)	9 (5.4%)
Grade 2	24 (14.5%)	12 (7.2%)	9 (5.4%)
Grade 3	10 (6%)	3 (1.8%)	2 (1.2%)
Rejection treatment with			
Corticosteroids only	40 (24.4%)	24 (14.5%)	26 (15.7%)
OKT3/ATG/ALG only	4 (2.4%)	4 (2.4%)	3 (1.8%)
OKT3/ATG/ALG and steroids	29 (17.7%)	13 (7.9%)	6 (3.6%)
Graft loss or death (6 months)	17 (10.4%)	9 (5.5%)	14 (8.5%)
Opportunistic infection	75 (45.7%)	74 (44.8%)	78 (47.0%)
CMV viremia/syndrome	25 (15.2%)	24 (14.5%)	22 (13.3%)
CMV tissue invasive	10 (6.1%)	15 (9.1%)	18 (10.8%)

[a]Aza versus MM (2 g), $p = 0.0015$; Aza versus MM (3 g), $p = 0.0021$

Table 5 Mycophenolate Mofetil Treatment in African Americans Versus Non-African Americans

	Aza	MM (2 g)	MM (3 g)
Patients (AA/Non-AA)	40/124	44/121	33/133
AA Rejections	19 (47.5%)	14 (31.8%)	4 (2.1%)
Non-AA Rejections	44 (35.5%)	19 (15.7%)	25 (18.8%)
AA OKT3/ATG use	7 (17.5%)	7 (15.9%)	1 (3%)
Non-AA OKT3/ATG use	26 (21.0%)	10 (8.3%)	8 (6%)
AA Rejection and/or TF	23 (57.5%)	17 (38.6%)	8 (24.2%)
Non-AA Rejection and/or TF	54 (43.5%)	33 (27.3%)	44 (33.1%)

Abbreviations: AA = African Americans; TF = treatment failure.

immunosuppressive agent with a relatively modest toxicity profile. However, because MM only acts in additive fashion with CsA, it is unlikely that MM will permit significant CsA dose reduction. The long-term beneficial effects of MM on chronic rejection must await analysis of 5-year treatment results.

III. SIROLIMUS

Sirolimus (SRL), a macrolide antibiotic produced by the actinomycete *Streptomyces hygroscopicus*, was isolated from a soil sample obtained on Rapa Nui (Easter Island) (40,41). Sirolimus is a 31-membered macrocyclic lactone (molecular weight 914.2, $C_{51}H_{79}NO_{13}$), which contains an unusual hemiketal masked α/β-diketo amide moiety, like TRL but with a larger ring structure that contains a unique triene segment (42).

A. Mechanism of Action

Sirolimus inhibits the proliferation of transformed T and B cells as well as cell lines of lymphoma, myelocytic, hepatoma, melanoma, and central nervous system origin (43). In vitro addition of SRL (1–300 nM) inhibits both Ca^{2+}-dependent and Ca^{2+}-independent, mitogen- and antigen-driven lymphocyte proliferation in response to stimulation in mixed leukocyte cultures (MLCs), PHA, anti-CD3 monoclonal antibodies (mAbs), phorbol myristate acetate (PMA), or anti-CD28 mAb (44,45). Whereas CsA inhibits T-cell progression from the G_0 to G_1 phase of the cell cycle, SRL acts during the G_1 progression (46–48). Sirolimus inhibits events triggered by a variety of stimuli, including those elicited by CD28, protein kinase C, and lymphokines (IL-2 and IL-6 signal transduction) (43,47,49–52). Although one study suggested that SRL partially decreased the cell surface expression of IL-2 and IL-4 receptors on mitogen-activated T cells (53), most studies show that SRL does not affect the numbers of receptors. There is also strong evidence that SRL inhibits proliferation of activated T cells in response to IL-12, a heterodimeric cytokine that regulates natural killer (NK) and T lymphocyte responses (54).

Although SRL binds to FKBP 25, its molecular effector mechanism is unclear; it has been proposed that $p70^{S6}$ kinase, $p34^{cdc2}$, and the cdk2/cyclin E complex are the target molecules. Although SRL does not directly inhibit purified $p70^{S6}$ kinase, it does block the phosphorylation of the kinase at the same concentrations at which it inhibits cell proliferation (6,43,55). The actual target of SRL action appears to be FKBP-receptor-associated protein (56).

SRL does not affect the transcription of early genes, including c-jun, c-myc, and c-fos, but does decrease c-myb production, thereby reducing cyclin A activity as well as the downstream serine-threonine protein kinases ($p34^{cdc2}$). The active $p34^{cdc2}$-cyclin heterodimeric complex forms the maturational promoting factor for progression of T cells from the G_1 to the S phase (57,58). The cyclin-dependent kinases (cdk), which associate with the G_1 cyclins D and E, are able to retard the rate of progression into the S phase of the cell cycle. During T-cell mitogenesis, antigen receptor signaling promotes the synthesis of cyclin E and its catalytic partner cdk2. IL-2 signaling activates the cdk2/cyclin E complex, which may be the triggering event required for progression into S phase. IL-2 triggers cdk activation by causing the elimination of the cdk inhibitor protein p27kip1, a step that may be prevented by SRL (59).

SRL also inhibits B-cell responses as well as Ig production both in vitro and in vivo (60–62). At concentrations of 1 nM SRL inhibits the stimulation of DNA synthesis by B cells, and at concentrations of 10 nM it inhibits IgM, IgA, and IgG secretion by B cells stimulated with *Staphylococcus aureus* (SA) or soluble CD40 ligand (CD40L). SRL also blocks the progression of human B cells during the G_1 to S phase (63), inhibits IL-4-stimulated IgE production by peripheral blood cells 1000 times more potently than CsA, and blocks the responses of stimulated B cells to IL-2 and IL-6 (62). SRL concentrations 10- to 100-fold greater than those needed to block T cell proliferation inhibit the toxic actions of NK, lymphokine-activated killer (LAK), and antibody-dependent cytotoxic cells (64).

B. Pharmacokinetics

The pharmacokinetics of SRL have been extensively studied in rats and rabbits using HPLC methods with a sensitivity of 1 μg/L on whole blood (65). After intravenous administration of SRL at doses of 0.005 or 0.5 mg/kg to 5 rabbits, the terminal half-lives ($t_{1/2}$) were 12.81 ± 2.14 and 15.33 ± 1.16 hours, respectively; the clearance rates were 0.98 ± 0.28 and 2.13 ± 0.16 ml/min/kg, respectively. After intramuscular administration of 0.1 mg/kg SRL, the $t_{1/2}$ was 10.87 ± 2.96 hours, and the clearance rate was 0.38 ± 0.04 ml/min/kg (66). In humans, 94% of the drug is contained in red blood cells, with less than 3% distributed in other cells. Only 3% of SRL is detected in plasma, and 0.175% of the total drug is free in blood (67). Currently, SRL pharmacokinetics in humans is under study. In a double-blind randomized phase I study (68), 40 stable renal transplant patients received either SRL (1 to 13 mg/m^2 for 13 days) or placebo in combination with CsA/prednisone. The blood/plasma ratio (mean = 38) of SRL was high, and SRL was rapidly absorbed (t_{max}=1.4 hours) and had a long half-life (62 hours), and a modest drug accumulation. The interindividual differences in pharmacokinetic parameters after SRL administration are great. Although the SRL dose does not correlate with any pharmacokinetic parameter, the AUC correlates well with trough concentrations. Although this study of a two-week course of administration failed to show any pharmacokinetic interaction between SRL and CsA, interaction was detected upon chronic therapy.

C. Animal Transplant Models (Table 6)

1. Prophylaxis of Rejection

Calne et al. (69) found that SRL prolongs heterotopic rat heart and pig kidney allograft survival. The toxicity of SRL in rats was mild; less than 10% of hosts lost weight. Three of nine pigs survived with normal creatinine values for six months after discontinuation of the

Table 6 Sirolimus Prevention of Allograft Rejection in Animal Experiments

Species (Organ)		Dose (Route)	Duration	n	Mean Survival Time (d)	*p* Value	Investigator
Mouse (heart)	BALB/c→C3H/km	Control		105	10.6		Morris et al. 1989
		6 mg/kg (p. o.)	0–13	11	14	<0.0001	
Rat (heart)	BN→LEW	Control		7	7		
		3 mg/kg (i. p.)	0–14	5	24	0.001	
Rat (heart)		Control		10	7.4		Calne et al. 1989
		50 mg/kg (IM)	0–10	4	100		
		10 mg/kg (IM)	0–10	4	82		
		1 mg/kg (IM)	0–10	4	47		
Pig (kidney)		Control			15.1		
		2 mg/kg (IM)	0–64	8	76		
Dog (kidney)		2 mg/kg (IM)	3–5	7	14		
Dog (kidney)		2 mg/kg (p. o.)	3–5	7	14		Collier et al. 1990
Pig (kidney)		Control			<10		
		2 mg/kg (p. o.)	0–64	9	76		
Rat (heart)	BN→LEW	12 mg/kg (i. p.)	1	5	148		Morris et al. 1991
	LBN→LEW	12 mg/kg (i. p.)	1	5	>200		

Rat (heart)	BUF→WF	Control		3	6.3		Stepkowski et al. 1991
		0.08 mg/kg (IV)	0–14	5	34.4	0.001	
		0.32 mg/kg (IV)	0–14	4	55.7	0.001	
		0.80 mg/kg (IV)	0–14	3	48	0.001	
Rat (kidney)	BUF→WF	Control		3	11.6		
		0.08 mg/kg (IV)	0–14	8	52.7	0.347	
		0.8 mg/kg (IV)	0–14	5	90.2	0.03	
Rat (small bowel)	BUF→WF	Control		3	10		
		0.8 mg/kg (IV)	0–14	5	26.8	0.001	
Mice (heart)	C57 BL→C3H	Control		6	7.7		Tu et al. 1995
		0.1 mg/kg	0–7	5	13	0.01	
		0.2 mg/kg	0–7	5	20	0.01	
		0.4 mg/kg	0–7	5	15.8	0.01	
Rat (small bowel)	LBN→LEW	Control		5	9.2		Chen et al. 1995
		16 μg/ml[a]		5	15.2	0.012	
		48 μg IV[b]	1	5	16.6	0.001	
Rat (kidney)	LBN→LEW	Control		5	8.4		
		48 μg IV[a]	1	5	26.4	0.0001	
		48 μg IV[b]	1	5	12.6	0.005	

[a]Intragraft SRL treatment
[b]Treatment of recipient
Abbreviations: IV = intravenous; IM = intramuscular.

drug. Thereafter Morris et al. (70) reported prolonged survival of nonvascularized heterotopic Balb/c heart grafts implanted in the ears of C3H/km mice as well as of primary vascularized abdominal BN heart grafts in LEW rats. Collier et al. (71) showed that SRL prolonged pig graft survival to 76 (±17) days compared to less than 10 days in the untreated group. However, in dogs, SRL induces a pernicious vasculitis, particularly in the gastrointestinal system.

Morris et al. (72) found that a single large intraperitoneal (i. p.) dose of SRL induced antigen-specific unresponsiveness in LEW recipients of BN hearts. A 14-day continuous intravenous infusion of 0.8 mg/kg/day SRL increased the survival of Buffalo (BUF; $RT1^b$) grafts in Wistar Furth (WF; $RT1^u$) rats (73), namely hearts from 6.3 (±0.5) to 48.0 (±3.6) days, kidneys from 11.6 (±1.5) to 90.2 (±62.4) days, and small bowel transplants from 10 (±0) to 26.8 (±3.7) days. Sirolimus (0.1 mg/kg/day) administered as a 7-day intravenous infusion prolonged the survival of vascularized C57 BL/10(H-2^b) heart allografts transplanted into C3H (H-2^k) mice recipients from 7.7 ± 1.4 to 13.0 ± 7.5 days (74).

In rat models, SRL induced the production of blocking antibodies, which mediated the prolongation of allograft survival (75). On the one hand, adoptive transfer of T cells from SRL-treated WF recipients did not prolong graft survival in irradiated (6 G) WF recipients of BUF hearts. On the other hand, the serum of SRL-treated WF recipients caused a significant prolongation of survival time from 9.8 ± 1.2 to 74.4 ± 31.1 days. The IgG fraction, probably IgG_{2c}, prolonged BUF heart allograft survival.

2. Reversal of Rejection

Wang et al. (76) documented that a 14-day intravenous infusion of SRL beginning 3 to 4 days after the transplant reversed the ongoing rejection of BUF hearts in WF recipients. Two 14-day courses of 0.8 mg/kg SRL delivered by continuous intravenous infusion before and after BUF heart allografting suppressed accelerated cardiac allograft rejection in WF recipients that had been presensitized by two consecutive BUF skin grafts.

3. Effects on Xenografts

Sirolimus is not as efficacious in xenotransplant as it is in allotransplant models. SRL (9 mg/kg/day i. p.) failed to prolong the survival of vascularized hamster hearts in LEW rats. Higher SRL doses (20 mg/kg) only minimally prolonged survival, namely from 4.1 days to 5 days ($p < 0.05$) (77). In combination with rabbit anti-mouse lymphocyte serum (on days −1, 2, and 4), a 12-day course of SRL (3.0 mg/kg) prolonged ACI skin xenografts in C57BL/6(F1) mice, from 9 to 47 days (78).

4. Effect on Chronic Allograft Vasculopathy

Sirolimus may reduce arterial intimal thickening. Meiser et al. (79) showed that LEW recipients of BN hearts did not display graft vessel disease when treated with SRL, whereas hosts treated with TRL (1.0 mg/kg) showed complete vascular occlusion. A recent study that administered the combination of SRL (1.5 mg/kg/day i. p.) and MPA (40 mg/kg/day per os [p. o.]) from day −3 to 13 showed an 85% reduction in arterial intimal thickening in response to mechanical stress to a rat carotid artery. Untreated rats did not show endothelial regrowth, whereas rats given the drug combination showed 68.01% ± 46.03% coverage of the luminal surface. Thus, SRL both reduced intimal thickening and allowed endothelial regrowth (80), presumably by inhibiting epidermal- and fibroblast-derived growth factors and/or cytokine-driven endothelial proliferation.

5. Toxicity Profile

Because the primary adverse reactions to CsA are renal dysfunction and histopathological changes, it was critical to show that kidneys from SRL-treated hosts were histologically unremarkable (81,82). Administration of SRL (1.5 mg/kg/day i. p.) for 14 days to male adult Sprague-Dawley rats did not produce any histopathological changes in the kidney, but did cause mild to moderate focal myocardial necrosis and medullary thymic atrophy (81). However, when administered for 14 days in combination therapy, SRL exacerbated the renal impairment produced by CsA (15 mg/kg) (83). In a subsequent study, DiJoseph et al. (84) found that high doses of SRL (up to 10 mg/kg) did not produce any histological change in the kidneys of Sprague-Dawley rats, whereas CsA 25 mg/kg p. o. caused proximal tubular damage. Recently, Thliveris and Yatscoff (85) treated rabbits with SRL (0.5 or 2 mg/kg/day p.o.) for 60 days, and found no change in renal function but did find an altered histopathological appearance of kidneys, including dose-related tubular atrophy, interstitial fibrosis, and arteriopathy.

D. Clinical Trials

1. Phase I

The phase I double-blinded clinical study administered a 14-day course of SRL (0.5–6.5 mg/m^2 twice daily) or placebo to 43 renal transplant recipients who were immunologically quiescent on a CsA-steroid regimen, and who had stable renal function for at least 6 months, glomerular filtration rates (GFR) >40 mL/min, and therapeutic CsA average steady-state blood concentrations for at least 3 months (86). Thirty patients received low (1–3 mg/m^2/day), medium (5–6 mg/m^2/day), or high doses (7–13 mg/m^2/day) of SRL; 10 patients received placebo. Patients receiving SRL showed a dose-dependent decrease in mean platelet and white blood cell counts (Table 7). The mean baseline platelet count (238.7 ± 62.9 × 10^3 cells/mm^3) for all SRL-treated patients decreased at day 9 to 202.7 ± 62.9 (p = 0.03), at day 12 to 185.6 ± 71.1 (p = 0.004), and at day 15 to 169.1 ± 74.9 (p = 0.0003). In contrast, there were no significant differences between the pre- and post-SRL treatment values for blood pressure, GFR, serum creatinine, liver function tests (serum glutamic oxaloacetic [SGOT] and serum glutamic pyruvic transaminase [SGPT]), or CsA steady-state average concentrations.

2. Phase II

The initial single-center phase II study administered SRL (0.5–7 mg/m^2/day p.o.) de novo to recipients of living-related renal allografts. Sirolimus reduced the incidence of acute rejection episodes, with a 3-month rejection incidence of 6.7% (2/30) compared with 36.9% among a historical cohort of 65 CsA/prednisone-treated recipients (p = 0.002). Furthermore, SRL administration permitted rapid steroid tapering as early as 1 month for patients on CsA/prednisone-based regimens (BD Kahan et al., 1996, unpublished data).

The phase II multicenter trial of SRL administration in combination with CsA/prednisone also documented a reduced incidence of acute rejection episodes within 6 months. Patients receiving open label treatment with 1 or 3 mg/m^2/day SRL experienced approximately a 10% incidence of acute rejection episodes compared with a 40% incidence among patients given placebo. The addition of SRL allowed the CsA dose to be substantially reduced with good therapeutic effect among all but black patients (Sirolimus Multi-Center Study Group).

Table 7 Phase I Study of Sirolimus—Effect of Sirolimus on Platelet and White Blood Cell Counts

		Placebo n = 10	All Sirolimus Groups n = 30	Low Dose n = 9	Medium Dose n = 9	High Dose n = 12
Mean age ± SD (yr)		42 ± 7.87	42.07 ± 9.35	43.67 ± 9.08	43 ± 9.8	41.6 ± 10.4
Platelet count (cells/mm^3)	Entry	250.8 ± 63.5	238.7 ± 62.9	223.1 ± 65.6	225 ± 103	253.3 ± 60.8
	Day 15	271 ± 84.6	169.1 ± 74.9	209.2 ± 89.61	143.8 ± 29.7	155.8 ± 76.8
White blood cell count (cells/mm^3)	Entry	10.58 ± 3.72	7.87 ± 2.45	7.27 ± 2.39	7.49 ± 1.9	8.51 ± 2.69
	Day 15	9.83 ± 2.95	5.96 ± 2.73	5.87 ± 1.74	5.05 ± 1.22	6.64 ± 3.83

IV. 15-DEOXYSPERGUALIN

15-Deoxyspergualin (Behringwerke, Germany), a synthetic, highly water-soluble peptide (molecular weight = 496 daltons) (87) derivative of spergualine that contains spermine and guanidine-like structures (88), is produced by *Bacillus laterosporus.*

A. Mechanism of Action

The precise mechanism of action of 15-DS is unknown. Preliminary studies suggest that the compound binds specifically to Hsp70, a constitutively produced member of the heat shock protein (Hsp) family, the members of which serve as immunophilins that are distinct from the cis-trans proline isomerases that bind CsA and TRL (89). Initial studies suggested that 15-DS exerts its immunosuppressive effects by primarily inhibiting: (1) monocyte lysosomal enzyme release, (2) macrophage superoxide production, and (3) class II major histocompatibility complex (MHC) antigen induction in response to immunological stimuli (90). However, subsequent studies suggested that 15-DS also exerts an antilymphocytic effect; it inhibits mitogenic or alloantigenic stimulation of human lymphocytes. In 1985, Umezawa et al. (91) observed that 15-DS reduced in vitro proliferation of mouse spleen cells upon concanavalin-A stimulation, inhibited the delayed hypersensitivity response of presensitized mice to subcutaneous footpad challenge with sheep erythrocytes, and almost completely blocked the induction of cytotoxic T-cell activity in mice sensitized toward P815 mastocytoma cells. A further study in AS rat hosts engrafted with DA rat kidneys showed that 15-DS (2.5 mg/kg) reduced IL-2R expression (92). Kerr et al. (93) observed that 15-DS reduced cytotoxic lymphocyte (CTL) activity of human PBL after alloantigenic stimulation without significantly affecting NK or LAK cell killing. In the most definitive studies, Tepper et al. (94) observed that CsA (0.5 μg/mL) inhibited mitogen- and MLR-stimulated responses of human T and B lymphocytes greater than did 15-DS (10.0 μg/mL). However, the expression of IL-2R was blocked by 15-DS (77.5% at 10 μg/mL), and IL-2–but not PHA- or OKT3-induced blastogenesis was inhibited by 15-DS. However, supernatants after MLR stimulation in the presence of 15-DS exposure did not show reduced IL-2 production (95). Because 15-DS inhibited MLR even when added 3 or 4 days after stimulation, it appears that 15-DS acts at a later point in cell maturation than CsA (92,95–97). When 15-DS (10.0 μg/mL) was added to the MLR either at the beginning of culture or every day, the generation of cytotoxic T lymphocytes was inhibited. Similarly 15-DS inhibits antibody production by interfering with B-cell differentiation: 15-DS (5μg/mL) reduced the expression of surface IgM in response to lipopolysaccharide or interferon (IFN)-γ (98) stimulation of the murine pre-B cell line 70Z/3.

B. Pharmacokinetics

Because of its low oral bioavailability (5%), which probably results from its highly polar properties, 15-DS must be given parenterally (99). A pharmacokinetic study of 25 advanced cancer patients who received 80 to 2160 mg/m^2/day 15-DS for 5 days by continuous intravenous infusion showed that plasma steady-state concentrations were achieved within 2 hours and maintained throughout the treatment. Moreover, steady-state plasma concentrations correlated with dose. 15-Deoxyspergualin displays a high clearance rate (364 ± 78 mL/min/m^2) with an average plasma half-life of 20 minutes (100). The major metabolite of 15-DS is desaminoprophyl-15-DS, which has been identified using an HPLC assay on human samples (101).

C. Animal Transplant Models

Umezawa et al. (91) showed the beneficial effect of 15-DS to prolong the survival of SHR rat skin grafts in F344 rats from 8 ± 2 to 17 ± 3 days. One year later, Walter et al. (102) noted that 15-DS (2.5 mg/kg) prolonged the survival of allogeneic rat islet transplants from 5.2 ± 2.3 to 39.3 days. In WKA rats, Ochiai et al. (103) showed that an 11-day course of 15-DS (3 or 10 mg/kg) prolonged F344 heart allograft survival, and, in the DA to LEW strain combination, 15-DS prolonged the survival of liver, pancreas, and pancreaticoduodenal transplants (104). In Chacma baboons, Reichenspurner et al. (105) found that 15-DS alone (4 mg/kg) prolonged heart allograft survival and, in combination with CsA, prolonged kidney allograft survival. However, compared with CsA, 15-DS was relatively ineffective in prolonging the survival of xenogeneic varvet monkey heart grafts in Chacma baboons (106) (Table 8).

Experimental animal studies suggested that 15-DS was more effective in reversing acute rejection episodes or blocking the induction of presensitization than it was for prophylaxis against rejection. Shorlemmer et al. (107) observed that 15-DS (0.5–2.0 mg/kg) administered on days 11 to 15 after rat skin allograft transplantation significantly prolonged survival. The administration of 1 mg/kg 15-DS (days 25–29) to hosts given 25 mg/kg CsA (days 1–15) markedly increased survival times. In another set of experiments, repeat donor-type skin grafts applied 63 and 98 days after long established primary grafts showed 25- and 36-day survivals, respectively, compared with 16 days in the control group. Third party (BN) skin grafts were rapidly rejected. Spleen cells from hosts bearing accepted skin grafts transferred tolerance.

D. Clinical Trials

1. Phase I

Phase I studies of 15-DS in cancer patients sought to define the maximal tolerable dose and to examine the pharmacokinetics of 15-DS. Tamura et al. (108) administered 3-hour infusions of 15-DS (20–600 mg/m^2) intravenously on 5 consecutive days to 33 cancer patients. Dose-dependent toxicities included mild nausea and vomiting, anorexia, alopecia, tongue and perioral numbness, and hypotension during or after the treatment. The maximal tolerated dose of 500 mg/m^2 produced granulocytopenia, leukopenia, and anemia, which tended to normalize within 21 days after initiation of the drug. The plasma peak concentration (C_{max}) was dose dependent. The drug clearance was rapid; 10% of the compound was directly excreted in the urine. There was no drug accumulation. A second phase I study administered 15-DS (80–2160 mg/m^2/day) to 25 advanced cancer patients by continuous intravenous infusion. This study observed the generation of the drug metabolite desaminopropyl-15-DS, which was extracted from patient plasma and urine (100).

The first clinical trial, which examined prophylaxis of renal allograft rejection, added 15-DS (2–5 mg/kg/day) for 5 to 14 days after transplantation to baseline triple-drug immunosuppression with prednisone, CsA (4–6 mg/kg/day), and mizorbine (2–3 mg/kg/day) in 10 patients (109). The 30% rejection rate within the first posttransplantation month was similar to that experienced by patients given a double-drug CsA–prednisone regimen. No long-term results were reported. Groth administered 15-DS (4 mg/kg for 5 days) in three cases of pig-to-man islet xenotransplantation. Two patients displayed pig islet cell survival for weeks to months, as manifested by urinary excretion of small amounts of porcine C-peptide (110). A year later, Gores et al. (111) reported that two type I diabetic patients who were given 15-DS (4 mg/kg) for 10 days after transplantation in addition to quadruple

Table 8 15-Deoxyspergualin in Experimental Transplantation

Species (Organ)		Dose (Route)	Duration	n	Mean Survival Time (d)	*p* Value	Investigator
Rat (skin)	SHR→F344	Control		9	8		Umezawa et al. 1985
		50 mg/kg	0–10	12	17	<0.01	
Rat (islet)	DA→LEW	Control		10	5.2		Walter et al. 1982
		2.5 mg/kg (i. p.)	0–9	10	39.3		
		1 mg/kg (i. p.)	0–9	5	27.4		
Rat (heart)	WKA→F344	Control		10	6		Ochiai et al. 1987
		3 mg/kg (IM)	0–11	8	>95	<0.01	
		10 mg/kg (IM)	0–11	8	>80	<0.01	
Rat (pancreas duodenum)	DA→LEW	Control		6	10.7		Theis et al. 1987
		2.5 mg/kg (i. p.)	0–9	7	17	<0.01	
Rat (pancreas)		Control		6	9.3		
		2.5 mg/kg (i. p.)	0–9	7	18.7	<0.01	
Rat (liver)		Control		6	13.3		
		2.5 mg/kg (i. p.)	0–9	10	>55.5	<0.01	
Baboon (heart)		Control		22	11		Reichenspurner et al. 1990
		4 mg/kg (IV)	0–9	5	28.2	<0.05	
Baboon (kidney)		Control		7	8.5		
		4 mg/kg (IV)	0–9	6	12.3	NS	
Rat (skin)	LEW→F344	Control		10	16		Schorlemmer et al. 1990
		0.5 mg/kg (i. p.)	0–9	10	26.8		
		1 mg/kg (i. p.)	0–9	10	25		
		2 mg/kg (i. p.)	0–9	10	28.2		
Rat (skin)	DA→LEW	Control		10	10.8		
		0.5 mg/kg (i. p.)	0–9	10	21.7		
		1 mg/kg (i. p.)	0–9	10	23.1		

Abbreviations: IM = intramuscular; IV = intravenous; NS = not significant.

induction therapy became insulin independent after transplantation of islets from a single donor. Both patients underwent simultaneous renal transplantation with organs harvested from the same donor, and they had good renal function and normal fasting blood glucose levels 9 months after the operation. In a pilot clinical trial for reversal of rejection with 15-DS, Amemiya et al. (112) documented a 79% remission rate after administration of 15-DS (40–220 mg/m^2) to 34 patients afflicted with rejection episodes within 6 months of transplantation.

2. Phase II

The pilot studies led to a trial of rescue therapy to treat rejection episodes by the Japan Collaborative Transplant Study Group (113). An initial dose finding study in 66 patients undergoing rejection episodes documented a 73% reversal rate using optimal doses of 3 to 5 mg/kg 15-DS. The combination of 15-DS with methylprednisone (MP), OKT3, or ALG increased the rejection reversal rate to 85% with the greatest degree of improvement (87.5%) being obtained in 40 patients who received 15-DS and MP. 15-Deoxyspergualin (3–5 mg/kg alone for 7 days) rescued 70% of patients refractory to steroids (n = 84), OKT3 (n = 9), or ALG (n = 7). Adverse effects included numbness of the face, lips, and limbs, as well as gastrointestinal toxicity, infections, and bone marrow depression (leukopenia, 54%; anemia, 26%; thrombocytopenia, 45%). These preliminary results have not been confirmed in blinded, randomized, controlled trials in large numbers of patients.

15-Deoxyspergualin may also reverse liver graft rejection episodes. Groth et al. (114) reported reversal of OKT3-resistant rejection in a patient given 15-DS (4 mg/kg for 5 days). By varying the duration of 15-DS (5 mg/kg) treatment in 53 patients, it was demonstrated that 5-, 7-, and 10-day courses produced similar degrees of efficacy; however, longer courses were associated with a higher incidence of leukopenia. The optimal duration of treatment was suggested to be 7 days (115).

A second potential indication for 15-DS therapy is inhibition of the antibody response after transplantation of ABO-incompatible or donor-presensitized recipients. Takahashi et al. (116) used CsA, MP, ALG, and 15-DS (5 mg/kg/day for 5 days) in addition to pretransplant plasmapheresis to treat 14 cases of transplants engrafted with ABO-incompatible organs. Five patients had no rejection episodes, one renal allograft was lost, and two patients died, including one of B-cell lymphoma and another of a cerebral hemorrhage. Another four patients who had documented preformed antibodies but negative immediate pretransplant T-cell cross-matches received 15-DS (5 mg/kg/day for 5 days) in addition to CsA, MP, and ATG; one patient experienced an acute rejection episode without graft loss. The authors attribute these findings to inhibition of B-cell activity. The clinical development of 15-DS has been limited.

V. BREQUINAR

Brequinar, a synthetic difluoroquinoline carboxylic acid derivative, inhibits pyrimidine biosynthetic pathways and was originally developed as an antineoplastic agent (117). The compound is rapidly absorbed from the gastrointestinal tract, metabolized by the liver, and excreted primarily, although not exclusively, via nonrenal routes (66% feces, 23% urine) (118).

A. Mechanism of Action

Chen et al. (117) discovered that a 48- to 72-hour exposure of cultured clone A human colon cancer cells to 25 to 75 μM BQR [6-fluoro-2-(2′-fluoro-1,1′biphenyl-4-yl)-3-methyl-4-

quinoline carboxylic acid sodium salt] caused 99% cell killing with depletion of the intracellular pools of uridine 5′-triphosphate and cytidine 5′-triphosphate. The levels were halved at 3 hours and undetectable by 15 hours. This study documented that BQR produces noncompetitive, reversible inhibition of the mitochondrial enzyme dihydro-orotate dehydrogenase (DHO-DH), the fourth enzyme in the pyrimidine biosynthetic pathway that catalyzes the conversion of dihydro-orotate into orotic acid, leading to the formation of uridine-MP. The marked dependence of lymphocytes on the de novo pyrimidine synthesis pathway renders them particularly sensitive to BQR during the cellular S phase (117,118). In addition to its antiproliferative activity, BQR inhibits the synthesis of glycoproteins, which depend on uridine-diphosphate intermediates such as the transfer of N-acetylglucosamine (Glc NA c) and of mannose to dolichol phosphate (119).

Simon et al. (119) found IC_{50} values between 22 and 185 nmol/L BQR for in vitro mitogen-induced human, rat, and minipig lymphocyte proliferative responses, whereas Jaffee et al. (120) documented that antigen-induced responses of human lymphocytes were most sensitive to BQR. The IC_{50} value of BQR to inhibit human MLR was low (10 ng/mL), and much more potent than the 1200 ng/mL IC_{90} required for CsA. In vivo in mice, DHO-DH activity was significantly inhibited by administration of 10 mg/kg BQR at the time of sensitization. Cytotoxic T-cell generation in C57BL/6 mice was suppressed by BQR at an IC_{50} value of 1.6 mg/kg, which was also more effective than CsA (IC_{50} = 88 mg/kg), Aza (IC_{50} > 100 mg/kg), or MM (IC_{50} = 140 mg/kg). In vivo BQR also reduced antibody responses to SRBC or to pneumococcal polysaccharide at IC_{50} concentrations of 1.6 or 0.3 mg/kg, respectively. The greater potency of the pyrimidine inhibitor BQR than purine inhibitors has been attributed to the 100-fold lower concentration of pyrimidine compared with purine precursors (121).

B. Pharmacokinetics

As a water-soluble compound, BQR can be administered orally or intravenously with a high bioavailability (90%), reaching peak levels at 2 to 4 hours (122). Arteaga et al. (123) determined plasma and urine concentrations estimated by a high-performance liquid chromatography assay in 28 advanced cancer patients given 135 to 300 mg/m^2 BQR doses as a single daily intravenous bolus for 5 days. The AUC increased linearly with the dose (r = 0.85) and with the length of treatment, namely, a 23% increase at day 5 compared with day 1. Brequinar had a volume of distribution of 15.5 ± 6.0 L/m^2 and a mean plasma clearance rate of 19.2 ± 7.7 mL/min/m^2. Only a minor fraction (0.13%–5.5%) of drug was excreted unchanged in the urine; the predominant excretion routes were nonrenal. In another study (124), 43 patients with solid malignancies received higher doses of BQR (200–2250 mg/m^2). These patients displayed nonlinear pharmacokinetics, which were different among patients given 600–1200 mg/m^2 as opposed to those given 1500–1800 mg/m^2. The latter group showed a terminal half-life of 15 hours. Because our animal studies suggested that the immunosuppressive effect correlates with the C_{max} (125) and because pharmacokinetic studies document a long half-life in humans, BQR has been administered on an alternate-day dosing schedule in clinical trials.

C. Animal Transplant Models

1. Prophylaxis of Allorejection

Cramer et al. (126) reported that BQR prolonged rat heart, liver, and kidney allograft survival. The ACI cardiac allografts in LEW recipients survived up to 54 days using BQR (12 mg/kg) administered for 30 days beginning 1 day before transplantation. Although some

allografts were rejected approximately 14 days after the discontinuation of the drug, 50% to 90% of liver and kidney transplants survived permanently. Donor-type but not third party BN heart grafts survived permanently in the long-term liver graft survivors. Makowka et al. (127) showed that BQR was also effective in the prophylaxis of allograft rejection in primates. Brequinar (4 mg/kg p. o.) treatment when initiated 1 day after transplantation and continued three times weekly prolonged the survival of cardiac allografts in cynomolgus monkeys from 8 ± 0.5 to 20.0 ± 21.5 days (Table 9).

Yasunaga et al. (128) reported that BQR prevented accelerated ACI cardiac allograft rejection in LEW recipients sensitized by placement of an ACI skin graft 7 days before challenge (see Table 9). The highest survival rate was observed in the rats given BQR (12.5 mg/kg/3 × week) during sensitization and from day −10 to +30 after transplantation. Cyclosporine (5–15 mg/kg/day for 30 days) was not as effective as BQR in prolonging graft survival. Brequinar initiated at the time of cardiac transplantation in low (3 mg/kg) or high (12 mg/kg/3 × week) doses reduced the IgM, but not IgG, antibody response to control levels. Brequinar doses (12 mg/kg/3 × week) initiated before the skin sensitization and continued thereafter prevented both IgM and IgG production.

2. Prophylaxis of Xenorejection

Brequinar inhibited IgM antibody-mediated rejection of hamster-to-rat heart xenografts (129). Four LEW recipients of hamster hearts that were given BQR 3 mg/kg daily survived more than 90 days. Brequinar blunted the IgM response: IgM levels increased from 218.3 ± 88.4 to 1631 ± 359.8 mg/L ($n = 6$, $p < 0.01$) in untreated animals, but showed no change in BQR-treated hosts. Brequinar treatment also abrogated the binding of rat antihamster IgM antibody to vascular endothelium.

Experimental animals showed toxic effects of BQR primarily in tissues with rapid cell turnover, including bone marrow, gastrointestinal tract, and lymphoid system (126, 130). Administration of daily oral BQR doses (5 mg/kg) to Sprague-Dawley rats for 1 month caused anemia, leukopenia, bone marrow hypocellularity, and gastrointestinal toxicity caused by inhibited epithelial growth and intestinal mucosal atrophy. Two of 32 rats died of toxicity. However, the combination of BQR with CsA (10 mg/kg) led to death in 22 of 32 rats (131).

Brequinar appears to be a promising immunosuppressive drug, based on the experimental studies that demonstrate that it prolongs the survival of allografts and xenografts, prevents accelerated allograft rejection, and inhibits both B-cell–mediated and T-cell–mediated immunity. Brequinar displays strong synergistic interactions with CsA and SRL, which suggests that it would be an effective immunosuppressant in combination therapy regimens.

D. Clinical Trials: Phase I

In an initial phase I study of 45 cancer patients, BQR (36–300 mg/m^2/day) was administered as a single daily intravenous infusion for 5 days. The toxicities included dermatitis, mucositis, gastrointestinal toxicity (nausea, vomiting, anorexia, diarrhea), phlebitis, reversible transaminase elevations, and thrombocytopenia (123). Another study in 43 cancer patients who received a total of 110 courses of BQR by short-term intravenous infusion every 3 weeks at escalating doses (ranging from 15 to 2250 mg/m^2) showed that, at doses of up to 300 mg/m^2, the only toxicity was mild leukopenia and thrombocytopenia, whereas at doses above 1200 mg/m^2 various dose-dependent toxicities, such as severe thrombocytopenia, leukopenia, mucositis, nausea, vomiting, and skin rash, were evident (124).

Table 9 Brequinar in Experimental Transplantation

Species (Organ)		Dose (mg/kg)	Duration	n	Mean Survival Time (d)	p-Value	Investigator
Rat (heart)	ACI→LEW	Control		11	7		Kramer et al. 1992
		6	−1–30	6	15	0.002	
		12	−1–30	8	45.5	0.002	
		24	−1–30	7	42	0.001	
Rat (liver)	ACI→LEW	Control		8	10		
		6	−1–30	8	13.5	0.36	
		12	−1–30	26	91.5	0.008	
		24	−1–30	8	9	0.49	
Rat (kidney)		Control		9	6		
		12	−1–30	6	>99	0.003	
		24	−1–30	9	13.0	0.006	
Rat (heart)	ACI→LEW[a]	Control		14	2.5		Yasunga et al. 1993
		3	−8–0	6	7	<0.001	
		3	0–30	12	12.5	<0.001	
		12	−10–30	7	40	<0.001	
		12	0–30	7	28	0.004	
Monkey (heart)		Control		4	8		Makowka et al. 1993
		4	Until rejection	5	20	<0.04	

[a]Presensitized LEW recipients

In a recent phase I study in 67 cancer patients, BQR was administered as a 10-minute intravenous infusion for 5 consecutive days. Dose-limiting myelosuppression with severe thrombocytopenia developed, the occurrence of which strongly correlated with the AUC value. The maximal tolerable doses were 210 mg/m^2 in poor risk patients, and 350 mg/m^2 in good risk patients. A phase II study conducted by Maroun et al. (132), in which BQR (1800 mg/m^2 tapering to 1200 mg/m^2) was administered to advanced lung cancer patients, confirmed the previously reported toxicity profile but failed to document significant benefit.

The findings of phase I trials of BQR have not yet been completely published. A large study in renal transplant recipients is presently in preparation for publication. A preliminary report by Sher et al. (133) described the use of BQR in 18 adult volunteer orthotopic liver allograft recipients. The initial single dose trial enrolled three to six patients at each of five oral dose groups (0.5–4 mg/kg). In a multiple-dose protocol (one intravenous dose followed by 15 alternate-day oral doses), three patients were enrolled in each of five dose groups (0.5, 1.0, 2.0, 3.0, and 4.0 mg/kg). This phase I study found that BQR doses between 0.5 and 4.0 mg/kg were tolerated with instances of mild diarrhea and infection. Brequinar did not affect the pharmacokinetics of CsA. The mean oral clearance of BQR was 16 ± 6 mL/min, and its mean terminal half-life was 15 ± 7 hours.

The clinical trials of BQR treatment for cancer, psoriasis, and rheumatoid arthritis document a side effect profile of myelosuppression, nausea, vomiting, diarrhea, mucositis, and skin rash. The major dose-limiting toxicities are mucositis and thrombocytopenia (123,124,134). Although an initial phase II study of BQR in renal transplant recipients failed to document a significant therapeutic effect, the study design was flawed. The utility of this powerful antiproliferative drug in organ transplantation needs to be reevaluated, because of the synergistic interaction of the agent with CsA and SRL, which may mitigate the intrinsic low therapeutic index of BQR.

VI. COMBINATION TREATMENT

A. Qualitative Analysis Using Limited Drug Combinations

1. Mycophenolate Mofetil Combination Therapies

The combination of MM (10 mg/kg p. o. from day 1 through day 50) with CsA (0.75 mg/kg i. p.) prolonged the survival of BN hearts in LEW recipients. Hosts receiving combination therapy showed a significantly longer survival time (median = 55 days; $p < 0.01$) than rats given only MM (10 mg/kg; median = 10 days) or CsA (median = 11 days) (25). Because of the limited dose–response data, it is not possible to evaluate the nature of the drug interaction. Another study in which a limited number of dosing ratios was used suggested that the combination of MM and CsA was more effective than that of MM and SRL. LEW recipients of BN heterotopic heart grafts treated with the combination of MM (10 mg/kg p. o.) and CsA (0.75 mg/kg i. p.) for 50 days displayed a mean graft survival time of 67 days compared with the 28-day survival achieved with MM (10 mg/kg p. o.) and SRL (1.5 mg/kg p. o.) (57). The combination of MM (20 mg/kg) with CsA (5 mg/kg) and MP (0.1 mg/kg) significantly increased canine renal allograft survival to 122.4 ± 38.75 days ($p < 0.002$) (7). The dual-drug combination of CsA and MP failed to prolong graft survival beyond that of the control group (8.1 ± 1.2 days; $n = 5$), and MM (40 mg/kg) alone increased graft survival to 36.0 ± 9.6 days ($p < 0.05$).

Mycophenolate mofetil appeared to potentiate the effects of BQR. In the rat (ACI-to-LEW) heterotopic heart allograft model, groups of hosts that received no treatment, MM (20 mg/kg/day × 3 weeks), BQR (3 mg/kg/day × 3 weeks), or BQR (6 mg/kg/day × 3

weeks) showed mean graft survival rates of 6.5, 11.5, 9.5 or 14.5 days, respectively. Hosts given combination therapy with MM and BQR (6 mg/kg) or MM and BQR (3 mg/kg) showed graft survivals of 133 or 121 days, respectively. In a model of acute rejection reversal therapy, MM (60 mg/kg/day) alone extended survival beyond 21 days in 4 of 5 LEW recipients of ACI hearts that had not received immunosuppressive drugs until 5 days after transplantation. Brequinar (12 mg/kg/day) reversed the rejection process in all five animals. The combination of BQR (6 mg/kg/day) and MM (40 mg/kg/day) produced graft survivals beyond 21 days in 4 of 5 hosts (135). In aggregate, these experiments do not allow one to conclude that the interaction is more than additive. Furthermore, there are no clinical data to suggest that MM permits reduction in the doses of CsA necessary to establish baseline prophylaxis of rejection.

2. 15-Deoxyspergualin Combination Therapies

The combination of 15-DS with CsA prolongs the survival of heart, kidney and islet allografts in experimental animals. For example, in canine islet transplantation, the addition of 15-DS (0.5 mg/kg/day) to a triple-drug regimen of CsA (20 mg/kg/day), ALG (2.5 mg/kg/day × 14 days), and Aza (2.5 mg/kg/day) prolonged the mean survival time from 10.8 ($n = 11$) to 32.4 days ($n = 10$; $p = 0.012$) (136); this interaction appeared to be additive. In Chacma baboons, treatment of cardiac transplant recipients with 15-DS alone (4 mg/kg for 9 days) prolonged survival from 11.0 to 28.2 days ($p < 0.05$), whereas CsA (10–40 mg/kg p. o. for 30 days) prolonged survival to 32.4 days ($n = 5$; $p < 0.05$). The two-drug combination produced a mean graft survival of 43.1 days ($n = 7$; $p < 0.025$ versus control). Cyclosporine, but not 15-DS alone, prolonged renal transplant survival to 30.4 days ($n = 5$). The 15-DS/CsA combination produced a mean graft survival of 148.9 days ($n = 8$; $p < 0.025$), with half of the hosts appearing to be immunologically tolerant at 340, 256, 244, and 164 days after treatment discontinuation (137). Both these experimental and clinical data suggest an additive interaction between 15-DS and other agents for prophylaxis of rejection (109) or reversal of refractory renal (112,113,116) or liver (114) allograft rejection.

3. Sirolimus Combination Therapies

In contrast, SRL and CsA show a synergistic interaction. Initial limited dose–response studies showed that SRL potentiated CsA immunosuppression. Ochiai et al. (138) found that SRL monotherapy (0.3 to 5 mg/kg for 11 days) produced dose-dependent prolongation of F344 cardiac allografts in WKA recipients. An 11-day course of SRL (0.1 mg/kg) combined with CsA (3.2 mg/kg) at individually ineffective drug doses prolonged graft survival beyond 100 days. A similar effect was observed in canine kidney transplantation. Subtherapeutic doses of SRL (0.3 mg/kg) and CsA (2.5 mg/kg) prolonged survival from 15 to 70 days and reduced the incidence of SRL-induced vasculitis and emaciation.

In contrast to CsA, TRL acts antagonistically with SRL. Addition of SRL reduced the inhibitory effect of TRL on the proliferation of C57B1/6 mice T cells stimulated with ionomycin and PMA. Sirolimus (1.1 μM) completely abrogated the inhibitory effect of TRL (10 nM) (139). The drugs interact adversely, most likely because they both compete to bind to FKBP12, a peptidyl propyl cis-trans isomerase to form a complex that inhibits calcineurin (140,141). The SRL and FKBP12 complex inhibits a variety of kinase activities (43,57).

4. Brequinar Combination Therapies

Combinations of BQR with CsA show prolonged allograft survival in animal transplant models. Oral treatment with BQR (6 mg/kg every other day [QOD]) in combination with

CsA (1 mg/kg for 30 days QOD) produced significant prolongation of ACI liver allograft survival in LEW recipients from approximately 10 to 220.6 ± 110.3 days (126). Kawamura et al. (135) combined BQR (3 or 6 mg/kg QOD) with MM (20 mg/kg) to prolong rat heterotopic heart allograft survival from 6.5 to 121 or 133 days, respectively. When the combination of MM (40 mg/kg/day p. o.) and BQR (6 mg/kg p. o. QOD) was initiated 5 days after transplantation, ACI heterotopic heart allograft survival was prolonged to more than 21 days in 4 of 5 LEW recipients (135). The combination of BQR (6 mg/kg p. o. QOD) with MM (20 mg/kg/day), but not BQR alone, inhibited intimal proliferation in LEW rat recipients of ACI or LEW infrarenal aortic grafts, in contrast to the marked intimal proliferation in untreated syngeneic grafts (142). Makowka et al. (127) found that administration of BQR (2 mg/kg QOD) in combination with CsA (2 mg/kg) prolonged the survival of heterotopic cardiac allografts in nonhuman primates from 8.0 ± 0.5 ($n = 4$) to 43 ($n = 2$) days. None of the recipients treated with CsA alone or the CsA/BQR combination exhibited toxic side effects (127). These results suggest that the addition of low doses of BQR to CsA therapy slows the progression of rejection.

B. Median Effect Analysis

1. Theory of the Analysis

The median effect equation (143), which is derived from the mass action law, relates drug dose to biological effect as follows:

$$\left(\frac{fa}{fu}\right) = \left(\frac{D}{Dm}\right)^m$$

where fa is the fraction affected (% inhibition); fu is the uninhibited fraction ($1 - fa$); D is the drug dose used in the reaction; Dm is the dose required for 50% inhibition (the median effect); and m is the slope (Hill) coefficient.

The power of the analysis stems from its logarithmic form, which allows the determination of the dose of each drug that is necessary to achieve a given effect:

$$\log\left(\frac{fa}{fu}\right) = m \log (D) - m \log (Dm)$$

An analysis of the log-log plot of log (fa/fu) versus log D allows the assessment of the correlation coefficient (r) between the observed data and the theoretical model. If the r value is greater than 0.75, the analysis can be applied to predict the drug dose (Dx) necessary to achieve an arbitrary effect level (fx). Thus, the amount of drug A necessary by itself to produce effect fx can be compared with that amount required when drug A is combined with a second agent B.

The combination index (CI) assess the interaction between immunosuppressive drugs by comparing the amounts of drug A necessary to produce an effect (x) when used alone versus in combination with a second agent B. The combination index to produce x% inhibition is defined as follows:

$$CIx = \frac{[A] \text{ combined}}{[A] \text{ alone}} + \frac{[B] \text{ combined}}{[B] \text{ alone}}$$

Combination index values are considered additive if values equal 1.0, synergistic if they equal less than 1.0, and antagonistic if they are greater than 1.0 (144). All agents tested to date display a high degree of correlation between the observed effect and the response predicted by the median effect equation.

2. Application of the Analysis

The median effect principle was applied to define the interactions between CsA and other agents. The initial study showed slight synergism with steroids, additive interactions with Aza or mizorbine, and antagonistic interactions with enisoprost (145). Cyclosporine–SRL drug synergy was documented by median effect analysis of in vitro and in vivo experiments. The combination showed synergistic inhibition of human PBL proliferation triggered by PHA, anti-CD3, or MLR at all doses in vitro (146).

Stepkowski et al. (147) and Tu et al. (74) applied the median effect analysis to document a synergistic interaction between CsA and SRL in rats and mice, respectively. BUF heart allograft survival in WF recipients was prolonged by treatment with a combination of CsA (0.5 mg/kg/day) and SRL (0.01 mg/kg/day) for 14 days, from 6.5 ± 0.5 to 35.4 ± 23.7 days (144). The combination of individually subtherapeutic doses of CsA (2.5 mg/kg) and SRL (0.05 mg/kg for 7 days) prolonged the survival of C57BL/10 hearts in C3H recipients from 7.7 ± 1.4 to 18.2 ± 2.9 days (CI = 0.14). Increased doses of CsA (5 mg/kg) and SRL (0.2 mg/kg) further prolonged survival to 23.0 ± 9.0 days ($p < 0.01$; CI = 0.01) (74). The clinical use of the combination of SRL and CsA greatly reduces the incidence of acute allograft rejection, and even small doses of SRL (3 mg/m^2) reduce CsA requirements by at least one-third.

Cyclosporine and BQR also display a synergistic interaction both in vivo and in vitro (125). When the CI equation was applied to analyze the inhibition of in vitro stimulation of human PBL with PHA, CI values ranged from 0.01 to 0.95 and, for MLR, CI_{95} from 0.003 to 0.06. Sirolimus and BQR also showed a synergistic interaction, with CI_{95} values ranging from 0.02 to 0.08. The greatest degree of inhibition was observed when BQR was added to the CsA/SRL combination, namely $CI_{95} < 0.0001$. Brequinar acted antagonistically with the purine nucleoside synthesis inhibitors Aza, mizoribine, and MPA. In vivo addition of BQR to the CsA/SRL regimen prolonged the survival of BUF hearts in WF recipients from 20.8 ± 2.7 (using CsA/SRL) to 56.6 ± 5.2 days, CI = 0.06 (with BQR 2 mg/kg QOD) (125). Brequinar also acted synergistically with CsA and/or SRL in the mouse heart transplant model. Addition of BQR (0.125 mg/kg) to CsA (0.25 mg/kg) and SRL (0.01 mg/kg) prolonged graft survival to 56.6 ± 22.1 days (CI = 0.01), whereas the CsA/SRL combination or BQR alone at the same doses had little effect (MST = 8.2 ± 1.5 days) (148).

VII. FUTURE PROSPECTS

Immunosuppressive therapy has progressed from the use of cytotoxic agents that produced a high morbidity to a strategy that alters specific host immunity to spare nonspecific resistance. New immunosuppressive modalities currently under investigation include drugs or monoclonal antibodies that target selective cell subsets, activation pathways, or cytokine mediators of effector function, membrane ligands that prevent intercellular interactions, or antimetabolites that preferentially affect lymphocytes.

Among the new agents, BQR is a potent antiproliferative agent that acts synergistically with CsA, but displays a low therapeutic index. 15-Deoxyspergualin may be effective for prophylaxis or treatment of acute rejection episodes as well as for blocking the synthesis of performed antibodies, particularly toward xenografts, but its administration is limited to brief treatment courses because of its requirement for the parenteral route and its low therapeutic index.

Currently the most effective immunosuppressive regimens include CsA as the primary agent. However, the wide array of toxic side effects of this drug led to a search for

immunosuppressive agents that could be used safely in combination with CsA, thereby allowing significant CsA dose reduction without an increased toxicity profile. The quantitative median effect analysis (149) documented that SRL displays strong synergistic interactions with CsA to reduce the incidence of acute rejection episodes. In animal models, both MM and SRL seem to reduce arterial intimal thickening produced by either immunological or mechanical stimuli, and they may have a beneficial effect for treating chronic allograft rejection. With the application of computerized drug design within the next decade, one can anticipate the development of a panoply of agents that display highly selective binding properties for intracellular targets critical for the expression of alloimmunity.

ACKNOWLEDGMENTS

This work was supported by a grant (NIDDK 38016-09) from the National Institute of Diabetes and Kidney Diseases.

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19

Immune Monitoring for Transplant Recipients

JAMES F. BURDICK
The Johns Hopkins University School of Medicine, Baltimore, Maryland
NOZUMI SHINOZUKA
Saitama Medical School, Saitama, Japan

I. INTRODUCTION

Historically, both principles of and pharmacological agents for organ transplantation immunosuppression were derived empirically, often from cancer therapy principles. In the 1970s, the only indications that the therapy was inadequate or too potent were the retrospective insights provided by the onset of rejection or infection. The desirability of a better way to individualize the prescription was obvious, but only when cyclosporine (CYA) became available was it possible to begin to determine the effect on the individual patient and adjust dose by the level of drug in the blood rather than by using a standard dose for every patient. Antilymphocyte antibodies and tacrolimus (FK) can also be monitored by following blood levels.

As an alternative to looking at the specific drug level, attempts have been made to generate nonspecific and donor-specific tests to provide better information about the patient's immunological status. Particular tests have included skin testing, certain in vitro tests of T-cell function, and flow cytometric analysis of circulating T cells. Monitoring in this way has not come into general use, both because the tests are cumbersome and because the data do not generally confirm robust clinical correlations. Another reason that individualization of dose by immune monitoring has not generally been done may be the remark-

able improvement that CYA produced in kidney graft survival by merely monitoring trough levels and adjusting dosage accordingly without use of more complex analyses.

In recent years, however, it has become apparent that chemical blood level analysis is imperfect. On occasion, infection or rejection may develop in patients with blood levels of CYA that are generally in the acceptable therapeutic range. It makes sense that the innate strength of a system as complex as that causing rejection is different in different patients, so that a given chemical blood level of immunosuppressant may produce different results in different patients. Analysis of the patient's nonspecific immunocompetence by assessment of mixed lymphocyte culture (MLC) stimulation or by measuring activity of immunophilins that provide the basis for action of CYA and FK may provide important information for individualization of immunosuppression.

II. SKIN TESTING

As transplantation was becoming generally practiced in the early 1970s, issues of immunocompetence were of great interest in the parallel field of oncology. Rejection of tumors was strong when they were allogeneic, but it gradually became clear also that certain experimental tumors were rejected on the basis of tumor-specific antigens rather than alloantigens. This led to the hypothesis that immunoincompetence could lead to, or potentiate growth of, cancers. Studies in which the ability to respond with contact sensitivity to a primary or recall challenge of dinitrochlorobenzene were used to index immune responsiveness (1,2), and these plus antigen-specific tests were used to assess the patient's prognosis (3). This nonspecific assessment of immunocompetence was extended to use for organ transplantation (4) and was used as an adjunct to guide therapy in some programs. The results were promising but not definitive, and eventually appeared not to warrant the relatively intensive resources required.

III. T-CELL SUBSET ANALYSIS

T-cell subset analysis has been reviewed previously (5) and has not changed substantially. When the dominant types of T cells were defined by their surface antigens using monoclonal antibodies, it seemed that the ratio of $CD4^+$ to $CD8^+$ cells, measured by flow cytometry in the peripheral blood, might be informative in evaluating the status of the transplant patient. Interpretation was complicated, however, by the association of CD8 with both cytotoxic T cells and suppressor T cells. A dominance of the former might be thought to be dangerous for the graft, whereas if an increased $CD8^+$ count represented mainly suppressor cells, the result would be protective. The suppressor idea initially seemed to be borne out by reports that low "helper to suppressor cell ratios" indicated less chance of rejection (6,7), but this finding did not prove durable, and early detection of rejection was similarly not reliable (reviewed in Ref. 5). A very low $CD4^+$ count is meaningful; in patients with preoperative total lymphoid irradiation, who were subsequently protected from rejection on relatively low-level immunosuppression, the $CD4^+$ levels stayed low for more than 2 years (8). A low $CD4^+$ cell level also correlates with progression of human immunodeficiency virus (HIV) infection, and before accurate testing for HIV became available, a very low $CD4^+$ count was the best way to diagnose progression of acquired immunodeficiency syndrome (AIDS).

One technical problem in studies of T-cell phenotype in transplant patients was the finding of large uncharacterized mononuclear cells irregularly present in some patients in

the first few weeks after transplantation (9). These interfered with flow cytometry studies, but they also should be considered in current cytokine assays, because these cells presumably could have direct and indirect effects on T-cell function analyses.

T-cell subsets have also been evaluated extensively, both in biopsies of the kidney allograft (10) and in fine-needle aspirates (11). There is not a general agreement regarding the relative patterns of lymphocyte types, although, as rejection intensifies, $CD8^+$ cells may become more dominant before there is a general increase in other varieties of leukocytes. Renal biopsies are not the ideal method for frequent routine monitoring. On the other hand, fine-needle aspiration can be done routinely, and analysis of T-cell subsets in these specimens can be part of such routine monitoring.

Additional information that might render analysis of either peripheral blood or graft infiltrate more useful is assessment of the degree of activation of the different subsets of lymphocytes. Two- or three-parameter analyses are possible with flow cytometric or immunohistological techniques, enabling detection of DR^+ or $CD69^+$ (12) cells. One study exemplifying analysis of activation status of cells in peripheral blood reported that an increase of DR^+ cells in the peripheral blood correlates with rejection (13). However, these studies are demanding for routine clinical use, and activation status in transplant recipients has not been explored extensively.

IV. DONOR-SPECIFIC IN VITRO RESPONSES

The major pathway for donor-specific T cell–mediated rejection, the in vitro analogy for which is mixed lymphocyte culture (MLC) stimulation leading to lymphocyte-mediated cytotoxicity (LMC), was described more than 20 years ago (14), and work since has, in essence, been directed at defining the details of the pathway. However, it has been frustrating that very little practical clinical utility has developed from this early understanding. In fact, several careful studies have documented that capacity to develop donor-specific reactivity in vitro by peripheral blood lymphocytes from transplant recipients can be linked with increased risk of rejection (15–21) However, other reports do not confirm the clinical relevance of these measures (22,23). A major exception is in patients given total lymphoid irradiation, who may tolerate their grafts long term without immunosuppression; such patients generally have absence of donor-specific reactivity in vitro (24–26).

One rigorous approach to quantitation of the immunological status in the patient is measurement of the frequency of precursor T cells capable of reaction with donor antigens using limiting-dilution analysis. This utilizes the same principle used for measurement of incidence of B cells secreting specific antibody and is applied to the measurement of the incidence of responsive T cells. However the B-cell frequency distinguishes positive responders from negative responders with an "all-or-none" outcome that is not true for T-cell responses. T-cell responses are usually assessed in bulk, for which a baseline of activity (typically chromium release or interleukin (IL)-2-generation) (27) usually exists, so that an arbitrary threshold must be defined to distinguish positive from negative wells. Also, the statistical analyses of the results to establish whether there is a sufficiently linear response and the statistical accuracy for the value for the precursor frequency require rigor that is not always observed (28). On the other hand, in practice, it has been possible to obtain clear reproducible data on this measure in transplant recipients. A decrease in frequency of specific precursors is often found to develop after transplantation, but in a careful longitudinal study of 33 patients after kidney transplantation, there was no correlation with a change in this activity and occurrence of rejection (29).

Reinsmoen et al. have evaluated mixed lymphocyte culture reactions against homozygous typing cells that express donor or third party DRw specificities (24,30,31). A double normalization technique was used to express results, allowing comparison among different responder cells and different experiments. They found that the living donor kidney transplant recipients that were hyporeactive, as they defined it in these studies, against donor-specific homozygous typing cells had a significantly lower average creatinine than those in the reactive group (31). The authors reviewed the relationship of long-term donor chimerism to graft survival to their assay for donor-specific hyporesponsiveness and found a relationship for lung recipients but not for kidney or liver recipients (32).

Although conventional T-cell–mediated cytotoxicity has been the usual focus of monitoring efforts, it may be that antibody-dependent cell-mediated cytotoxicity (ADCC) is an additional mechanism of rejection. The general issue of antibody in rejection is discussed in Chap. 6. However, in one recent report, analysis of ADCC was shown to correlate with clinical status after transplant (33).

There are multiple likely explanations for the absence to date of a routine clinical test for donor-specific reactivity in the transplant recipient. The assays are time consuming and may not be able to provide prediction of rejection sufficiently in advance to be useful (34). Preservation of donor-strain cells for stimulators and targets, or the use of homozygous typing cells (24), involves numerous logistical problems. Finally, there is an unresolved debate as to the degree to which donor-specific events in the peripheral blood mirror those in the graft (see Chap. 2).

V. CYTOKINE ANALYSIS

There has been a good deal of work characterizing biopsies for their expression of certain types of cytokines. It has recently become possible to measure some cytokines that are likely to be related to rejection by enzyme-linked immunosorbent assay, allowing assessment in blood, urine, or culture supernatants. How cytokine measurements relate to rejection correlation or prediction is controversial (35,36). Analysis by polymerase chain reaction (PCR) of cytokine gene expression in peripheral blood monocytes did not produce useful correlations with rejection (37).

On the other hand, the capacity for monocytes to be stimulated to release IL-2 and interferon-γ was found to be correlated with the development of acute cellular rejection after kidney transplantation (38). Elevated tumor necrosis factor (TNF)-α occurs in heart transplant recipients (39) and several cytokines were found to be elevated in coronary sinus compared with peripheral blood after heart transplantation (40).

Urinary cytokine mRNA measurements revealed TNF-α and IL-6 elevations during rejection of kidney transplants, and decreased IL-10 and increased IL-5 as rejection resolved (41). In another report, a very large number of samples and variety of cytokines were assessed in kidney transplant recipients, and it was possible to identify values for threshold serum levels indicating increased risk for rejection (42).

In general, increased serum levels of IL-6 and interferon-γ seem most indicative of increased immunological reactivity (rejection or infection), and decreased levels seem most correlative of successful rejection treatment (42–45). It has been shown that the interferon-γ receptor chain that confers responsiveness to that cytokine is downregulated when the level of interferon-γ increases, complicating the inferred effect of an increased circulating level of interferon-γ in a patient (46). Overall, for many reasons related to the complexity

of immune responses, direct measurements of the cytokine proteins, even though possible, have not become generally applicable in kidney or other transplant recipients (47,48).

There has been interest in the measurement of soluble IL-2 receptor (IL-2R), which is elevated in patients on dialysis (49–52). Although some cytokines have relatively short half-lives in serum, the larger size and therefore slower clearance of receptors such as IL-2R makes their measurement less promising for a short-range correlate of rejection (36,51,52). If followed up serially in the urine (in patients with adequate kidney function), the level of soluble IL-2R correlates with rejection (50), although it seems that this test does not provide major incremental improvement in clinical management over use of routine clinical criteria (53).

Another circulating class of molecules that could possibly be related to immune status of transplant recipients is the soluble human leukocyte antigen (HLA) molecules. These are elevated in transplant recipients, in amounts correlated with specificity, and may be related to rejection episodes (54). It has been noted that these circulating molecules may have immunosuppressive capacity (49,54), which complicates the interpretation of the finding of an increased level in a patient. The cytokine human interleukin for DA cells/leukemia inhibitory factor (HILDA/LIF), studied in urine and blood of kidney transplant recipients, was found to be increased in infection and rejection (55,56). Endothelin, another mediator, was present in increased levels in the plasma when rejection involved vascular damage (57). As with other larger molecules such as IL-2R that are cleared by the kidneys, endothelin clearance is decreased, and levels are increased during any episode of impaired renal function.

A recent development with considerable potential is the use of labeled monoclonal antibodies and cellular saponification to detect intracellular cytokine by flow cytometry (49). Using this technique following appropriate cell separation, it was shown that it was possible to distinguish the helper cell subtypes in peripheral blood (58).

A major issue in general with measurement of immunologically significant proteins is the extent to which elevated levels reflect nonspecific rather than immunologically relevant processes. In the blood, there is the question of to what degree the level measured is merely a reflection of generally decreased clearance. For urine measurements, increased renal inflammation and/or proteinuria may cause an increase in measured levels.

Another major issue in the measurement of circulating cytokines is that the mechanism of interest may generally be paracrine or even autocrine, and detection of systemic changes may not reflect these local events in the region of the secreting cell. Clinical correlations have been observed that would at least support further investigation in these areas. Regardless of the immediate clinical utility of these measurements, they promise to illuminate mechanisms relevant to rejection in a way that should help direct development of therapeutic agents or strategies for the future (46,56,57,59).

VI. NONSPECIFIC IMMUNOCOMPETENCE

Blastogenesis phytohemagglutinin (PHA) responsiveness, and other in vitro responsiveness measures have been tested for generalized immunocompetence quantification, although they have not become generally used (5). "Spontaneous" blastogenesis (which could result from a response induced to the fetal calf serum in the culture media in these experiments) was found to increase significantly on the average during rejection (51). It seems clear that patients vary in their degree of underlying immunocompetence, which should affect the result when standard doses of immunosuppressive agents are used.

Trauma has long been known to be immunosuppressive (60,61), as is anesthesia (62). Experimentally, the vascularized murine heart graft operation results in nonspecific decreased IL-2 production in MLC for days postoperatively (63). Spontaneous proliferation of peripheral blood mononuclear cells was reported to be associated with rejection episodes or low tacrolimus (FK) levels in liver transplant recipients (64). An important principle is the distinction between day-to-day monitoring to predict rejection 1 to 2 days earlier versus use for long-term characterization of general status to characterize the overall likelihood of stability, or of rejection or infection.

Nonspecific general capacity for responsiveness in MLC can be used to assess the immunocompetence of transplant recipients on cyclosporine (CYA) (65). Higher doses of CYA or FK are required to inhibit interleukin-2 generation in the MLC response when allogeneic antigen presenting cells are present (66) than when stimulators are depleted of the antigen presenting cells. This principle was used with third party, not organ donor–derived, stimulator cells to determine the general level of unresponsiveness in a series of kidney transplant recipients receiving cyclosporine. In that report, patients whose immunocompetence was inadequately suppressed by this test were more likely to have rejection, whereas the incidence of infection was greater when depression of immunocompetence was more profound (67,68). Longitudinal analysis revealed that there was considerable tendency for the level to fluctuate, but when the patients were categorized by average of their results, those with above the middle range had a significantly increased tendency for rejection to develop (69). Furthermore, when patients previously found to have relatively strong immunocompetence were followed up, there was an increased tendency toward increased graft loss. The generation of a significantly increased "spontaneous" blastogenesis in rejection (51) may reflect increased activity of the indirect pathway of antigen presentation (fetal calf serum) and therefore be analogous to capacity to respond to depleted stimulating cells.

We have investigated whether a simplification of this measure of immunocompetence could be used to characterize the degree of immunosuppression in transplant recipients by measuring proliferation rather than generation of interleukin-2. Stimulator cells were prepared from lymphocytes from a Ficoll-Hypaque gradient and saved either not depleted (ND) or depleted (D) of antigen presenting cells. At the time of preparation of the stimulator cells, unirradiated D and ND aliquots were used as responder cells, using as stimulators two different D and ND populations. As shown in Fig. 1, the ND population was able to respond to either D or ND stimulator cells, whereas the D population showed no response when tested against the other D stimulator populations.

We have confirmed that such patients had the predicted tendency toward depressed immunocompetence, unlike the normal responses found in normal individuals or in allograft recipients that were not receiving CYA or FK. Normal individuals or patients with functioning grafts from 3 months to several years after liver or kidney transplantation were studied. These were patients who were thought to be generally on optimized cyclosporine or tacrolimus doses, based on clinical criteria and conventional levels. A few long-term kidney transplant recipients were on only prednisone and azathioprine. For the period from 1 week before to 6 weeks after drawing the blood for the most recent immunocompetence assay, clinical status was determined as either stable or as infection or rejection having occurred. No patient had concurrent infection and rejection during this interval.

Eighteen normal individuals were studied, and all had normal responses in this assay, with the pattern shown in Fig. 2A. Although some patients immunosuppressed with CYA or FK were also normal, patients given one of these drugs were commonly subnormal in immunocompetence, with the pattern as shown in Fig. 2B, or they were so immunoincompetent that they produced no response to either stimulus. As shown in Table 1, patients

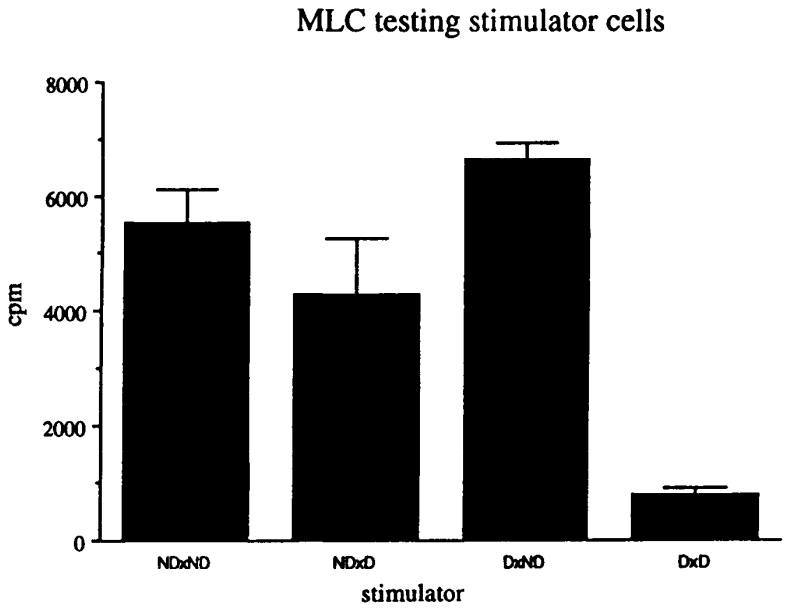

Figure 1 The MLC results with normal responders and stimulators not depleted (ND) or depleted (D) of antigen presenting cells. *Abbreviations:* Cpm = counts per minute; error bars = SEM.

receiving FK adjusted by conventional clinical parameters were generally more immunosuppressed than those on CYA; the fraction of patients on CYA with marked depression of immunocompetence (less than +/–) was 0.0213, significantly less than the 0.318 of the patients on FK with immunocompetence less than 0.318 (95% confidence limits for difference of proportions = 0.496 to 0.0979).

The overall relationship between degree of immunoincompetence as measured by this assay and clinical events is shown in Table 2. A large fraction of the patients fall in the midranges, with relatively few patients being either +/+ or –/–. There is a trend toward the previously reported correlations of immunocompetence with outcome, because the incidence of rejection was higher in +/+ patients compared with the rest (18% versus 13%) and the incidence of infection was higher in the –/– patients than in the rest (100% versus 18%). Comparison of the 100% occurrence of infection in the two –/– patients versus the results with less suppressed patients was significant (difference between proportions = 0.817, 99% confidence interval 0.688 to 0.946).

The pattern of responses shown in Tables 1 and 2 is similar to that described by Shearer and colleagues (67,68). However, this assay, using assessment of proliferation, is simpler than measuring total interleuking-2 produced as described previously, because it avoids the necessity for the antiinterleukin-2 antibody required in that assay, and proliferation is easier to measure than interleukin-2 production. The patterns were as predicted from the reports looking at interleukin-2 production instead of proliferation, with for example, no –/+ outcomes, and the results demonstrated a significant relationship to infection and a trend toward correlation with rejection status among the patients on cyclosporine or tacrolimus. This suggests that this simplified assay could be used in practice to supplement other information used in prescribing these immunosuppressive agents.

If allogeneic antigen presenting cells carried by the graft eventually succumb, then graft-specific stimulation by direct antigen presentation, the potential for which is measured by stimulation by undepleted stimulators in this assay, can no longer occur. Thus, the

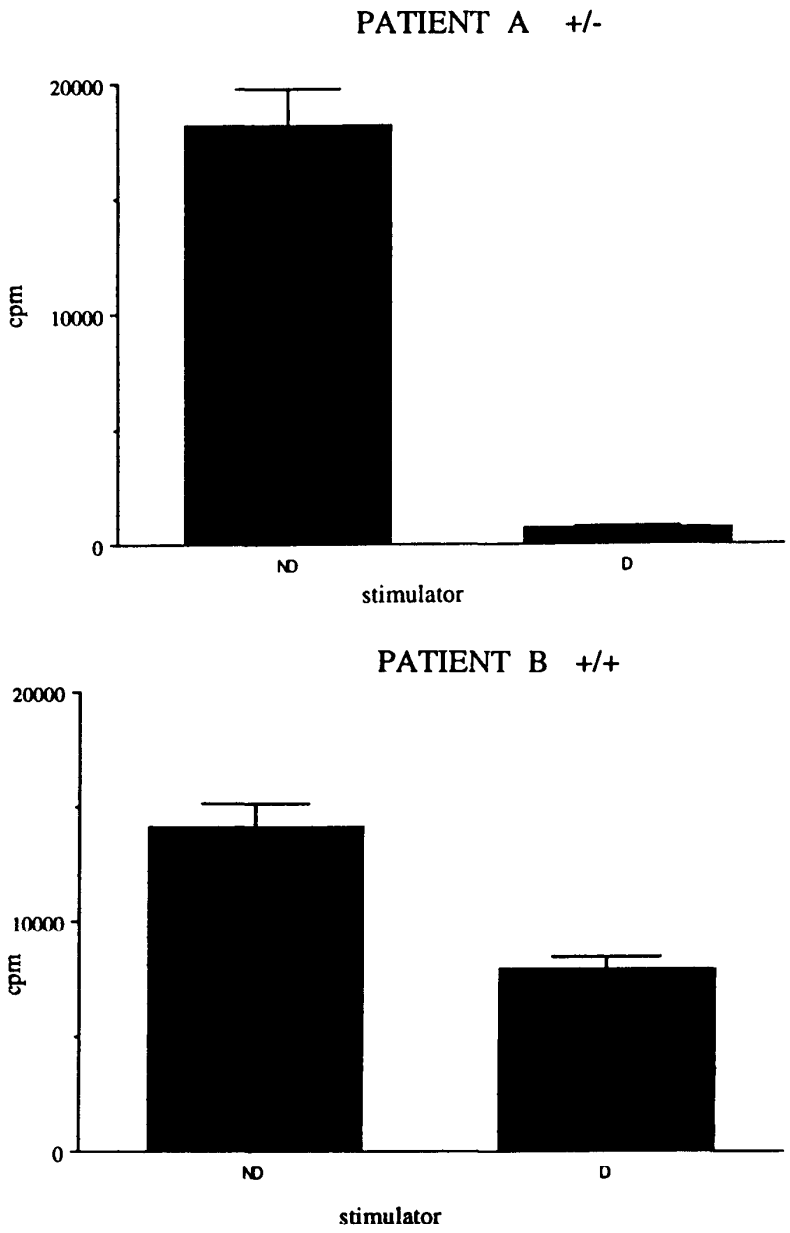

Figure 2 The MLC results for two patients, A and B, using responders and stimulators not depleted (ND) or depleted (D) of antigen presenting cells. *Abbreviations:* Cpm = counts per minute; error bars = SEM.

intermediate +/– degree of immunoincompetence, indicating responsiveness only to stimulators containing allogeneic antigen presenting cells, would theoretically be adequate to preclude rejection while retaining some immunocompetence against infection.

One example of application of this assay is the finding that patients given ketoconazole to decrease the dose and expense of cyclosporine have, on the average, more suppressed immunocompetence for conventional trough levels than patients treated conventionally (70). This presumably is due to a higher area under the curve resulting from altered pharmacodynamics, but this needs further analysis. It may be that analysis of the cytochrome P450 system activity in difficult patients could provide help with dose adjust-

Table 1 Distribution of Immunocompetence by Immunosuppressive Regimen[a]

	Number of Patients					
	+/+	+/±	+/–	±/–	–/–	Total
CYA-based	22[a]	3	21	1		47
FK-based	4	5	6	4[b]	3[b]	22
AZA-based	3	1		1		5
Total	29	9	27	6	3	74

[a]Patients were identified as having responsiveness either to both ND and D stimulators (+/+), to ND but not D (+/–), or to neither stimulator (–/–).
[b]$p < 0.95$ compared with ±/– and –/– patients on CYA.

Table 2 Relationship of Immunocompetence to Clinical Status[a]

	Number of Patients			
	Rejection	Normal	Infection	Total
+/+	4	14	4	22
+/±	2	3	2	7
+/–	2	20	4	26
±/–	1	3	1	5
–/–			2[b]	2
Total	9	40	13	62

[a]Patients were identified as having noted clinical situation as defined in text.
[b]$p < 0.99$ compared with all others.

ment. This is possible with a test that involves administration of ^{14}C-erythromycin, and sampling the breath shortly thereafter for the level of C14, which reflects P450 activity (71).

VII. FOR THE FUTURE

Knowledge of immunology relevant to a general understanding of the process of rejection is extensive, specific, and relatively complete. Why has this understanding not been applied more rewardingly to specific clinicial events? One factor may be that the processes that may result in accommodation to the graft (tolerance, peripheral suppression, stable chimerism) are less well understood. In addition, the clinical situation is complicated by other immune responses, the redundancy of the immune response, the complexity of immunosuppresives used, and the varied physiological responses of different patients. Accurate analysis of the patient's status by one narrow parameter cannot be predictive in the presence of unanalyzed additional influences. It seems predictable that only a broad measure that is likely to reflect the result produced by the interaction of several of these influences, or a detailed series of factors analyzed concurrently, will be found useful.

Possible improvements in immunocompetence analysis may come from advances in

measurement and understanding of the Th1/Th2 dichotomy (72,73), in the simplified analysis of donor-specific T-cell reactivity, and in the use of reagents that improve measurement of interference with the intracellular signaling processes in T lymphocytes.

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Cancer in Recipients of Organ Allografts

JAMES F. WHITING and DOUGLAS W. HANTO
University of Cincinnati Medical Center, Cincinnati, Ohio

I. INTRODUCTION

It is well recognized that immunodeficiencies of various types predispose the patient to the development of de novo cancers. McKhann (1) and Penn et al. (2) independently first reported an association between cancer and immunosuppression in renal allograft recipients in 1969. The reported incidence of cancer arising in individuals with genetically determined immunodeficiency disease is 4% (3). In immunosuppressed renal allograft recipients, the incidence ranges from 1% to 16% at different centers (4–7) averaging 6% (6), far greater than that in the general population matched for age (3). The most frequent neoplasms in genetically affected patients are those of the lymphoreticular system (8,9) and, in transplant recipients, nonmelanotic skin and lip cancer followed by lymphoproliferative diseases (6,7,10,11). The incidence of malignancy following transplantation increases with time. A follow-up of 6067 renal transplant patients in Australia and New Zealand revealed a 3% incidence of cancer at 1 year, 17% at 5 years, and 64% at 20 years (Fig. 1) (11). Likewise, a study of 124 cardiac transplant recipients at Stanford showed an actuarial risk of cancer development of 2.7% ± 1.9% at 1 year and 25.6% ± 11.0% at 5 years (12).

In 7869 organ transplant recipients reported to the Cincinnati Transplant Tumor Registry (6), skin and lip cancer and malignant lymphomas accounted for 56% of the total can-

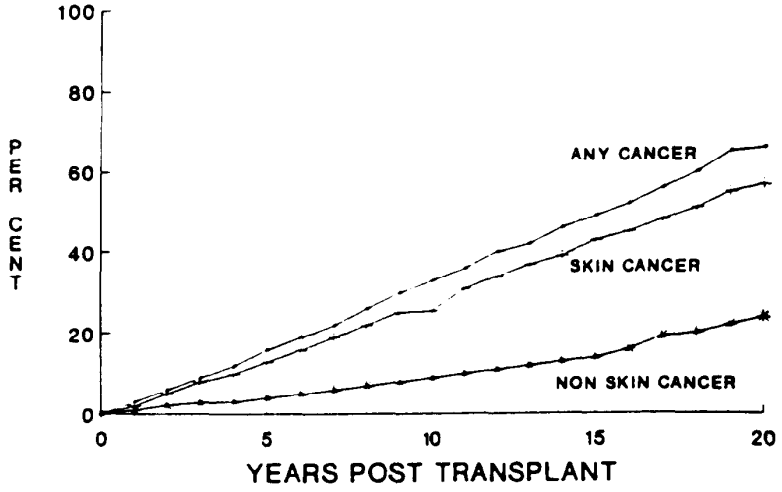

Figure 1 Cumulative risk for development of a malignancy after renal transplantation. (From Ref. 107.)

cers (Table 1 and Fig. 2). By excluding the recipients with nonmelanotic skin cancer, the remaining results can be compared with cancer statistics from the general population (3). Malignant lymphomas and leukemias account for 19% of the tumors in transplant recipients, compared with 7% to 8% in the general population (3). Conversely, carcinoma of the prostate accounts for 19% of cancers in males in the general population but for less than 2% of the cancers in transplant recipients. Similarly, carcinoma of the colon and rectum, carcinoma of the female breast, and lung cancer account for 15%, 13%, and 16%, respectively, of all cancers in the general population (3) but only 3%, 3%, and 6% of the tumors in transplant recipients (6). These differences are related in part to the relative youth of transplant recipients (average age in mid-30s) but may have another explanation in the probable different pathogenesis of cancer following transplantation. It should be noted, however, that, although these tumors comprise a smaller percentage of cancers in any particular cohort of transplant patients, population-based studies demonstrate that there is still an increased risk of contracting these tumors in transplant patients compared to the general public (7,13).

Systematic investigation has been difficult because of the sporadic nature of the problem. Several theories have been proposed for the increased incidence of cancer in immune-deficient individuals, however, including impaired immune surveillance mechanisms (14), chronic antigenic stimulation (15), reactivation of latent oncogenic viruses (16), and the direct oncogenic effects of immunosuppressive drugs. Some of these theories are discussed in this chapter as the different types of malignant neoplasms found in transplant recipients are examined.

II. SKIN AND LIP CANCER

Skin cancers are the most common malignant neoplasms. Nonmelanoma skin cancer accounts for approximately 30% of all new cancers in the United States (3,17). In transplant recipients approximately 37% of de novo cancers are of the skin and lips, and the risk increases with the duration of immunosuppression (6,13,18,19). There is also an increased frequency of premalignant actinic keratoses among transplant patients, 28% at 4 years (20), and these lesions may develop into carcinomas more rapidly. Furthermore, the skin cancers are commonly multiple, metastasize more frequently, and are associated with an increased mortality rate compared with those in the general population, in whom skin cancer accounts

Table 1 De Novo Tumors in Organ Transplant Patients

Type of Tumor[a]	Number of Tumors
Cancers of the skin and lips	3087
Lymphomas	1406
Carcinomas of the lung	469
Kaposi's sarcoma	341
Carcinomas of the uterus (cervix 283; body 45; unknown 4)	332
Carcinomas of colon and rectum	290
Carcinomas of the kidney (host kidney 247; allograft kidney 25; unknown 11)	283
Carcinomas of the breast	264
Carcinomas of the head and neck (excluding thyroid, parathyroid and eye)	237
Carcinomas of the vulva, perineum, penis, and scrotum	214
Metastatic carcinoma (primary site unknown)	186
Carcinomas of the urinary bladder	186
Leukemias	161
Hepatobiliary carcinomas	145
Carcinomas of prostate gland	129
Carcinomas of thyroid gland	103
Sarcomas (excluding Kaposi's sarcoma)	98
Stomach cancers	97
Testicular carcinomas	72
Ovarian cancers	60
Miscellaneous neoplasms	218
Total	8378

[a]There were 7869 patients of whom 475 (6%) had two or more distinct tumor types involving different organ systems. Of these, 32 patients each had three separate types of cancer and one had four.
Source: From Ref. 6.

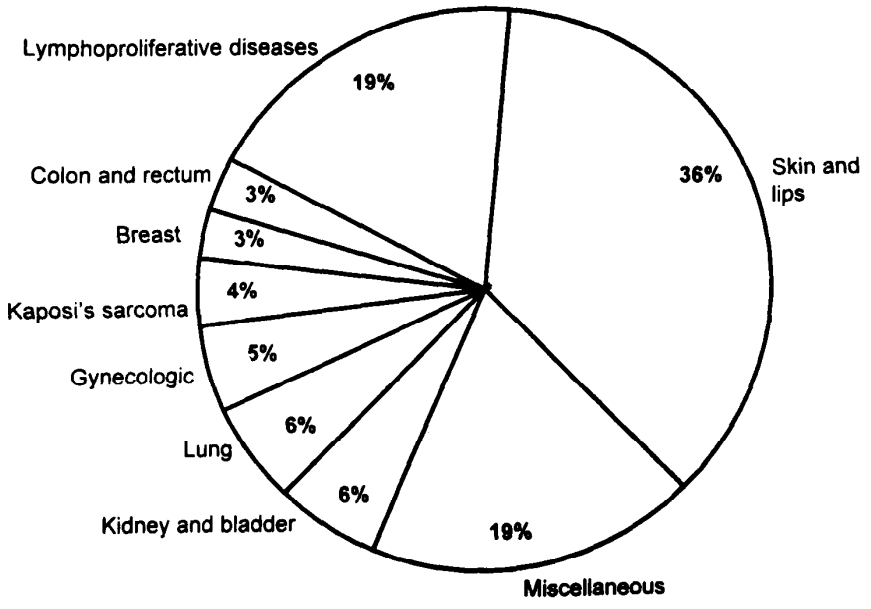

Figure 2 Distribution of the most common malignancies arising in organ transplant recipients. (From Ref. 6.)

Table 2 Types of Skin Cancer Reported to the Cincinnati Transplant Tumor Registry

Types of Cancers[a]	Number of Tumors
Squamous cell carcinoma	1546
Basal cell carcinoma	866
Squamous and basal cell carcinoma	453
Malignant melanoma	164[b]
Type not specified	29
Merkel's cell tumor	18
Malignant sweat gland tumor	9
Miscellaneous	2
Total	3087

[a]Keratoacanthomas, Kaposi's sarcomas, and lymphomas are excluded.
[b]41 patients also had basal or squamous carcinomas or both.
Source: From Ref. 6.

for less than 1% to 2% of all cancer deaths, the majority secondary to malignant melanomas (3). Squamous cell and basal cell carcinomas are the most common types of skin and lip cancers (Table 2) and often cause significant morbidity as reflected in loss of limbs and disfigurement (Fig. 3).

A. Incidence and Risk Factors

The incidence of nonmelanotic skin cancer is determined by the amount of exposure to ultraviolet light, which depends on a person's latitude, altitude, occupation, clothing, and outdoor recreational activities (21). When exposure is equal, skin cancer is more likely to develop in fair-skinned people than in those with dark skin. Other risk factors include exposure to chemical carcinogens (e.g., arsenic and organic hydrocarbons), exposure to x-rays and γ-rays, chronic scars, and the presence of ulcers, and sinus tracts.

In the general population, the incidence of nonmelanotic skin cancer varies from 0.15% per year in Minnesota to 0.38% per year in Texas (17). The first report of new skin cancer in renal transplant patients came from Sydney, Australia, and showed a 14% incidence (22). Since then, many reports have emphasized the increased incidence of skin and lip carcinoma in these patients, ranging from 1.4% in Minnesota to 20% in Australia and New Zealand (6,7,11,13,18,19,23–31).

The incidence of various cancers is usually calculated as the number of patients in whom the condition is developing divided by the total number of patients receiving transplants. Because the risk of skin cancer development has been demonstrated to increase with increasing duration of immunosuppression, early estimates of skin cancer incidences with short follow-up periods of 1 to 5 years may have significantly underestimated the severity of the problem. Life table analysis of a cohort of patients from Australia and New Zealand has demonstrated a 57% probability of skin cancer development after 20 years of graft survival (13). Even in relatively "low risk" populations for sun exposure such as in the Netherlands, a 10% probability of development of skin cancer at 10 years and 40% probability at 20 years have been estimated (26). Combined data from Denmark, Sweden, and Norway on follow-up of 5692 renal transplant patients have demonstrated a relative risk of devel-

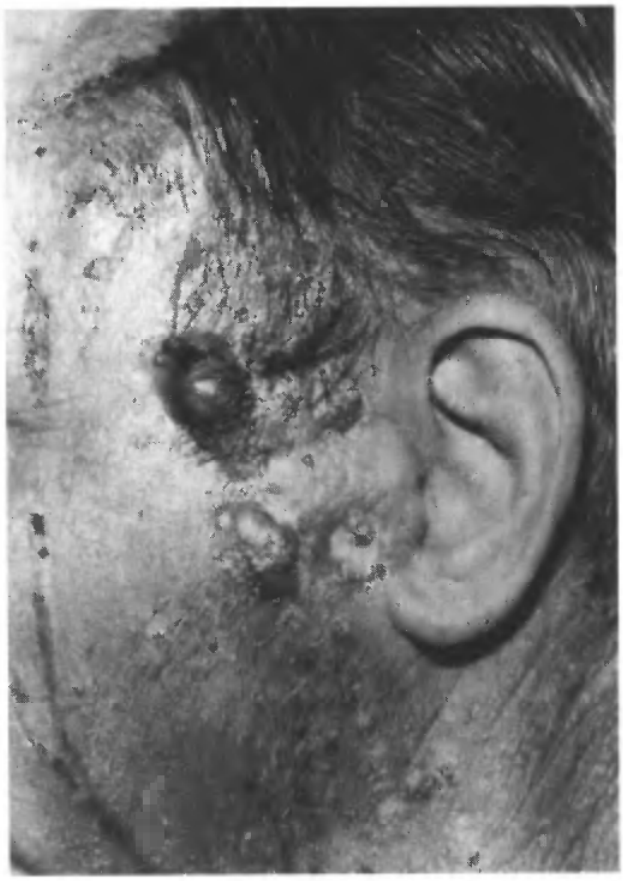

Figure 3 Multiple recurrent squamous cell carcinomas of the face.

opment of skin cancer of 10 to 20 times that in the general population (7). Similarly, a single-center study of 523 renal transplants from Canada demonstrated an 18-fold risk of squamous cell carcinoma development, although only a 1.4-fold risk of basal cell carcinoma development (25).

Skin cancers are by far the most common malignancy in all reports of transplant patients. Skin cancer made up 93% of the total cancers in Brisbane (18) and 37% in the Cincinnati Transplant Tumor Registry (CTTR) (6). A more recent report from Australia and New Zealand revealed that skin cancer made up 73% of 1752 cancers seen in a group of 6596 renal transplant recipients (11). Furthermore, premalignant skin lesions are common, occurring in 28% of patients who survived for 4 years in one series.

B. Age and Sex

In the general population, the average age at the time of diagnosis of skin cancer is approximately 64 years (19), but the peak incidence is between 75 and 84 years of age. In transplant recipients, the diagnosis is usually made during their 40s (18,20,22,26,32). Children as young as 13 years old have had squamous cell carcinoma of the face (19).

The male to female ratio for skin cancer in the general population is approximately 3:1 for squamous cell carcinoma and 2:1 for basal cell carcinoma. In transplant recipients, the male predominance persists, with overall ratios ranging from 1.5:1 (18) to 3:1 (19). The increase in risk has frequently been reported to be greater for females than males (18,25), although a recent large population–based series has cited equal incidences (7).

C. Interval from Transplantation to Diagnosis

In immunosuppressed patients, cancer can develop within 2 months (19), although longer intervals averaging 60 months (29) are seen in the United States. In Australia, the average interval is 18 (22) to 34 months (18,22), probably the result of more intense sun exposure in a susceptible population.

D. Cell Type, Location, and Multiple Tumors

The most common tumors are squamous cell and basal cell carcinomas. In the general population, the ratio of basal cell to squamous cell carcinoma varies from 3:1 (Texas) to 6:1 (Minnesota) (27). In the transplant population, this ratio is reversed to 1:1.5–1.9 because of the much increased incidence of squamous cell carcinoma with only a small or insignificant change in the incidence of basal cell carcinoma (18,19,25,26).

Most lesions are located on the sun-exposed areas of the head, neck, lips (vermilion border of the lower lip exclusively), and upper extremities (especially dorsal surfaces of the forearm, hands, and fingers) (18,19,26). Extensive actinic damage is common, and premalignant actinic kerotoses probably progress to invasive squamous cell carcinoma more frequently in transplant patients (20). There also appears to be more rapid progression from actinic keratosis, keratoacanthoma, and Bowen's disease to invasive squamous cell carcinoma.

According to the CTTR, 43% of patients had multiple skin cancers (6), as did 75% of the Australian patients (18). Of the Australian cases, 59% had more than one lesion at initial diagnosis. The combined Australian and New Zealand study showed multiple lesions in 69% of patients with skin cancer (11). Primary skin cancers in young patients with multiple warts have been reported (19,33).

E. Diagnosis, Treatment, and Prognosis

Frequent skin examinations and self-examination, particularly of skin exposed to sun, should be regular features of posttransplant care. Because the differential diagnosis of skin lesions in these patients by gross examination alone is often misleading, a biopsy should be performed on all new lesions. For example, carcinoma of the lip often appears as a superficial ulcerated lesion without other characteristics of cancer and is frequently misdiagnosed as a herpes simplex "cold sore." If a cold sore persists for 3 to 4 weeks, a biopsy should be performed (19). Other unrelated neoplasms must be looked for because the relative risk is increased substantially. All patients should be instructed in the diligent use of sunscreen when outdoors.

Conventional therapy usually includes local excision, cryosurgery, radiotherapy, or topical 5-fluorouracil cream, depending on the size, location, and extent of the lesion. There is no evidence that reduction of immunosuppressive medications causes spontaneous regression, delays spread of this cancer, or reduces the incidence of new lesions.

In recent years, considerable attention has been devoted to the role of retinoids for treatment or prevention of skin cancers. Retinoids have long been known to induce cellular differentiation or suppress proliferation of numerous cell lines in vitro (34,35), and dietary manipulations of retinols have been known to influence progression and regression of preneoplastic lesions in various experimental models (36). Although anecdotal reports of regression of squamous cell cancers do exist (37,38), the only systematic benefit of retinoids has been demonstrated in premalignant lesions such as actinic keratoses (39,40).

In the immunocompromised host, squamous cell carcinoma appears to be more vir-

ulent than in the general population (18,19,25,41,42). Although local recurrence is unusual, the CTTR notes that 6% (63 patients) of transplant patients with squamous cell carcinoma had regional lymph node or distant metastases (6). In the general population, the incidence of metastases of squamous cell carcinomas that arise in actinic damaged skin is 0.1% to 0.5% (43,44). In contrast, squamous cell carcinoma of the lip has a high risk of metastases (5% to 37%) in both groups (21). Because we cannot predict which tumors will metastasize, all patients with squamous cell carcinoma of the lip must be evaluated regularly.

Although few transplant patients die because of skin cancer, a 10-fold increase in the risk of death from squamous cell carcinoma of the skin has been reported (28). Sheil et al. (11) reported a 7% incidence of recurrence and metastasis with 37 deaths (5% of patients with squamous cell carcinoma). In the CTTR, 5% of patients with skin cancer died of metastatic disease, with 61% of deaths secondary to squamous cell carcinoma and 34% secondary to melanoma (6).

F. Pathogenesis

Many factors interact to cause the increased incidence of skin and lip carcinoma in immunosuppressed transplant recipients. Both epidemiological and experimental evidence implicate ultraviolet light in the pathogenesis of skin carcinoma in the general population and in immunocompromised patients (21,45). Tobacco exposure is an important risk factor in lip cancer (46). Other factors that may also be important, either alone or in combination, include impaired immune surveillance mechanisms, chronic antigenic stimulation from the transplanted allograft, reactivation of latent oncogenic viruses, and direct oncogenic effects of immunosuppressive drugs.

Although none of these have been proved, a few clues are available. Skin cancer develops with greater frequency in hairless mice, a strain prone to the development of ultraviolet light–induced skin cancer, when the mice are exposed to a combination of ultraviolet light and azathioprine (47). Thymectomy plus the chemical carcinogen 3,4-benzopyrene produces a higher incidence of skin tumors in mice than 3,4-benzopyrene alone (48). Furthermore, under certain circumstances, herpesviruses are oncogenic viruses. Ultraviolet-irradiated herpes simplex virus type 1 (HSV-1) transforms both the mouse L cell (49) and hamster embryo fibroblasts (50) and can cause chromosomal changes in vitro (51,52). This ability of ultraviolet light to change this virus from the infectious to the oncogenic form is intriguing because (1) most skin cancers occur on sun-exposed areas of the body, and (2) the lower lip is a frequent location of both herpes simplex type 1 infection and squamous cell carcinoma in transplant patients. A case of squamous cell carcinoma of the lower lip that developed at the site of a chronic herpes simplex infection has been reported in a non-immunosuppressed patient (53). Eglin et al. detected RNA complementary to herpes simplex virus in 14 of 24 human oral squamous cell carcinomas (54). However, they do not state how many were positive for HSV-1 or HSV-2, although both were tested. Other herpesviruses may also be important. Analysis of 33 proliferative skin lesions from four organ transplant patients demonstrated that 82% were positive for a polymerase chain reaction (PCR) product known as KS330, originally found in specimens from patients with Kaposi's sarcoma (55). This DNA sequence is homologous to the minor capsid and tegument protein genes of several well-described herpesviruses. Thus, lip cancer in transplant patients may be related to a combination of herpesvirus infection, immunosuppression, and ultraviolet light.

Evidence also suggests an oncogenic role for human papillomavirus (HPV) (2,6,10, 19,29,30,33,55–64). Patients with the rare, chronic flat wart disease, epidermodysplasia

verruciformis, have detectable HPV in skin lesions (65). Squamous cell carcinoma develops in 35% of these patients during the second and third decade, usually on sun-exposed skin (66–68). Several studies have attempted to assess the role of HPV in benign, premalignant, and malignant skin lesions in immunosuppressed patients. From Germany, Gassenmaier et al. reported that warts developed in 36 of 148 (24.3%) of renal allograft recipients after transplantation. Only five patients (3%) had warts before transplantation. Biopsies were examined from 16 of these lesions, and 10 of them were found to contain HPV viral DNA (33). In a series of 202 renal transplant patients from Edinburgh, 77% of patients with graft survivals of more than 5 years had warts, with HPV viral DNA detected in 16% of the warts examined, but HPV viral DNA was found in 60% of squamous cell carcinomas (56), and a recent French study demonstrated HPV viral DNA in 13 of 24 (54%) squamous cell carcinomas examined (69). The Scottish study also noted a correlation between skin warts and genital warts, with eight of the women in the study having genital warts containing HPV viral DNA. Overall, 25% of female transplant patients with warts of any kind can be expected to have genital warts as well (61). With the proven association between genital warts and cancer (70) it is especially important for all female transplant patients to be closely followed up with full colposcopic examination. At the University of Minnesota, 90% of specimens examined from 36 renal transplant patients with a history of wartlike lesions were found to contain HPV viral DNA (63). These studies have also shown an increased incidence of warts and other skin lesions developing over time following transplantation. The oncogenic potential of certain HPV types clearly plays a critical role in the development of premalignant and malignant skin lesions in immunosuppressed transplant patients.

III. OTHER CANCERS INVOLVING THE SKIN

A. Melanoma

The risk of melanoma after organ transplantation is unclear because the patient population is small (71). The CTTR has accumulated data on 164 patients with melanoma, 5.3% of all patients with skin cancer (6) compared with 2.7% in the general population (3). Sheil reported an incidence of 3% of 6596 patients with melanoma (11), with 10 deaths, or 24% of those afflicted. There is some evidence to suggest that melanoma in some of these patients may evolve from dysplastic nevi (72) and may have enhanced malignant growth potential with increased immunosuppression (73). Other reports have cited the development of melanoma after the use of immunosuppressive therapy not associated with transplantation (74–76). The Australian and New Zealand experience revealed that malignant melanoma occurred five times more frequently in transplant recipients than expected in the age-matched general population (11), whereas the risk was estimated to only be 1.7 times that of the general population in the Nordic countries (7).

B. Kaposi's Sarcoma

Kaposi's sarcoma is a rare tumor that comprised only 0.2% to 0.7% of all cancers in the general population in the United States (77) before the acquired immunodeficiency syndrome (AIDS) epidemic. A total of 341 cases (4%) of Kaposi's sarcoma in organ transplant recipients were reported to the CTTR (6), more than the number of patients with either colon or breast cancer. The Australian and New Zealand transplant registry reported 14 cases of Kaposi's in follow-up of 6596 patients, representing a greater than 1000-fold risk as compared to the general population (11).

Kaposi's sarcoma is an angiomatous neoplasm derived from vasoformative mesenchyma that primarily involves the skin (78). On gross examination the characteristic lesions are multiple, irregular, bluish dermal plaques or nodules that occasionally resemble raised pyogenic granulomas. Although the disease usually begins on the feet and legs, the lesions can be found on the mucous membranes of the mouth or oropharynx. The skin is almost always involved, and visceral involvement, particularly in the gastrointestinal tract, can also occur (79). The diagnosis of Kaposi's sarcoma is made primarily on gross appearance and histological confirmation.

Approximately two-thirds of transplant recipients in whom Kaposi's sarcoma develops have the "benign" nonvisceral variety of the disease, involving the skin, conjunctiva, or oropharyngeal mucosa. Treatment has resulted in complete remissions in half these patients with benign disease. Treatment usually involves reduction in immunosuppression (80,81–84), radiotherapy (80,84,85), or chemotherapy (80,84), and up to one-third of successful treatment involves only reduction or withdrawal of immunosuppression (86). Half of patients with nonvisceral Kaposi's sarcoma can be expected to respond to treatment with a prolonged remission (86). Reinstitution of immunosuppression has been reported to quickly produce a recurrence of the disease (81,87).

The remaining patients have "malignant" disease with involvement of internal organs, mainly the gastrointestinal tract. Only 20% can be expected to have complete remissions, but therapy can stabilize the disease (86). Early reports of posttransplant visceral Kaposi's sarcoma reported exceptionally high case fatality rates with 9 of 13 patients dying in one study (84). More recent reports as well as data from the CTTR have been more optimistic (86,88) with mortality rates ranging from 20% to 49%. In general, when compared with AIDS patients, transplant recipients with Kaposi's sarcoma have less visceral disease and lymphadenopathy as well as a better overall chance of survival (78,89–92).

The cause of this disorder is obscure, although a viral origin and relationship to the herpesvirus family has been proposed. Cytomegalovirus (CMV) has been isolated in an in vitro established tumor cell line (93). A serological association between American Kaposi's sarcoma (but not the African form) and cytomegalovirus has been reported (94). More recently, important links have been established between other herpesviruses and Kaposi's sarcoma (95,96). Analysis of tissue samples from patients with AIDS-associated Kaposi's sarcoma demonstrated a herpesvirus-like DNA sequence referred to as KS330 by PCR in 10 of 11 tissue sample's, whereas only 1 of 21 control samples were positive. Patients with classic Kaposi's sarcoma or HIV-negative homosexual men with Kaposi's sarcoma also were positive in all tumor samples tested (96).

IV. LYMPHOPROLIFERATIVE DISEASES

A. General Considerations

"Malignant lymphomas" are the second most common malignancy after skin and lip carcinoma and make up 19% of the total, compared with 2% to 4% of all cancer seen in the general population (see Fig. 2 and Table 1). The risk of development of malignant lymphoma in a renal allograft recipient is said to be increased 10 times (7,11). Approximately 85% of these lymphomas are of B-cell lineage and, although the precise cause remains obscure, Epstein-Barr virus (EBV) has been implicated by the demonstration of the EBV genome or its products in the majority of posttransplant lymphoproliferative disease (PTLD) cases. Because a morphological spectrum of B-cell proliferation exists, we prefer the general term "lymphoproliferative diseases," which encompasses proliferations ranging from reactive,

polyclonal hyperplasias to true monoclonal malignant lymphomas, as discussed later. The overall incidence of lymphoproliferative diseases reported in renal, liver, heart, and heart-lung transplant recipients is 0.5% to 2.5% (97,98), 0.5% to 2.3% (97,98), 0% to 11.8% (98,99), and 4.6% to 9% (98,100), respectively. If only patients at risk (i.e., those alive with functioning grafts) were included, the incidence would be higher. Because of the widespread dissemination of molecular techniques in recent years, enormous strides have been made in the classification and understanding of these diseases with relevance to potential treatment. In the past 15 years, we have attempted to systematically characterize the clinical, histological, immunological, cytogenetic, and virologic features of these lymphoproliferative diseases (101–110). We have suggested previously that a combination of clinical syndrome, standard histopathology, and molecular analysis can be used to separate transplant recipients into distinct groups, both to further our knowledge as well as to guide potential therapy. Our results are summarized here and compared with those of others.

B. Clinical Syndromes

In Penn's study of 1406 patients with lymphoproliferative disorders, extranodal involvement occurred in 69% of patients compared with 24% to 48% of patients in the general population (6). The single organ most frequently involved was the central nervous system (CNS) (22%), although the disorders were frequently multicentric. In a recent French series, PTLD developed in 24 of 1385 (1.7%) organ recipients; while 5 of the 21 patients (23%) with B-cell PTLD had CNS involvement, and all manifested extranodal disease (111). In our series, CNS involvement occurred in 45% of patients and is usually indicative of widespread disease. Disease confined to the CNS occurred in only 14%. Hodgkin's disease is only slightly more common in transplant patients than in the general population (7), and it comprises only 2.7% of the lymphomas reported to the CTTR (6), yet, not surprisingly, it is entirely absent in most single-center series (97,98,111). The average time of presentation in the French study was 210 days (range 60–4140 days) (111). In the first Minnesota series, two main clinical groups were identified (Table 3) (106,107,110). First,

Table 3 Summary of Patient Groups with Posttransplantation Lymphoproliferative Diseases

Group 1: Infectious Mononucleosis-like Illness (42%)	Group 2: Localized Solid Tumor Masses (58%)
Young patients	Older patients
Mean age 21 years	Mean age 47 years
Range 11–46 years	Range 22–68 years
Short interval from transplantation to diagnosis	Long interval from transplantation to diagnosis
Mean interval 7.8 months	Mean interval 5.3 years
Range 1 week to 2.8 years	Range 1.1–11 years
Symptoms of viral illness (fever, pharangitis, lymphadenopathy)	Symptoms related to solid tumor masses (confined to CNS in 14%)
Widespread disease	More localized, primarily extranodal disease
Mortality rate (50%)	Mortality rate (75%)
Short clinical course when fatal	Longer clinical course when fatal
Mean survival 2.3 months	Mean survival 6.6 months
Range 10 days to 8 months	Range 2 weeks to 15 months
Response to acyclovir	No response to acyclovir

young patients (mean age 21 years) present soon after transplantation (mean interval from transplantation to diagnosis 7.8 months) or antirejection therapy (i.e., during periods of maximal immunosuppression) with an infectious mononucleosis-like syndrome characterized by fever, pharyngitis, and lymphadenopathy. Second, older patients (mean age 47 years) present at longer intervals after transplantation (mean interval 5.3 years) with localized, extranodal tumor masses, often with unusual clinical and radiological manifestations (112,113). In some patients, uncomplicated infectious mononucleosis also develops and should be included in the spectrum of Epstein-Barr infections (114). In general, pediatric centers report higher incidences of PTLD (115–119). This may be secondary either to the more intense immunosuppression administered to pediatric organ recipients or to the fact that up to 50% of pediatric organ recipients are estimated to be EBV seronegative and that, in up to one-third, primary EBV infection will develop after transplantation, which may also be important (111). At Pittsburgh (98), 18 of 43 patients had a syndrome of fever and generalized lymphadenopathy resembling infectious mononucleosis. Of the 18, however, six had more extensive disease and five eventually died secondary to complications of their PTLD.

C. Histology

Morphologically, all these EBV-related lymphoid disorders have the features of monomorphic or polymorphic B-cell proliferation and produce extensive architectural obliteration of the organs involved (98,101). However, some cases classified as polymorphic diffuse B-cell hyperplasia (PDBH) showed marked plasmacytic differentiation without atypia and necrosis, and others classified as polymorphic B cell lymphomas (PBL) are characterized by nuclear atypia of the large cells and extensive necrosis. The lymphoproliferative diseases in cardiac allograft recipients from Stanford have all been classified as diffuse large cell lymphomas (118). No disorders resembling PDBH were apparently seen. Polymorphic lesions, whether classified as hyperplasia or lymphoma, can be polyclonal, whereas monomorphic lesions are invariably monoclonal. Nalesnik et al. first noted a general progression from polymorphic to monomorphic as the virulence of the tumors increased, and they proposed that this progression was more important than the presence of necrosis and atypia per se (98). Standard histology is otherwise of little help in distinguishing between polyclonal and monoclonal proliferations, which are important determinants of biological behavior.

D. Molecular Analysis

All patients with PTLD should have clonality of the lymphoproliferation (polyclonal, oligoclonal, or monoclonal) determined. Strategies available include (1) immunological cell typing (surface and cytoplasmic immunoglobulin -α, -γ, -μ, and -δ heavy light chains and κ and λ light chains) on frozen sections and cell suspensions, (2) cytogenetic analysis of metaphase preparations, (3) immunoglobulin gene rearrangement analysis, and (4) analysis of tandem terminal repeats of EBV genomes. Classically, malignant B-cell lymphomas have been designated as monoclonal on the basis of staining for a single immunoglobulin light-chain isotype. However, immunoglobulin light-chain staining for determination of clonality may not permit the detection of a monoclonal malignant population if it constitutes only a small percentage of the visualized cells, especially when there is a significant admixture of normal, polyclonal B cells. This limitation can be overcome by the detection of clonal immunoglobulin gene rearrangements by blot hybridization, which is sensitive enough to detect a monoclonal population constituting as little as 1% to 2% of the

total cell population (119,120). Benign polyclonal B-cell proliferations contain many different immunoglobulin gene rearrangements, such that each is below the threshold of sensitivity of a blot hybridization and a single rearranged band is not detected. These can therefore be differentiated from monoclonal lymphomas (121).

Using fluorescein-conjugated monospecific antibodies against each heavy and light Ig chain (101,103,106,107,110), we have shown that 8 of 11 lymphoid lesions studied were polyclonal B-cell proliferations. True monoclonal B-cell tumors (IGG-K in all three cases) were documented. However, cytogenetic analysis and immunoglobulin gene rearrangement demonstrated that, of the three lesions classified as polyclonal lymphomas, two actually had evidence of monoclonal proliferations by the more sensitive techniques (Table 4). Sub-

Table 4 Morphological, Surface Marker, Cytogenetic, and Immunoglobulin–Gene Rearrangement Characteristics of Posttransplant B-Cell Lymphoproliferative Diseases

			Surface and/or cytoplasmic immunoglobulin			Immunoglobulin–Gene Rearrangements		
Patient	Sample	Histological Diagnosis	Heavy Chain	Light Chain	Karyotype No./total[a]	J_H	C_κ	$C\lambda$
1	LN	PDBH	μ, γ, α	κ, λ	46, XY, 20/20	G[b]	---	---
2	LN	PDBH	μ, γ, α	κ, λ	46, XY, 30/30	G[b]	---	---
3	aLN	PBL	μ, γ, α	κ, λ	---	G	G	R
	bLN	IS	γ	κ	---	---	---	G
4	aLN	PBL	μ, γ, α	κ, λ	46, XY/47, XY, +3 4/18	R	G	R
	bLN	NPD	---	---	---	G	G	G
5	a L Tonsil	PBL	μ, γ, α	κ, λ	46, XX 11/11	R1	R	R
	b R Tonsil	PBL	μ	κ	46, XX 14/14	R2/R3	---	---
6	Soft tissue	PBL	μ	κ	45,XY,–1,–2,–6,–8,+9 –14,–21, +der (1).t(1;6).(36;q21.,+der(14).t(9;14).(q13;q32). + mar 1 9/15 45,XY,–1,–2,–6,–8,+9,–14,–21,+ der (1).t(1;6).(p36;q21).,+ der (2).t(2;?).(q13;q32).+ mar 2+ + mar 3 2/15	R	R	---

[a]Indicates number of metaphases with karyotype designated/total number of metaphases studied. Remainder of metaphases were normal, diploid.

[b]With both BamHI and HindIII-digested samples.

Abbreviations: LN = lymph node; PDBH = polymorphic diffuse B-cell hyperplasia; PBL = polymorphic diffuse B-cell lymphoma; IS = immunoblastic sarcoma; NPD = no pathogerm line; (---) denotes not done.

Source: From Hanto DW, Birkenbach H, Frizzera G, Gajl-Peczalska KJ, Simmons RL, Schuback WH. Confirmation of the heterogeneity of posttransplant Epstein-Barr virus associated B-cell proliferations by immunoglobulin gene rearrangement analyses. Transplantation 1989; 47:458–464.

sequent studies have confirmed that, excluding lesions that would be considered PDBH, the majority of PTLDs are monoclonal. In the French study, 16 of 21 patients with PTLD had monoclonal tumors by both immunoglobulin staining and gene rearrangement (11). No differentiation between PDBH or PBL was made; PTLDs were histologically classified as either monomorphic or polymorphic. In the Pittsburgh series, the lymphoproliferations were found to be polyclonal in 13 and monoclonal in 17, and the clonality was indeterminate in 13: again, histological classification was between monomorphic and polymorphic (98). Cleary et al (122) reported that clonal immunoglobulin gene rearrangements were present in all the lymphoproliferative lesions that developed in 10 cardiac allograft recipients. These had been classified as diffuse large cell lymphoma, and 9 of 10 were negative for immunoglobulin, thereby preventing determination of their clonality using light-chain phenotyping (118). A subsequent report from the same group demonstrated that, in 5 of these patients, tissues obtained at different times or from different sites at the same time contained different clonal immunoglobulin gene rearrangements and therefore concluded that such lesions were "oligoclonal" rather than monoclonal (123).

Clonality has been demonstrated to be an important prognostic indicator. In our study, all patients with PDBH had polyclonal tumors. Leblond et al. reported that, in their group, all patients with polymorphic polyclonal tumors were cured, in contrast to 7 of 16 cures in the patients with monoclonal tumors (111). Kaplan et al. reported on seven patients with monoclonal or oligoclonal tumors; all died (124). The Pittsburgh series noted that, of five patients who exhibited no response to therapy, all were monoclonal (98).

Perhaps the most interesting groups of patients are those with polymorphic histology and/or polyclonal immunoglobulin profiles, yet who turn out to have evidence of clonal populations on further investigation by cytogenetic analysis, gene rearrangement studies, or EBV genome studies. Close examination of these patients suggests that polyclonal B-cell proliferations may be precursors of monoclonal malignant lymphomas and may undergo malignant transformation identified by clonal cytogenetic abnormalities. We have examined metaphase preparations from biopsy specimens in five patients (Fig 4) (107). In two patients with polyclonal PDBH, the karyotypes were normal diploid so that these tumors meet all the criteria for a benign, EBV-induced B-cell proliferation (polymorphic hyperplasia, polyclonal, and diploid). In a second set of patients with polyclonal PBL, clonal cytogenetic abnormalities indicative of early malignant transformation were identified in a subpopulation of cells (trisomy-3 and trisomy-14). In biopsies from an intermediate group of three patients that we have also studied with morphologically malignant proliferations that were composed predominantly of a polyclonal population of B cells as determined by immunoglobulin profile, clonal gene rearrangements were also found, consistent with early malignant transformation in a subpopulation of cells. The increasingly aggressive biological behavior of these tumors from polymorphic, polyclonal tumors to monomorphic monoclonal tumors also supports this progression from benign hyperplasia to diffuse lymphoma.

E. Epstein-Barr Virus Studies and Pathogenesis

The link between EBV and these lymphoproliferative diseases has been firmly established by serological studies, Epstein-Barr nuclear antigen (EBNA) staining of tumor tissue, and EBV DNA hybridization studies that demonstrate the presence of EBV-specific DNA sequences in biopsy specimens. In the Minnesota series, tumor-bearing patients with an infectious mononucleosis-like illness had serological evidence of a primary (33%), reactivation (33%), or long-past EBV infection (33%) (107). In older patients with tumor masses,

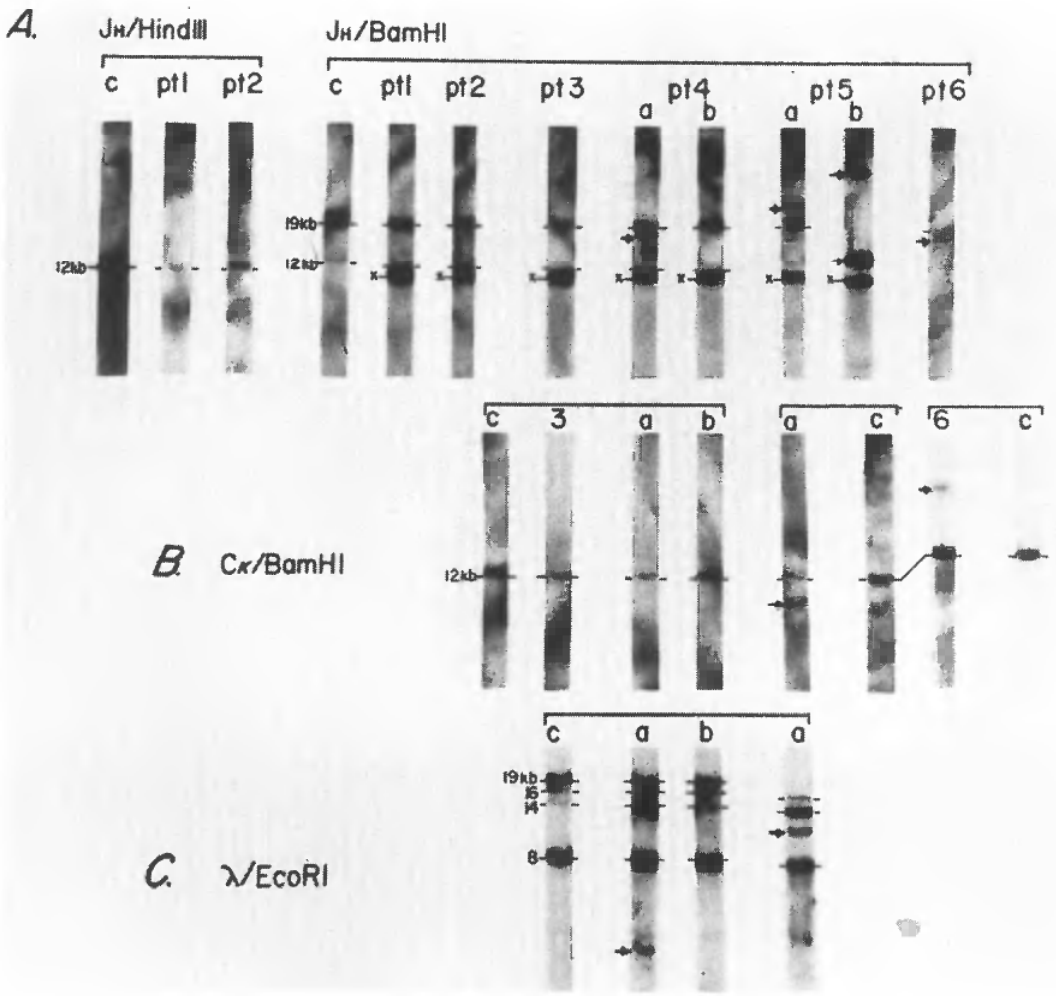

Figure 4 Blot hybridization analysis of immunoglobulin gene arrangements in various patient samples. Tissue DNA (10–15 μg) was digested with HindIII, BamIII, and EcoRI, electrophoresed, blotted, and probed with the probes indicated. The patient numbers correspond with those in Table 4. Lanes designated c contain samples from human granulocyte DNA used as a germline control. A: Samples digested with HindIII and BamHI were probed with the J_H probe. HindIII digestion produced a germ line band of 12 kb; BamHI digestion produced a major 19-kb and a minor 12-kb germ line band. Restriction fragments indicative of the germ line configuration are indicated by thin lines; novel bands indicative of a ^{J}H rearrangement are indicated by an arrow to the left of the strip. A cross-reacting genomic fragment denoted x was present in BamHI-digested samples from patients 1 through 5. B: Samples from patients 3 through 6 digested with BamHI and probed with the C_κ probe. A 12-kb germ line control band c is indicated for patients 3 and 4; different germ line controls are noted for patients 5 and 6. C: Samples from patients 4 and 5 were digested with EcoRI and probed with the λ probe. The control lane demonstrates germ line bands of 8, 14, 16, and 19 kb. DNA from patient 5 lacks the 19-kb germ-line band, which is polymorphic and absent from a significant proportion of normal DNA samples. (From Hanto DW, Birkenbach H, Frizzera G, Gajl-Peczalska KJ, Simmons RL, Schuback WH. Confirmation of the heterogeneity of posttransplant Epstein-Barr virus associated B-cell proliferations by immunoglobulin gene rearrangement analyses. Transplantation 1989; 47:458–464.

no primary infections occurred, but serological evidence of reactivation infection (63%), or long-past infection (37%) was found. Staining of tumor tissue for EBNA was positive in several instances (106,125,126), but false-negative results can occur for technical reasons, and EBV DNA hybridization techniques have provided a more sensitive method of detecting the EBV genome within tumor cells (127). Lymphoid lesions from 19 of 20 renal trans-

plant recipients in the Minnesota series (97) and from four cardiac transplant recipients from Stanford (128) contained EBV-specific DNA sequences using EBV cRNA/DNA filter hybridization, vDNA/DNA reassociation kinetic analysis, or the Southern blot test. In the Pittsburgh study, 12 of 12 patients exhibited EBV-specific DNA sequences by in situ hybridization (129). In the French study, EBV was detected in 13 of 19 B-cell tumors studied by either Southern blot or in situ hybridization analysis (111). Seven of eight PTLDs analyzed by Kaplan and associates with DNA blot hybridization were positive for EBV, and six of the seven were clonal for EBV (124).

Despite this impressive association, the exact mechanism of the development of PTLD and evidence of a true cause and effect relationship between EBV and PTLD remains enigmatic. The EBV is known to replicate in oropharangeal epithelial cells where free infectious virions can be recovered from virtually all otherwise healthy seropositive individuals (130). Viral infection and transformation of B cells result in the release of latently infected, immortalized B cells found in the circulation and lymphoid tissue. In the immunocompetent host, EBV is believed to be controlled by virus-specific, human leukocyte antigen (HLA)-restricted cytotoxic T lymphocytes (CTL) capable of lysing EBV-infected B cells, a process that has been shown to be defective in some transplant recipients (131,132). In a prospective study of pediatric renal transplant patients, 12 of 22 (54%) exhibited episodes of appearance of EBV positive cells detected by PCR, whereas 24 age- and serologically matched control specimens exhibited no such episodes (133). Decreased CTL surveillance coupled with other factors that can cause increased B-cell proliferation such as chronic antigenic stimulation or Th2 cytokine shifts (134) may lead to the development of polyclonal polymorphic hyperplasia or uncomplicated infectious monucleosis-type syndromes. Once established, these syndromes, initially responsive to therapy, could then progress to a more malignant monoclonal tumor following a cytogenetic event that confers malignant growth potential to a small clone. At this stage, the tumor would resemble the polymorphic monoclonal tumors of intermediate aggressiveness described previously. Eventually, outgrowth of the malignant clone becomes the dominant characteristic of the tumor. This hypothetical progression from benign EBV infection to malignant tumor is diagrammed in Fig. 5.

F. Therapy

Because PTLD represents a wide spectrum of lymphoid proliferative tumors, clearly effective therapy should be directed by using the available clinical, histological, and molecular data to categorize patients according to risk and likely response to therapy. Four broad groups of patients can be identified (Fig. 6).

Group 1 patients are those with uncomplicated mononucleosis-type syndromes and group 2 patients are those with benign polyclonal polymorphic B-cell hyperplasia. No molecular or immunocytochemical indicators of oligoclonality or monoclonality are present in these patients Because these syndromes are ostensibly still dependent on active viral replication, acyclovir, a synthetic antiviral agen that inhibits EBV DNA replication by blocking the EBV-associated DNA polymerase, is effective therapy (104). A reduction in immunosuppression may or may not be necessary, depending on the severity of EBV infection and response to acyclovir.

Group 3 cases are the most difficult to diagnose and characterize. These consist of patients who may have benign clinical syndromes such as fever and adenopathy. Histological examination of involved tissue reveals a polymorphic pattern and further examination by light-chain phenotyping may demonstrate a polyclonal pattern. Further analysis of these

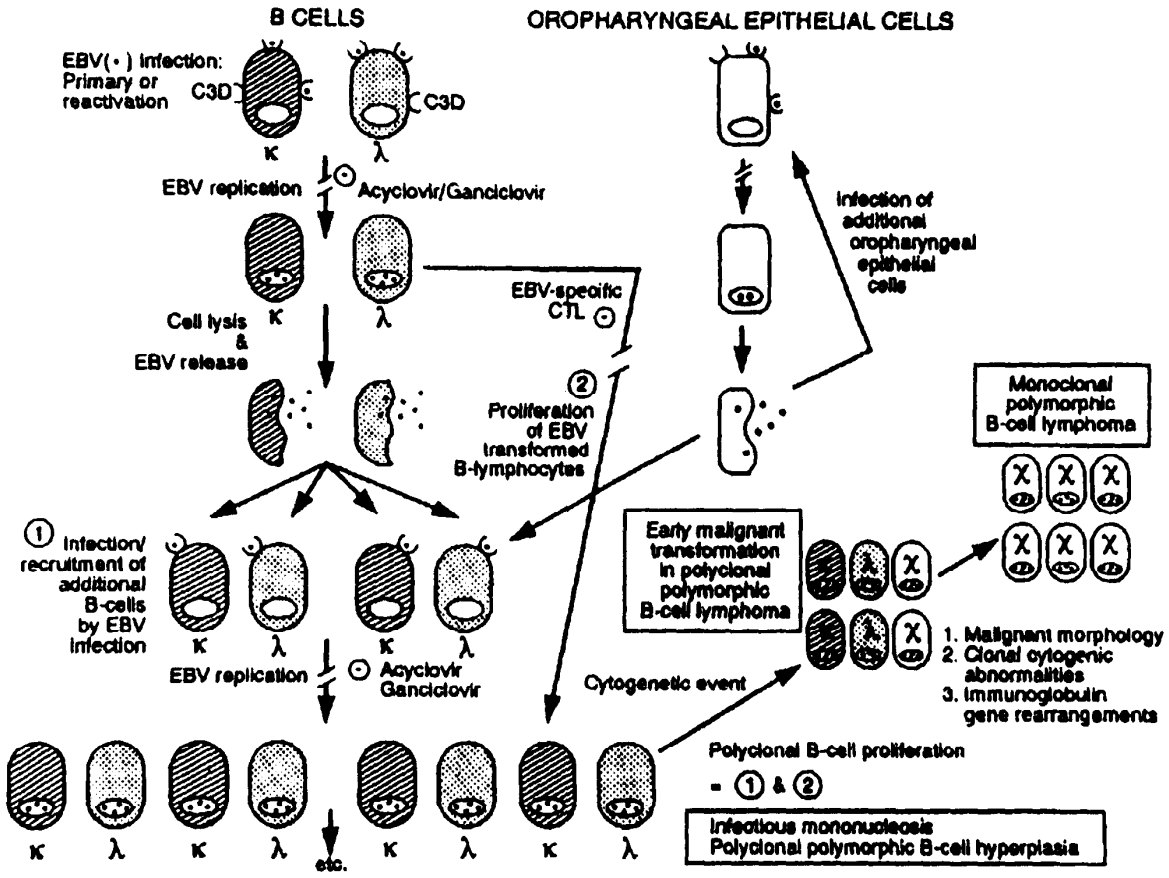

Figure 5 Proposed pathogenesis of EBV-induced posttransplantation lymphoproliferative diseases.

patients by molecular methods reveals evidence of clonal populations or cytogenetic abnormalities. Because of the risk of progression from polyclonal to a monoclonal B-cell proliferation, immunosuppression should be reduced or stopped in most kidney transplant patients with this lymphoproliferative disease, and rejection should be allowed to proceed, if it occurs. Acyclovir may control the polyclonal aspect of the proliferation and reduce the risk of further malignant transformation of proliferating B cells, but it does not alter the synthesis of the latent EBV genome in nonproducer cell lines (104) and therefore would not be expected to treat the clonal population of cells in these tumors.

It is in this group of patients and the group 4 patients that a variety of immunologically mediated therapies based on imperfect understanding of the pathogenenesis of this disease have been advocated.

Interferon-α and intravenous immunoglobulin have been used as a treatment of EBV-related PTLD in immunosuppressed patients, including one child with PTLD following liver transplantation and five patients in the Minnesota series (97,131). It has been proposed that, because interferon possesses antiproliferative activity and antiviral activity and it is capable of augmenting EBV CTL activity, it could be a potent therapy for EBV-associated PTLD. The child in question, who had a polymorphic, monoclonal tumor, did respond and was free of tumor as of the date of the report, but the therapy, which also included cessation of cyclosporine and intravenous acyclovir, did precipitate a rejection that was subse-

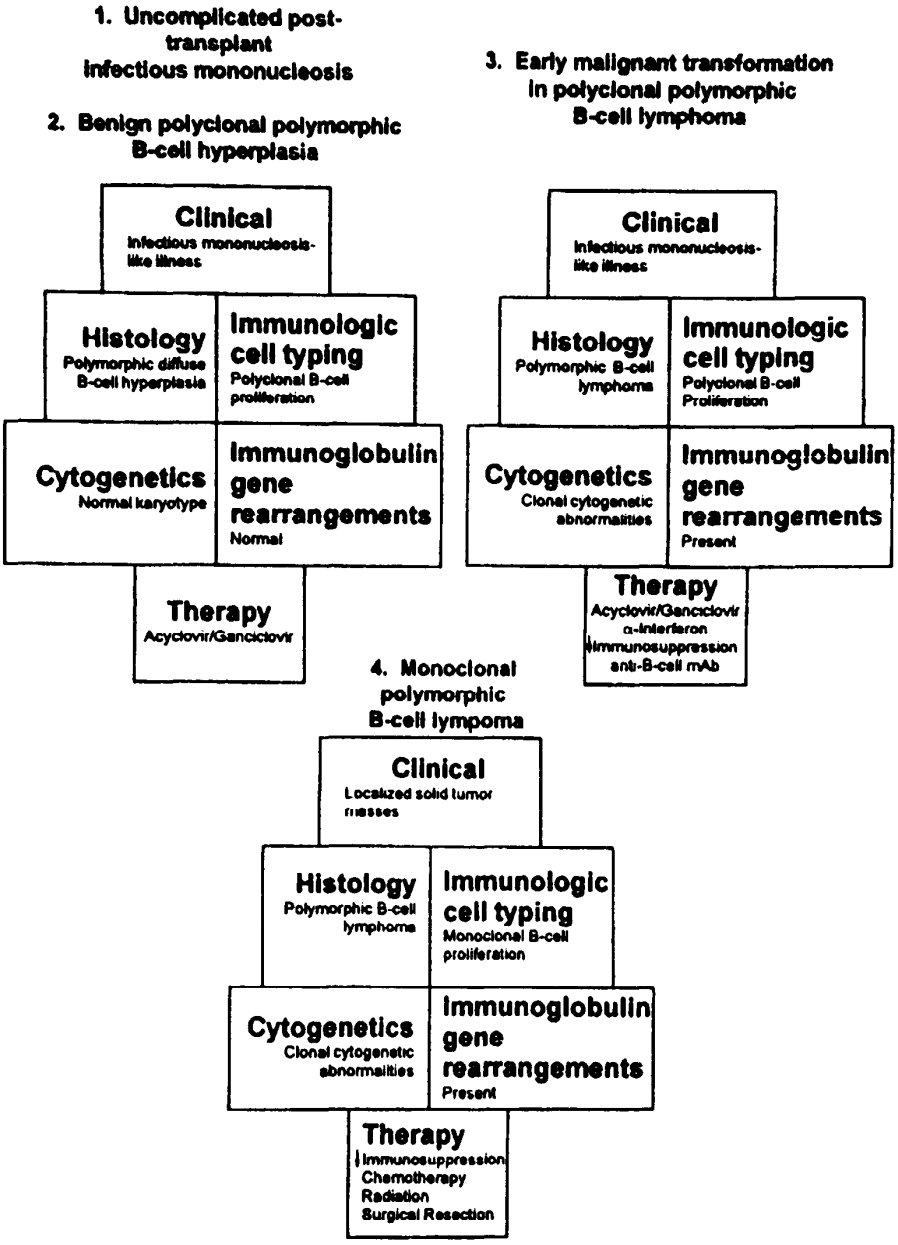

Figure 6 Summary of renal transplant recipients with EBV-related lymphoproliferative diseases.

quently controlled (131). Results in the Minnesota series were equivocal; one patient obtained a complete response, one patient died, one patient had therapy discontinued secondary to toxicity, and two patients had partial responses (97).

Anti–B-cell antibody therapy has been advocated as a potential treatment for PTLD and there are anecdotal reports of treatment (135). In the only larger series available from a multicenter trial in Europe (136), 12 organ transplant recipients were seen. Although the overall response was encouraging, no patient with a monoclonal tumor evidenced a response, and clonality was determined by surface immunoglobulin staining only. It seems that anti–B-cell antibodies will therefore be most useful as an adjunct in patients with polyclonal tumors who are unable to undergo a reduction in immunosuppression.

Lastly, attempts to bolster immune surveillance by infusions of virus-specific CTL have produced promising results in bone marrow transplant patients (137). Rooney et al.

followed up patients after bone marrow transplantation with serial peripheral blood determinations of EBV DNA levels. Three patients were identified by screening or development of lymphoproliferative disease. The patients were then given infusions of virus-specific CTL of donor origin prepared in anticipation of development of EBV-related disease. All three patients did well clinically and EBV DNA levels declined to normal (Fig. 7). Significant hurdles remain before such a strategy could be adapted to solid organ transplant patients, but the general strategy in bone marrow transplantation of prospectively examining patients for the appearance of EBV DNA in peripheral blood may allow identification of patients earlier in the proliferative process (138,139).

Group 3 patients include older patients in whom there is development of extranodal solid tumors that are morphologically malignant (PBL) and monoclonal and generally contain clonal cytogenetic abnormalities and immunoglobulin gene rearrangements in a majority of cells. These lymphomas may begin as EBV-induced proliferations in association with cytogenetic transformation and are composed of latently infected (and therefore acyclovir-insensitive) B cells. Standard chemotherapy or radiation therapy combined with discontinuation of immunosuppressive drugs should be used in these patients, whose mortality rates remain greater than 80%.

Other forms of treatment may be applicable in selected patients. Radiation therapy combined with a reduction in immunosuppression is still the treatment of choice in primary CNS lymphomas, although the recurrence rate is high and survival time is short (107). They are histologically identical to the other EBV-related lymphomas and may be caused by EBV (140). Therefore, the addition of acyclovir therapy may be helpful in these patients, depending on clonality.

V. T-CELL LYMPHOMAS

Although as many as 14% of PTLDs are purported to be of T-cell origin (86,111), detailed examination of these cases has been sparse, with fewer than 30 cases being documented (97,111,124,141–144). Van Gorp recently reported on 22 cases of posttransplant T-cell

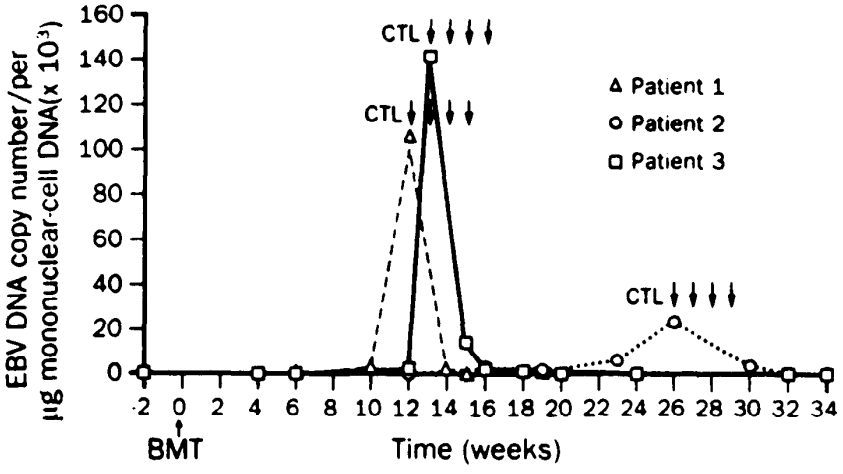

Figure 7 Epstein-Barr virus DNA concentrations before and after CTL infusions in three patients with evidence of EBV-related lymphoproliferations. (From Ref. 138.)

lymphomas (144). Nineteen of the 22 cases were peripheral T-cell lymphomas, with extranodal involvement in all cases except one. In nine cases in which T-cell receptor rearrangement was investigated, monoclonal tumor populations were found in eight, in contrast to the frequently polyclonal arrangements of B-cell lymphomas mentioned previously. The tumors were extremely virulent, resulting in 14 deaths despite reduction in immunosuppression, and tumors in patients on cyclosporine-based immunosuppression appeared earlier, primarily in the first 2.5 years after transplantation, as opposed to tumors in patients on azathioprine and steroids in whom tumors were more likely to appear 6 to 10 years after transplantation. In a single-center experience from France (111), three more T-cell lymphomas were reported, representing 12% of cases of PTLD at that center. All three tumors were monoclonal and all three patients died, despite reduction of immunosuppression in two.

That EBV contributes to the increased incidence of high-grade non-Hodgkin's lymphoma in chronically immunosuppressed individuals is unquestionable. This represents an exaggeration of an otherwise normally controlled biological relationship of EBV and host B cells. It seems unlikely that a similar relationship exists between EBV and T-cell lymphoma. In 22 T-cell lymphomas reviewed by van Gorp, only four exhibited strong evidence of EBV (144). The relatively diminished frequencies of Hodgkin's disease and T-cell non-Hodgkin's lymphoma compared with B-cell non-Hodgkin's lymphoma in immunodeficient patients probably reflect the incidence of sporadic cases in the general population, which are, at present, of unknown origin. How immune deficiency contributes to the cause and aggressiveness of these lymphomas needs to be investigated as additional cases are recognized.

VI. GENITOURINARY CANCERS

McKhann's 1969 report of a patient in whom squamous cell carcinoma of the cervix developed 2.5 years after renal transplantation (1) was the first of its kind. Subsequently, other reports of genitourinary cancers after transplantation appeared (145,149–152). Grouped together, genitourinary cancers include tumors of the bladder, kidney, and ureter as well as gynecological cancers such as cervical dysplasia, carcinoma in situ and invasive squamous cell carcinoma of the cervix, and metastatic malignant dysgerminoma of the ovary. Sheil reported genitourinary cancers to be the most common non-skin malignancy in transplant patients, accounting for 34% of all tumors, and with an increased risk of four times the general population (11). In the CTTR, genitourinary tumors account for 12% of the tumors reported (6).

Uterine cancers occur in 4% of patients in whom de novo neoplasms develop after transplantation and they are the fifth most frequent type (6). Cervical carcinoma was most common, comprising 11% of posttransplantation cancers in women; 203 (73%) of the women with cervical lesions had carcinoma in situ. The risk of development of carcinoma of the cervix in situ has been calculated to be 14 times that of an age-matched control population (145). Endometrial carcinoma was rare, reflecting either the younger age of transplant recipients or the different mechanisms responsible for development of disease.

Renal cancers in transplant patients are almost equally divided between lymphoma or carcinoma (6). Although in the general population, 5% to 10% of renal cancers originate in the renal pelvis, this proportion increases to 14% in transplant patients (86). The risk of cancer of the bladder, kidney, or ureter is 4.2, 8.9, or greater than a 1000 times that of the general populations, respectively (11). These increased incidences and risks are undoubtedly

at least partly due to the fact that the population of patients receiving renal allografts is much more likely to possess serious genitourinary tract abnormalities that are known to, by themselves, predispose the patient to malignancy.

Conventional therapy for cervical carcinoma, including simple hysterectomy for minimally invasive disease and cone excision of the cervix or cryosurgery for carcinoma in situ, has been effective. As in most epithelial cancers, a reduction in immunosuppression does not seem to be essential or even advisable. The morbidity and mortality rates are low. In one report, only 2 of 18 patients with cervical carcinomas (16 carcinoma in situ and 2 invasive) who received conventional therapy had progression of their disease (145). One patient did well after a hysterectomy for recurrent cervical carcinoma after conization, but the other died of metastatic disease following a hysterectomy for invasive carcinoma and radiotherapy for a vaginal vault recurrence. All female transplant recipients who receive standard immunosuppressive therapy should have frequent, regular gynecological examinations, including Papanicolaou smears. Likewise, conventional therapy of cancer of the kidney, ureter, and bladder is recommended in transplant patients afflicted with these tumors. No evidence suggests that reduction of immunosuppression is helpful although it often can be attempted. Tumors in transplant patients generally exhibit aggressive biological behavior (146), yet analysis of 80 patients with a variety of malignancies in the CTTR suggests that transplant patients whose tumors carry a good prognosis secondary to cell type and stage can undergo treatment in the same fashion as nontransplant patients with similar expectations of success (41).

The pathogenesis of cervical carcinomas in these patients is undoubtedly also multifactorial. There are many reports of cervical dysplasia and carcinoma developing in patients who receive therapy with busulfan, cyclophosphamide, azathioprine, prednisone, and methotrexate (145). In recent years, evidence has emerged strongly implicating human papillomavirus, especially HPV types 16 and 18, in the development of cervical cancer (147). Particular attention has been focused on the E6 and E7 gene products of HPV types 16 and 18, proteins capable of interacting with tumor suppressor mechanisms and thereby altering normal growth and differentiation. Women undergoing renal transplantation demonstrate virus-specific decreases in IgG titers to HPV antigens while manifesting increased IgA seroreactivity against early proteins of HPV within 3 months of transplantation (148). This has been interpreted as evidence suggesting reactivation of latent HPV following transplantation. Immunosuppression may act as a cofactor in the progression from viral infection to neoplastic transformation.

VII. TRANSPLANTATION OF KIDNEYS FROM DONORS WITH CANCER

Rarely, a malignant neoplasm is transplanted with the donor organ. Southam et al. reported the results of homotransplantation of human neoplastic cell lines into normal volunteers and terminal cancer patients (153) 8 years before the first report of a transplanted cancer (154,155). An inflammatory response was evoked in the volunteers, and the transplanted tumor implants were rejected within 4 weeks. In the cancer patients, however, the inflammatory response was depressed, the tumor implants often grew for 1 to 3 weeks before regression occurred at 4 to 6 weeks, and the tumor implants of several patients continued to grow until excised several weeks later. In 1965, McPhaul and McIntosh described a recipient of a cadaver kidney from a patient who died of squamous cell carcinoma of the piriform sinus (155). The recipient died 8 months after transplantation of squamous cell carcinoma that had metastasized to the liver and transplanted kidney. The inadvertent trans-

plantation of organs from donors with cancer has been reported many times since then (156–167).

Penn has recently updated the CTTR experience with tumors inadvertently transmitted by organ donors (168). The most common tumors in the donors were metastatic melanoma, bronchogenic carcinomas, which are the most frequent metastatic tumor found in the kidney in the general population (169), and primary renal carcinomas. Transplanted cancers appeared in 103 recipients of patients receiving organs from donors with malignancies. One very notable finding of the study was the transmission of five primary CNS tumors to organ recipients. In four of these patients, there were factors that may have contributed to such spread, including craniotomies or ventricular shunts. Particularly virulent were organ donors who transmitted malignant melanoma. Twenty organ recipients received allografts from donors with malignant melanoma and in only three the tumor did not develop. Of the 103 patients, 39 had tumors confined to the allograft, six had invasion of surrounding structures, and, in 58, there were distant metastases.

In summary, transplanted tumors appeared in 103 of 248 patients (42%) receiving organs from donors known to have cancer. As Penn has pointed out, the chance of transmission of cancer to the recipient is less for low-grade cancers that rarely metastasize and highest for bronchial and renal carcinomas. In 15 of 24 patients, widespread metastases occurred and resulted in death in a majority. Only three cases of "immunologic" rejection of the transmitted cancer after cessation of immunosuppressive medication have been reported. Prevention is critical.

To prevent transmission of malignant cells by the transplanted organ, the first priority is to not accept any donor with a known cancer, other than low-grade skin cancers or primary central nervous system neoplasms without evidence of tentoral violation. Because occult primary tumors, especially of the bronchus, often appear to be a primary brain tumor, a firm tissue diagnosis should be obtained before transplantation from such a donor. If a suspicious lesion is found at the time of a donor nephrectomy, a biopsy and frozen section diagnosis should be obtained. If the lesion is malignant, the organ should not be transplanted. If histological evidence of cancer is not available or is later revised on permanent histological section, a transplant nephrectomy (preferably) or wide excision of the lesion should be performed.

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Impact of Cytomegalovirus Infection on Renal Transplantation

ROBERT H. RUBIN

Harvard-Massachusetts Institute of Technology Center for Experimental Pharmacology and Therapeutics and Massachusetts General Hospital, Boston, Massachusetts

ROBERT B. COLVIN

Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts

I. INTRODUCTION

Cytomegalovirus (CMV) remains the single most important microbial pathogen among organ transplant recipients, with evidence of viral replication being found in at least 50% of patients. The consequences of CMV replication in these patients are, in large part, related to three major characteristics of this virus: latency, cell association, and potential oncogenicity (1,2).

A. Latency

The concept of latency refers to the observation that CMV seropositive individuals without active replicating virus harbor latent virus in various tissues that is capable of being reactivated under certain conditions. The most frequent of these conditions that lead to viral reactivation are allograft rejection, treatment with antilymphocyte antibodies (both polyclonal antithymocyte globulin [ATG] and the monoclonal anti T-cell antibody [OKT3]), and sepsis, particularly that caused by gram-negative organisms. Over the past several years, it has become apparent that the common denominator in the reactivation of CMV from latency is tumor necrosis factor (TNF). The interaction of TNF, generated in response to any clinical process, with the TNF receptor of cells results in the production of the transcription factor NFkB, which, in turn, initiates the transcription and subsequent reactivation of CMV from

latently infected cells. The clinical impact of such reactivation is then determined by the immune status of the individual (1–3).

B. Cell Association

The virus is highly cell associated, which means that transmission between individuals occurs as a result of cell-to-cell contact, and systemic spread (e.g., viremia) occurs via infected cells. As a result, humoral immunity is relatively inefficient in the control of the virus, and the key host defense is major histocompatibility complex (MHC)-restricted, virus-specific, cytotoxic T cells, precisely the aspect of host defense most inhibited by current immunosuppressive strategies. The MHC restriction of this host response raises the possibility that the MHC-mismatched allograft could serve as a privileged site for viral replication, consistent with the observation that replicating virus may be excreted in the urine of renal allograft recipients for many years. Careful studies in renal allograft recipients have demonstrated that a major consequence of CMV viremia is lytic infection of capillary endothelial cells, thus causing an "infectious vasculitis," accounting for the myriad of clinical syndromes caused by this virus in transplant patients (1,2,4).

C. Potential Oncogenicity

Like all herpes group viruses, CMV is regarded as potentially oncogenic. At present, the clearest evidence of a role for CMV in transplant patients is in the pathogenesis of Epstein-Barr virus (EBV)-associated posttransplant lymphoproliferative disease (PTLD). The risk of PTLD is more than seven times greater in patients who have had symptomatic CMV disease than in those without such overt manifestations of this infection (1).

The clinical impact of the virus can be divided into two general categories: the direct causation of a variety of clinical infectious disease syndromes and the indirect contribution of CMV to the pathogenesis of other, multifactorial disease processes. This latter group of effects includes oncogenesis, a rather global immunosuppressive effect that contributes to opportunistic superinfection with a variety of organisms (e.g., *Pneumocystis carinii*, *Listeria monocytogenes*, and a variety of fungi), and a role in the causation of allograft injury. The indirect effects of CMV appear to be mediated by cytokines and growth factors released in response to tissue-invading replicating virus and the resultant inflammatory response. There is thus the recognition that cytokines, particularly TNF, elaborated in one condition, will modulate the extent of CMV infection; conversely, cytokines elaborated in response to CMV will modulate other important processes. This bidirectional trafficking in cytokines is the main focus of this discussion on the interaction between this virus and the renal allograft. The question that has been asked for more than 2 decades—"Which comes first, allograft injury or CMV?"—has been answered: either or both (1–3,5).

II. PATTERNS OF TRANSMISSION OF CYTOMEGALOVIRUS IN RENAL TRANSPLANT PATIENTS

There are three patterns of transmission of CMV in the transplant patient: primary CMV infection, reactivation CMV infection, and CMV superinfection (1,2).

A. Primary Cytomegalovirus Infection

Primary CMV infection occurs when seronegative individuals receive latently infected cells from seropositive donors, with subsequent reactivation of the virus after transplantation. In more than 90% of instances, these latently infected cells lie within the allograft

(donor positive and recipient negative [D+R–]); however, viable leukocytes in blood products drawn from seropositive donors can also transmit the virus. The attack rate for the direct clinical manifestations of CMV is 50% to 65% for D+R– transplants, which account for approximately 10% to 15% of renal transplantations performed. Rarely, seronegative recipients of a kidney from a seronegative donor acquire primary CMV disease through intimate contact in the general community.

B. Reactivation Cytomegalovirus Infection

Reactivation CMV infection occurs when seropositive individuals reactive endogenous virus after transplantation (donor either seropositive or seronegative and recipient positive [D?R+]). Overall, an estimated 60% to 70% of individuals coming to transplantation are CMV seropositive, with approximately 10% to 20% of these individuals becoming ill as a direct consequence of viral reactivation. However, the indirect effects of CMV may be occurring in the absence of recognizable clinical symptoms of infection.

C. Cytomegalovirus Superinfection

In at least 50% of the instances in which an allograft from a seropositive donor is transplanted into a seropositive recipient (D+R+), the virus that is reactivated and disseminates systemically is of donor rather than recipient origin. This reflection of the genomic and antigenic diversity among CMV isolates in nature is associated with an attack rate of 20% to 40% for clinical disease. D+R+ transplants account for approximately 25% of renal transplantations performed.

III. IMPACT OF IMMUNOSUPPRESSION ON THE PATHOGENESIS OF CYTOMEGALOVIRUS INFECTION IN THE RENAL TRANSPLANT RECIPIENT

Other than the past experience of the donor and recipient with CMV (the laboratory marker of this being seropositivity), the most important determinant of the effect of CMV on the transplant patient is the nature and intensity of the immunosuppressive therapy being administered. Careful clinical and experimental studies have demonstrated that different immunosuppressive drugs, both alone and in combination, have very different effects on the three key steps in the pathogenesis of CMV disease: reactivation from latency, systemic dissemination, and host control by the virus-specific, cytotoxic T-cell response (1,2,6).

Immunosuppressive agents can be divided into three categories in terms of their ability to reactivate CMV from latency: (1) antilymphocyte antibodies, both ATG and OKT3 are the most potent reactivating agents, presumably because of the TNF elaborated in response to their administration; (2) cytotoxic agents, namely azathioprine, cyclophosphamide, and mycophenolate, have moderate activity in terms of reactivating latent virus; (3) cyclosporine, tacrolimus (FK506), rapamycin, and prednisone have no ability to reactivate latent CMV. Because allograft rejection is also a potent stimulus for reactivating latent virus, it seems that the use of an antilymphocyte antibody to treat acute rejection would be a particularly potent stimulus for reactivating latent virus. Indeed, in studies of seropositive renal allograft recipients, it was demonstrated that if immunosuppression consisted just of cyclosporine, azathioprine, and prednisone, the incidence of clinical disease was approximately 10%, but if an antilymphocyte antibody was used as induction therapy before initiation of the three oral drugs, the incidence of clinical disease increased to 24%. However,

if an antilymphocyte antibody was needed to treat steroid-resistant acute allograft rejection, then the rate of clinical CMV disease increased to 65% (6–8).

Once there is active, replicating virus present, there is a marked change in the impact of immunosuppressive drugs. There is a reversal in the potency of the three categories of immunosuppressive agent in terms of the magnitude of their effects on the course of the infection: (1) cyclosporine, tacrolimus, rapamycin, and prednisone amplify the extent and effects of the replicating virus by inhibiting the cytotoxic T-cell antiviral response (we refer to cyclosporine and tacrolimus as "in vivo PCRs [polymerase chain reactors]" of CMV) and (2) the cytotoxic agents and the antilymphocyte antibodies are both moderately active in terms of increasing the impact of CMV infection (6–8). There are several implications of these observations as follows:

1. Because immunosuppressive drugs impact different aspects of CMV pathogenesis, it is the net effects of the entire regimen that should be considered. Thus, both in animal models and in transplant patients, the greatest problems from CMV is in patients treated with antilymphocyte antibodies, particularly for rejection (this combination providing the most potent viral reactivating stimulus), followed by cyclosporine or tacrolimus immunosuppression ("the PCR effect") (1,8).

2. The benefits of anti-CMV preventive strategies tend to be attenuated in the face of antilymphocyte antibody therapy, unless the antiviral regimen is intensified in conjunction with the antilymphocyte antibody therapy. For example, administering the antiviral ganciclovir during antilymphocyte antibody therapy of CMV seropositive individuals decreases the incidence of clinical disease by a factor of three. This and other similar observations have led to the concept of the "therapeutic prescription" for the transplant recipient. This has two components, an immunosuppressive component to prevent and treat rejection and an antimicrobial component to make it safe. In the case of CMV, the intensity of the immunosuppressive regimen determines the intensity of the antiviral preventive strategy that is needed, with particular attention to the administration of antilymphocyte antibody therapy in conjunction with cyclosporine or tacrolimus therapy (1,8).

3. Before the cyclosporine era, when immunosuppression consisted primarily of azathioprine and prednisone with or without antilymphocyte antibody therapy, relapsing CMV infection was rare. In the present era, relapsing disease (defined as clinically symptomatic infection diagnosed and treated with a 2- to 3-week course of intravenous ganciclovir, with an appropriate response, followed within 6 weeks by a recrudescence of full-blown clinical disease) is not uncommon, with an incidence of 10% to 20% in seropositive individuals and an incidence of 50% to 65% in those with primary infection. This relapse in renal transplant recipients is not due to antiviral resistance; rather, it appears to be due to persistence of a small amount of virus at the end of the course of antiviral therapy, and then an amplification of the extent of this infection because of the profound inhibitory effect of cyclosporine and tacrolimus on the critical CMV-specific cytotoxic T-cell response (1,2,8).

IV. CLINICAL EFFECTS OF CYTOMEGALOVIRUS IN THE RENAL TRANSPLANT PATIENT

Whatever the pattern of transmission, if clinical CMV syndromes resulting from the direct tissue invasive effects of the virus occur, most instances occur 4 to 16 weeks after transplantation, with a peak incidence at 4 to 6 weeks. As with most viral infections, CMV infection usually begins insidiously with constitutional symptoms of anorexia, malaise, and fever, often accompanied by myalgia and arthralgias. In many patients, unexplained fever

and constitutional symptoms, often with accompanying neutropenia with or without thrombocytopenia, subclinical hepatitis, and a mild atypical lymphocytosis (a mononucleosis-like illness frequently referred to as the CMV syndrome), is the sole manifestation of this systemic infection. The most feared complication of CMV infection is pneumonia (Fig. 1). Typically, a nonproductive cough develops within a few days of the onset of the constitutional symptoms. Initially, dyspnea and tachypnea are not noted, but over several days progressive respiratory distress can ensue. The attack rate for pneumonia is highest in those patients with primary infection (D+R–) and in those who have undergone intensive immunosuppression for severe rejection without a concomitant increase in antiviral preventive therapy (1,2).

The gut is commonly involved with CMV, resulting in diffuse inflammation with functional disturbances, hemorrhage, frank ulceration, perforation, and, possibly, the development of pneumatosis intestinalis. The stomach appears to be the most frequent site of symptomatic CMV infection, not infrequently causing subjective complaints of nausea, a sense of abdominal fullness, and dysphagia, with evidence of poor gastric emptying as a consequence of the infection. Cytomegalovirus infection at other sites in the upper gastrointestinal (GI) tract is also not uncommon, with both esophagitis and duodenitis being well described. Colonic ulceration, often involving the right colon, presenting as GI hemorrhage, perforation, or even pseudomembranous colitis is also not uncommon. Gastrointestinal manifestations of CMV infection can occur in the absence of fever, leukopenia, or other manifestations of systemic CMV disease (1,2,9–14).

Presumably related to the endothelialitis produced by CMV (1), a wide variety of other clinical syndromes due to the direct effects of CMV have been reported uncommonly in transplant recipients, including endometritis, encephalitis and transverse myelitis, and a cutaneous vasculitis. Chorioretinitis is the major late manifestation of CMV infection, usually being noted for the first time more than 6 months after transplantation (1,2).

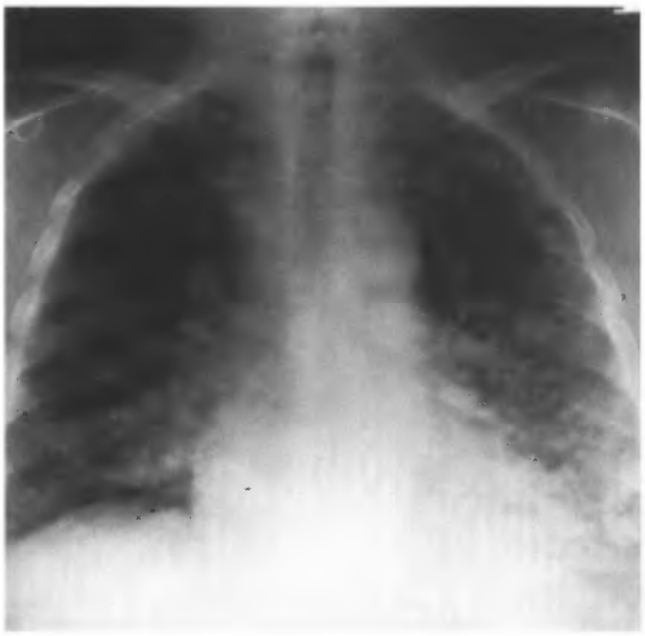

Figure 1 Biopsy-proven cytomegalovirus pneumonia in a recipient of a cadaver donor renal allograft. Chest x-ray and lung biopsy performed 8 weeks posttransplant.

V. DIAGNOSIS OF CYTOMEGALOVIRUS INFECTION

Asymptomatic CMV replication is not uncommon in transplant patients, as manifested by isolation of the virus from urine or respiratory secretions. However, it is unclear whether the indirect consequences of CMV replication, particularly the effects on allograft function, can proceed in the absence of a systemic inflammatory response. In any case, cultures of these sites of possibly inconsequential CMV replication are not a reliable means for making a diagnosis of clinically important infection. There are two approaches to diagnosis: (1) demonstration in tissue biopsies of the virus histologically (e.g., recognition of the pathognomonic CMV cells with their characteristic inclusions), immunochemically, by in situ hybridization, or on culture, or (2) demonstration of viremia. Although asymptomatic viremia can occur, the demonstration of viremia in patients with a clinically compatible syndrome is sufficient evidence to institute antiviral therapy. The absence of viremia is strong evidence against CMV being a cause of pneumonia, hepatitis, or virtually all manifestations of CMV infection, except for local infection of the gut, particularly the stomach, which can be present in the absence of viremia (1,2,15).

As befits the importance of demonstrating viremia in the diagnostic approach to CMV infection, a great deal of attention has been devoted to developing sensitive and specific assays whose results are available in timely fashion. The gold standard has traditionally been viral isolation by tissue culture, the endpoint being the development of a characteristic cytopathic effect. However, this technique can require several weeks for reliable interpretation, which is unacceptable in this era of antiviral therapy in which therapeutic decision-making requires more timely information. The first major advance was the development of the shell vial technique. With this approach, the clinical specimen is centrifuged onto the fibroblast monolayer, which assists adsorption of any virus present, resulting in a fourfold increase in the infectivity of the viral inoculum. Twenty-four to 48 hours later, when there is no visible evidence of viral replication, the fibroblast monolayer is stained with a monoclonal antibody to the 72-kD major immediate early protein of CMV, which can be demonstrated by immunofluorescence hours after inoculation. This is a highly specific technique that is very sensitive for demonstration of virus in tissue, urine, or respiratory secretions. However, its sensitivity is less than 50% for detecting viremia, causing its abandonment at most centers. The most accessible sensitive and specific technique for testing for viremia is the CMV antigenemia assay, as originally developed by The and colleagues in the Netherlands (16). In some laboratories, a quantitative PCR assay can reliably provide the same information (1,2,15,17,18). Quantitative antigenemia also has been proposed as a diagnostic test for symptomatic infection (19) and guide to therapy (20).

Serological testing is useful for defining the presence of latent virus in both donor and recipient before transplantation and defining the subsequent risk of symptomatic CMV disease. However, it is both insensitive and too delayed to provide useful information after transplantation in terms of diagnosing clinical infection. In particular, serial determinations of IgM anti-CMV antibody have proven to be of little value in the diagnosis and management of most patients, and they have been abandoned by many centers (1,2,15).

VI. EFFECT OF CYTOMEGALOVIRUS INFECTION ON RENAL ALLOGRAFTS

In 1970, Simmons and colleagues first suggested that CMV infection was associated with an increased risk of allograft dysfunction and loss (21). Many studies have ensued, and the association is still debated (22,23). The major difficulties that prevent clear resolution of

this issue have been (1) the ubiquity of CMV infection, (2) the lack of an adequate laboratory test for distinguishing clinically important from trivial CMV infection, and (3) the risk of CMV infection increasing as a consequence of treatment for rejection.

Cytomegalovirus infection has been associated with a higher rate of renal allograft loss in many series from individual centers (24,25). One study showed a 1 year graft loss of 24% among CMV-positive patients versus 12% for CMV-negative patients (25). Two studies showed a decreased renal allograft survival rate among patients with primary and reactivation CMV infection as opposed to those without infection with the virus (26,27). When "tissue-invasive" (symptomatic) infections were separated from asymptomatic infections, only the tissue-invasive group had an increased rate of graft loss (28).

In renal allografts, CMV infection has been associated with (1) asymptomatic infection, (2) acute tubulointerstitial nephritis, (3) acute tubulointerstitial rejection, (4) allograft glomerulopathy, and (5) allograft arteriopathy. It is useful to categorize these by pathogenetic mechanisms, namely, whether they arise from a direct infection or an indirect effect of a systemic CMV infection.

A. Direct Infection

1. Asymptomatic Infection

The kidney is one of the more common organs infected by CMV infection, somewhat less than the lung and GI tract, as judged by autopsy (29,30). Cytomegalovirus genome can be detected by in situ hybridization in the tubular epithelium in approximately 40% of renal allografts, independent of whether the patient had an active CMV infection at the time (31). Renal tubular cells in culture support persistent and prolonged CMV expression without significant cell death for more than 6 weeks, which may account for chronic viruria occurring in individuals with asymptomatic CMV infection (32). Other renal cell types are rarely infected in asymptomatic patients, although in some instances infection of glomerular endothelial and epithelial cells, peritubular endothelium, and infiltrating mononuclear cells has been demonstrated. Outside the kidney, smooth muscle cells as well as stromal and epithelial cells have been reported to contain viral antigens (33).

2. Acute Tubulointerstitial Nephritis

Clinically significant acute CMV tubulointerstitial nephritis has been well described in nontransplanted kidneys, more commonly among children than adults. Typically, a prominent infiltrate of mononuclear cells and plasma cells is present in the interstitium, accompanied by characteristic cytomegalic cytopathic changes of the tubular epithelium (intracytoplasmic and intranuclear inclusions) (34). Minor glomerular lesions have been noted (30), but generally no severe glomerular or vascular lesions have been detected (35,36). A few allografts with prominent CMV cytopathic changes and an interstitial nephritis have been described in which the graft failure was attributed to CMV infection rather than rejection (35–37). Cytopathic changes have been reported in glomerular and peritubular endothelial cells in addition to the usual tubular sites (37).

The key question is how to distinguish an infiltrate of T cells caused by CMV from that caused by rejection. The issue is further complicated by the common occurrence of asymptomatic renal CMV infection. In CMV nephritis in nontransplants, the infiltrate is largely $CD8^+$ (paralleling the CD8 lymphocytosis in the blood) (34). However, in rejection, the infiltrate may also be predominately $CD8^+$, particularly in irreversible rejection (38,39). Tubular cell expression of CMV early nuclear protein and HLA-DR distinguished systemic

CMV infection from rejection in fine-needle aspirates (FNA) (40). Cytomegalovirus antigen was positive in all cases of CMV infection with only 4% false-positive results. Tubular cell HLA-DR was never present in the absence of rejection. Another FNA study reported that HLA-DR was invariably expressed by tubular cells during CMV infection and was regarded as a reason for the associated increased frequency of late rejection (41).

3. Detection of Cytomegalovirus in the Kidney

Several ways to detect CMV infection of the kidney are listed as follows in approximate order of sensitivity: (1) electron microscopy, (2) histology for inclusions, (3) viral culture, (4) immunohistochemistry, and (5) PCR or in situ hybridization for CMV genome or viral mRNA. Cytopathic changes indicate productive infection but are not as sensitive an indicator as RT-PCR or immunological techniques (42). Electron microscopy for viral particles is even less sensitive (37). Viral culture is slow and tedious, and requires fresh tissue.

Polymerase chain reaction or in situ hybridization for CMV genome does not distinguish productive from latent infections (43). Cytomegalovirus genome is detected in the absence of cytopathic changes. Overall, 41% of renal transplant biopsies showed CMV genome by in situ hybridization, whether or not an active CMV infection was present at the time of the biopsy. The presence of CMV genome was not associated with any perceptible change in the pattern of rejection or the phenotype of the infiltrate (44). In latent infections, the virus is largely present in tubular epithelial cells. In apparent contrast, during active CMV infection, the viral genome was detected primarily in focal clusters of infiltrating mononuclear cells (45).

Detection of synthesis of viral mRNA for late antigen by RT-PCR showed a 100% correspondence with CMV disease in six cases (as opposed to seropositivity); only 33% had detectable viral inclusions (46). The authors concluded that the PCR test is more sensitive and specific for CMV disease than either serological or histological testing and faster than viral cultures.

B. Indirect Renal Effects

It is well known, since the writings of William Councilman in 1898 (47), that certain viral and bacterial infections can exert an indirect effect on the kidney, producing tubulointerstitial inflammation. The effects have been attributed to immunological cross-reaction, systemic cytokine release, and microbial toxins (48). It is not surprising, therefore, that CMV might also cause renal injury by indirect means (49). Cytomegalovirus appears to exert most of its effects through activation of the rejection process, which commonly becomes focused on glomeruli or vessels.

1. Acute Tubulointerstitial Rejection

Several prospective studies document the increased risk (from twofold to fourfold) of acute rejection in patients with CMV infection. In three such studies, the frequency of acute rejection among CMV-negative versus CMV-positive patients was 27% versus 60% (50), 25% versus 48% (25), and 11% versus 45% (51). The frequency of late rejections was fivefold higher in patients with proved CMV disease (86%) compared with those without CMV disease (17%) (41). Overall, 75% of 144 patients in whom a CMV infection developed had a coincident increase in serum Cr of at least 25% (25), which returned to baseline in 75%. A temporal relationship to viremia has been documented in approximately 70% of cases (52). The issue is whether the "acute rejection" is truly rejection activated by the CMV infection,

a "pseudorejection" caused by CMV cytopathic effects, or an immunological reaction to CMV antigens.

Perhaps the most convincing evidence that CMV promotes graft injury is the study of Reinke et al. who showed that 17 of 21 patients who had "late-acute rejection" on biopsy responded to ganciclovir (53). The outcome with conventional immunosuppression was considerably worse, with 80% graft failure at 1 year. These patients had no symptoms of CMV infection, although virus was detectable in the blood in 80% by PCR and in 42% by the antigenemia test. Curiously, the patients did not demonstrate a humoral response, as judged by a lack of change of the anti-CMV titer. A characteristic feature was the expansion of $CD57^+LFA\text{-}1(bright^+)$ $CD8^+$ cells in the blood to greater than 20%, which also occurs in symptomatic infections.

2. Allograft Glomerulopathy

In 1981, Richardson and colleagues described a distinctive pattern of glomerular injury in renal allografts that they associated with CMV viremia (54) and which they subsequently termed acute allograft glomerulopathy (39). Clinically, these patients had progressive azotemia a few weeks to months after transplantation, sometimes with proteinuria. The salient pathological features were diffuse endothelial hypertrophy and necrosis accompanied by accumulation of fine fibrillar webs of periodic acid–Schiff (PAS)-positive material and mononuclear cells that resulted in obliteration of the glomerular capillaries (Fig. 2).

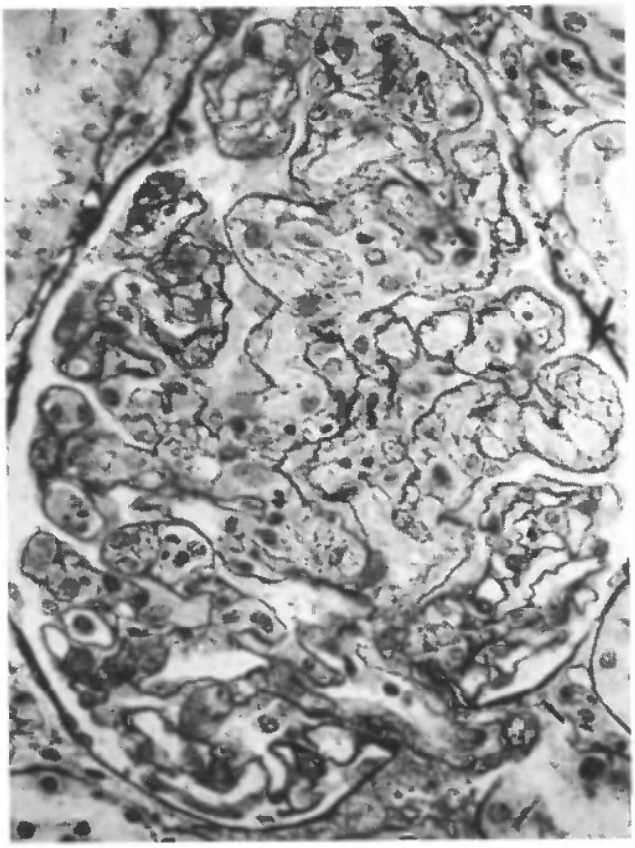

Figure 2 Glomerulopathy in a patient with CMV infection. Most of the capillary loops contain swollen endothelial cells and mononuclear leukocytes. The arrow points to a capillary with webs of material reactive with periodic acid-Schiff (PAS stain) (×400). (Reprinted from Ref. 63 with the permission of the *New England Journal of Medicine.*)

Fibrin and small amounts of IgM and C3 were found by immunofluorescence, and occasional subendothelial deposits were found by electron microscopy. Raji cell reactivity, presumed to be immune complexes, was present in the serum of the two patients studied. No viral particles were detectable by electron microscopy, nor were any viral antigens detected using polyclonal anti-CMV antibodies or two monoclonal antibodies to CMV early and late antigens. An important feature was the minimal degree of interstitial mononuclear infiltrate that usually accompanied the glomerulopathy. Associated vascular injury was seen in one of the original cases and several subsequent cases (55). One patient showed evolution of the acute glomerulopathy into a chronic allograft glomerulopathy. The prognosis was poor, with 66% graft failure.

Subsequent studies have confirmed the distinctive pathology noted by Richardson and colleagues and have confirmed many other key features, namely occurrence in the absence of tubulointerstitial rejection (56), the association with vascular rejection (30,35, 39,56–59), the lack of CMV in the lesions (30,35,42), and the poor prognosis (35,57,58). An essential requirement in the glomerulopathy, however, is the renal allograft itself. This lesion does not develop in patients without a renal allograft who contract CMV infection (30,35), although a mild mesangial proliferative glomerulonephritis has been reported (60).

The glomerulus is normally relatively spared in acute cellular rejection. The accumulation of a few mononuclear cells in glomerular capillaries can sometimes be seen (61), but the number of lymphocytes is greater in the glomerulopathy and the endothelial reaction is more marked. In our experience, PAS-stained 2-μm sections are best for demonstrating the characteristic features. Glomerular thrombosis and necrosis can accompany severe vascular rejection (62), but, in the glomerulopathy, global necrosis and thrombosis are not prominent.

Although the absence of interstitial rejection helped in the initial recognition of this injury, subsequent cases have been seen in association with typical interstitial infiltrates. One example is particularly instructive (Fig. 3). The initial biopsy showed a combination of interstitial rejection and the glomerulopathy. The patient was given OKT3 and initially responded, but renal function worsened after therapy was completed. A biopsy showed resolution of the interstitial process but a progression of the glomerulopathy, a strong argument that the two types of injury have a different pathogenesis.

Progression of acute to chronic allograft glomerulopathy in sequential biopsies has been noted in several cases (54,63,64). Thus, prior episodes of acute allograft glomerulopathy could account for some of the cases of duplication of the glomerular basement membrane that is seen commonly in late biopsies of renal allografts. In serial biopsies, 13 of the 34 patients with chronic glomerulopathy had acute allograft glomerulopathy documented on a prior biopsy (64).

How strong is the evidence that the glomerulopathy is related to CMV? Many studies have confirmed an association of the glomerulopathy with CMV infection (39,57,59, 63,65–68), but several studies did not find an association (30,35,42,56,58,69,70). The consensus is that CMV is neither necessary nor sufficient for the lesion to develop. However, if all the data are aggregated in a meta-analysis, there have been 175 cases of acute allograft glomerulopathy reported, and 67% have had CMV infection (compared with the 47% frequency of CMV in the 146 cases without glomerulopathy). Although this is statistically different ($p < 0.001$ by chi square analysis), there are major issues in the interpretation of these heterogeneous published data:

1. The criteria for infection with CMV vary, from viremia to seropositivity and from disease to asymptomatic infection. The criteria for the timing accepted for a causal relationship also vary.

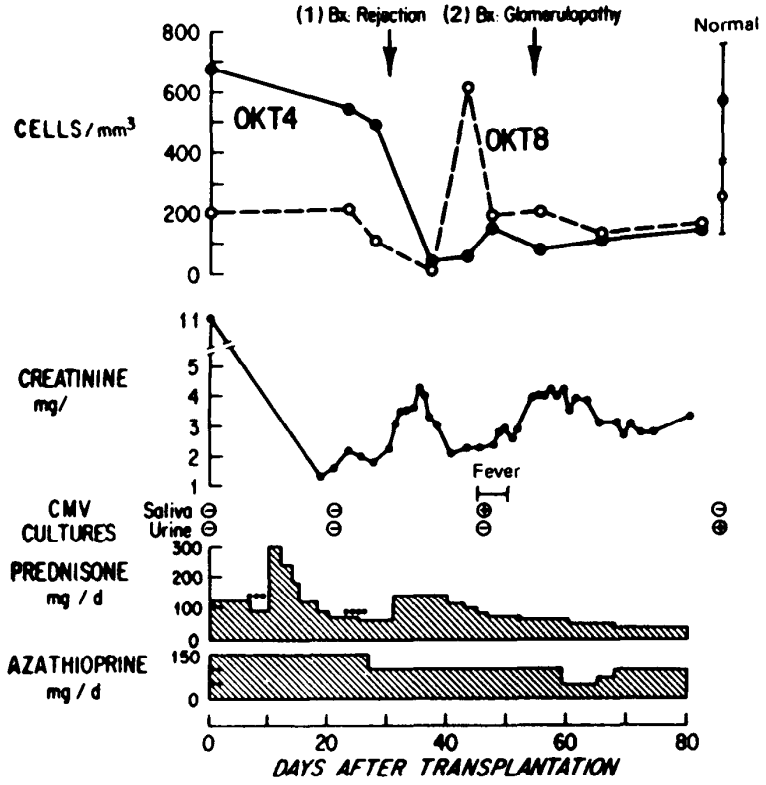

Figure 3 Clinical course of a patient with early rise in creatinine and biopsy 1 showing rejection and glomerulopathy. After initial response to immunosuppression, creatinine rose and biopsy 2 revealed only glomerulopathy.

2. The criteria for the glomerulopathy differ, in that some investigators include mild lesions and some recognize only severe lesions. The difference in criteria are reflected in a marked range of incidence of the glomerulopathy in transplant biopsies (2.5% to 28%). Centers differ in the frequency and intensity of the glomerulopathy even in the same study (56).

The frequency and sensitivity of the viral cultures influence the number of CMV infections detected, particularly the buffy coat cultures. In our original series, five of eight patients with viremia had the glomerulopathy (67%), whereas none of seven without viremia had the lesion, results comparable to those from another transplant center (65). Subsequent studies (55,63) showed that the glomerulopathy could occur in the absence of demonstrable CMV viremia (seven patients with the glomerulopathy had negative buffy coat cultures within 4 days of the renal biopsy) (63). Overall, in our experience CMV-infected recipients who are viremic have about twice the frequency of glomerulopathy as those who are nonviremic (58% versus 32%). Thus, viremia is a major risk factor but is neither necessary nor sufficient for the development of the glomerulopathy, which has not been found in patients in the absence of CMV. Because more than 50% of the patients in our institution have an active CMV infection in the first 100 days after transplantation (63), there remains the possibility that the association is fortuitous. Of the 36 biopsies taken for renal allograft dysfunction, however, the glomerulopathy was seen in 12 of 25 (48%) of those with CMV and 0 of 11 of those with negative CMV cultures or antibody titers within 3 weeks of the biopsy ($p < 0.005$) (63).

In addition, the T-lymphocyte subset changes induced by viral infection (the previously described "inverted CD4/CD8 ratio") have been associated with the glomerulopathy. In our series, 9 of 11 patients (82%) with the glomerulopathy had CD4/CD8 ratios of less

than 1.0, compared with 7 of 26 (27%) without the glomerulopathy ($p = 0.003$) (55); similar data have been reported by others (68). To determine what cells were directly involved in the glomerulopathy and their nature, we performed an immunoperoxidase analysis of the intraglomerular cells for surface markers (39). We found a 10-fold increase in $CD3^+$ T cells in the glomeruli affected with the glomerulopathy compared with the glomeruli within biopsies exhibiting interstitial rejection. All these T cells could be accounted for by the $CD8^+$ cells, which were selectively concentrated from the blood, in which 65% of the $CD3^+$ cells were $CD8^+$. CD4 was present on a minority of the cells within the glomeruli, some of which had the appearance of macrophages. The interglomerular mononuclear cells displayed activation markers, including HLA-DR, HLA-DC, and the interleukin (IL)-2 receptor. These data provide evidence that the glomerulopathy is mediated by a subset of T lymphocytes, namely CD8 cells, that are activated within the glomerulus. This subset includes both suppresser-effector cells and cytotoxic cells primarily reactive to HLA class I antigens whose absolute numbers increase in severe CMV infections (71,72).

The occurrence of the glomerulopathy is independent of the particular type of immunosuppressive therapy (although probably not the intensity), the histocompatibility match, and the patient's original disease. We have observed the glomerulopathy in patients receiving ATG, OKT3, cyclosporine, and azathioprine and steroids alone, in patients with transplants from HLA-identical siblings as well as mismatched cadaveric donors, and in patients whose original disease was nonglomerular (e.g., tuberculosis).

Pathogenesis of Glomerulopathy

There is no animal model of the glomerulopathy known to us. Cytomegalavirus infection of cyclosporine-treated rats bearing renal transplants has no obvious effect on the pathology of rejection, in particular no glomerular lesions were noted (73). Smith and Wehner (74) have described a mesangiopathic glomerulonephritis in mice in which cells in the mesangium are infected with CMV, as indicated by their characteristic cytopathic changes on light microscopy and viral particles on electron microscopy. In addition, glomerular mesangial staining with anti-CMV antiserum has been described in a variety of glomerular disorders, particularly IgA nephropathy (75). These are clearly different from the CMV-associated glomerulopathy seen in renal transplant recipients, in which direct viral infection of the glomerular cells is not demonstrable. Cytomegalovirus productively infects podocytes in vitro only after exposure to retinoic acid (32) and is detected only rarely in glomerular cells in transplant biopsies by in situ hybridization (31).

How, then, can this variety of information, both pro and con, be put together in a unitary hypothesis? The current evidence is adequate to rule out a direct effect of the virus or immune complex deposition in causing the allograft injury. We propose instead that certain indirect effects of the CMV infection, but effects not restricted to CMV infection, are involved. Other virus infections might produce similar systemic effects. Recent case reports of allograft glomerulopathy in patients infected with hepatitis C may represent such a phenomenon (76).

Cytomegalovirus infection has profound systemic effects, indicative of immune activation, including an elevation of blood levels of IL-6 (77), soluble forms of vascular cell adhesion molecule (VCAM)-1 (78), and IL-2R (79), and a marked increase in the numbers of circulating $CD2^+CD8^+HLA\text{-}DR^+$ cells compared with rejection (80). A humoral response is evident by the presence of circulating immune complexes (81,82), rheumatoid factor (83), and circulating products of complement activation C3d and C3a des-arg (82), which are all more commonly elevated in patients with CMV infection than in conventional

rejection. Cytomegalovirus infection of cells in vitro leads to increased levels of ICAM-1 and class I MHC antigens, but not class II MHC antigens (84). Murine CMV infection causes an increase in alloreactivity in some strains of mice, as judged by in vitro assays of T-cell–mediated cytolysis, coincident with emergence of autoreactivity (85).

A number of studies in recent years have emphasized that the upregulation of MHC antigen expression on allografted tissue is an important step in the pathogenesis of immune-mediated injury. A variety of cytokines, particularly α-interferon (INF-α) and γ-interferon (INF-γ) both of which are elaborated in response to CMV infection, can accomplish this upregulation. Thus, INF-α can enhance the expression of MHC class I antigens on spleen cells and glomeruli when given to mice (86), and the surface expression of MHC class I antigens and susceptibility to T-cell lysis by human cells are increased by INF-α in vitro (87). The administration of recombinant INF-α systemically to renal transplant patients was associated with graft loss and glomerular and vascular injury (88). One patient had nephrotic range proteinuria and intracapillary hypercellularity, consistent with acute allograft glomerulopathy. The INF-γ activates macrophages and increases the expression of HLA class II antigens on vascular endothelial cells (89). Thus, either of these mediators produced in response to such viruses as CMV has the potential for increasing alloantigen expression in the glomerulus. As discussed, MHC class I antigen is increased in glomerular capillary endothelium affected by this glomerulopathy (39) and CMV infection is accompanied by an upregulation of tubular MHC class II antigen in grafts (40,41).

We, therefore, suggest that certain forms of allograft injury are triggered by the interferon (or other cytokine)-induced upregulation of MHC antigens on glomerular, arterial, or endothelial cells, with alloreactive cytolytic $CD8^+$ lymphocytes being the major effectors of the injury. Although local cytokine release could be caused by a variety of processes, in clinical renal transplantation, CMV infection is one of the most important initiating factors. The absence of evidence of CMV infection in some patients with glomerulopathy and other forms of allograft injury could be because of the initiation of this process by other inducers of cytokine release (49). In this regard, it is notable that patients with CMV "disease," as opposed to asymptomatic CMV infection, have a higher frequency of co-infection with human herpesvirus 7 (90) and reactivation of EBV (91). It is possible that some of the effects attributed to CMV may actually derive from co-infecting viruses.

Molecular virologic studies of CMV have suggested other factors as well that may play a role in CMV-associated allograft injury. Sequence homology and immunological cross-reactivity between an immediate early antigen of human CMV and the HLA-DR β-chain have been demonstrated (92). In addition, CMV-infected cells produce a glycoprotein homologous to MHC class I antigens (93). Thus, it is not unreasonable to speculate that immune injury triggered by the virus could be directed at cells that bear either the appropriate HLA-DR antigen or the particular class I antigen.

3. Allograft Arteriopathy

Several studies have shown an association between CMV infection and acute and chronic vascular rejection in the kidney (30,35,39,56–59,94). Similar associations have been noted in heart transplants, both the chronic allograft arteriopathy (95,96) and the endothelialitis (97,98). Immunopathological analysis of cardiac transplant biopsies has shown that the onset of CMV viremia (antigenemia) is accompanied by an induction of VCAM-1 on capillary endothelial cells, which exceeded that in acute rejection (99). ELAM-1 induction was more characteristic of rejection than CMV. Perhaps related is the release of soluble VCAM-1 into the circulation during CMV infection (78).

The pathogenesis of the lesion is not established. One study reported the presence of CMV infection locally in the affected arterial wall in the heart (100), but a careful study of chronic allograft arteriopathy in kidneys was unable to detect CMV antigens by immunohistochemistry or CMV DNA by in situ hybridization or PCR (101). A preliminary report has described the presence of CMV-infected endothelial cells in the circulation (102).

Animal studies with rat CMV (RCMV) have been instructive and may apply to the lesions in humans. Rat CMV was reported to infect rat endothelial cells, as judged by demonstration of viral antigens, and to induce class II expression (103). In the mouse, in situ hybridization (murine CMV) has also suggested endothelial infection (104), but no endothelial infection was seen in another study using in situ hybridization (105). However, infection of autologous smooth muscle cells in the neointima (but not medial smooth muscle cells) was demonstrated; infection of these cells occurred only when the endothelium was denuded and in immunocompromised hosts (105).

The key findings using rat aortic allografts were (a) RCMV increased the intensity of the endothelialitis, smooth muscle cell proliferation, and medial destruction, which are characteristic of rejection in that model (106,107); (b) RCMV increased the severity of the chronic arteriopathy and smooth muscle proliferation in the neointima (106); (c) the RCMV effect was seen only in allografts and not isografts and, therefore, acted by enhancing the rejection process (106); (d) prophylaxis with DHPG inhibited infection and the vascular lesions (107); however, (e) treatment with immunosuppressants decreased the progression of the lesion, indicating the major process was rejection and not infection (108).

Overall, these results are compatible with the postulated mechanism for allograft glomerulopathy, namely that CMV infection intensifies and modifies the rejection process, which may focus on the glomerulus, arteries, or the tubules and interstitium. These indirect effects all probably develop through common pathways, involving increased cytokine release and vascular endothelial activation.

C. Miscellaneous Renal Lesions Related to Cytomegalovirus

1. Immunotactoid Glomerulopathy

A case of de novo immunotactoid (fibrillary) glomerulopathy in a renal allograft has been associated with CMV infection (109). In one patient, hematuria, proteinuria, and acute renal failure developed 6 weeks after transplantation. During this time, CMV viremia with acute hepatitis and bone marrow suppression developed. The clinical manifestations of CMV illness and the renal disease subsided following the withdrawal of immunosuppressive agents and treatment with ganciclovir and did not recur in the subsequent 20 months of follow-up. Immunotactoid glomerulopathy is of unknown pathogenesis, but generally immunoglobulin is demonstrable in the deposits, sometimes with light chain restriction. Although there was no direct proof that CMV infection was responsible, the circumstantial evidence strongly suggested that these two diseases were causally linked.

2. Immune Complex Glomerulonephritis

Despite the evidence of circulating immune complexes in CMV infection, there are few, if any, convincing reports of immune complex glomerulonephritis induced by CMV. One series notes small IgG granular glomerular deposits by immunofluorescence which were associated with active CMV infection, but not glomerular inflammation (30). A mild immune complex mesangial glomerulonephritis has been described in a nonrenal transplant patient (60).

3. Antiviral Drug Toxicity

Foscarnet, a well known nephrotoxin, is another etiological agent that needs consideration in CMV-associated renal failure. In one report, foscarnet caused acute renal allograft dysfunction and fever in five patients (110). The biopsies showed tubular degenerative changes, intratubular calcium deposits, and an infiltrate of mononuclear cells and neutrophils. The creatinine returned to baseline after withdrawal of the drug. A crescentic glomerulonephritis developed in a patient with acquired immunodeficiency syndrome (AIDS) treated for cytomegalovirus retinitis with foscarnet (111). The renal biopsy showed crystals within the glomerular capillaries consistent with the drug. Acyclovir also has nephrotoxic effects, in which intratubular drug crystallization or simply acute tubular necrosis is seen (112). Patients with acute renal allograft failure while on acyclovir have responded to discontinuation of the drug (112,113). Similarly, ganciclovir, the mainstay of antiviral therapy against CMV can be nephrotoxic, although bone marrow toxicity is usually the dose-limiting adverse effect of this agent (1).

VII. TREATMENT AND PREVENTION OF CYTOMEGALOVIRUS INFECTION

Given the protean effects of CMV in the transplant patient, a great deal of effort has been devoted to the treatment and prevention of CMV disease. However, virtually all the reports of antiviral strategies for CMV are based on the prevention or treatment of the direct infectious disease effects of the virus, and there is a notable lack of information on the effect of antiviral strategies on the occurrence of allograft rejection or the pathogenesis of PTLD.

The cornerstone of treatment for symptomatic CMV disease is intravenous ganciclovir (at a dose of 5 mg/kg twice daily, with dosage modification in the face of renal dysfunction) for 2 to 3 weeks. Because of the previously discussed risk of relapse, we advocate following up with a 2- to 4-month course of oral ganciclovir (1 g two to three times daily), with a longer course being given to those with primary disease or patients with relapsing infection. Ideally, the virus should have been cleared from the blood before cessation of the intravenous therapy. For patients with relapsing disease or for those with life-threatening manifestations of CMV disease (e.g., pneumonia, severe GI disease), many centers add anti-CMV hyperimmune globulin to the therapeutic regimen (1,2,6).

Although ganciclovir-resistant CMV has been documented to occur in AIDS patients and in lung and bone marrow transplant patients, it has not been an issue in renal transplant patients. For this reason, there is little experience with such other drugs as foscarnet, which is far more toxic than ganciclovir, in this patient population (1,2,6).

A major goal of the transplant community has been the prevention of CMV disease. Two approaches can be used: (1) preemptive, in which anti-CMV therapy is deployed in patients who manifest one or more markers connoting an especially high risk of CMV disease, and (2) prophylactic, in which the entire population receives an antiviral agent without regard to any specific risk factor (6,114).

Two approaches have been taken to preemptive therapy. First, as previously noted, seropositive patients treated for rejection with an antilymphocyte antibody have a threefold greater incidence of CMV disease (7). If intravenous ganciclovir is administered once daily during the course of the antilymphocyte antibody therapy, the incidence of CMV disease is decreased from 65% to approximately 20% (8). We have found that if this is followed by a 2- to 3-month course of oral ganciclovir that this risk is decreased further to less than 5%. Second, Singh et al. (115) reported a different approach (1,2,3): high-dose oral acyclovir

was compared with no prophylaxis except viremia monitoring and institution of preemptive ganciclovir with the onset of asymptomatic viremia. The group of patients in the monitoring/ganciclovir group had a significantly lower incidence of CMV disease than did the acyclovir group.

A variety of approaches to prophylaxis have been attempted in organ transplant recipients (with the effects in one organ transplant population being more or less transferable to the others, with the possible exception that lung allograft recipients appear to require more prolonged and intensive regimens), with the following results:

1. High-dose oral acyclovir (,3200 mg/day) or anti-CMV hyperimmune globulin for 3 to 4 months was moderately effective in providing protection against CMV disease in seropositive allograft recipients. This protection was attenuated in patients given antilymphocyte antibody therapy for rejection and was less effective in patients at risk for primary disease (1,2,6).
2. A variety of combination regimens, in which sequential therapy (ganciclovir followed by acyclovir) or combination therapy with hyperimmune globulin and acyclovir with or without ganciclovir early in the course, have been tried, with better success than oral acyclovir or globulin alone (1,2,6).
3. Prolonged courses of intravenous ganciclovir (4 to 6 months, requiring a central intravenous access device) are quite effective in preventing CMV disease, with short courses (e.g., less than 1 month) only prolonging the incubation period (1,2,6).
4. In a recent study, oral ganciclovir was administered for 4 months, which successfully prevented CMV disease in seropositive recipients and appeared promising in patients at risk for primary disease and those treated with antilymphocyte antibodies for rejection. However, the number of patients in these latter two groups were small enough to cause uncertainty about this point (116).

What is lacking is a pharmacoeconomic assessment of the impact and costs of viremia monitoring and preemptive therapy versus prophylaxis. Pending the availability of more data, our approach is to use ganciclovir prophylaxis (10 days intravenously and then 3 to 4 months orally) for patients at risk for primary disease (D+R–). For seropositive individuals, intravenous ganciclovir for the duration of antilymphocyte therapy is administered, followed by oral therapy for 2 to 3 months. For the remainder, viremia monitoring or oral prophylaxis appears reasonable. As more cost-effective techniques for viremia monitoring become available, it is likely that this approach will gain more appeal. Finally, all patients should receive CMV-free blood products. This can be accomplished in two ways: (1) using seronegative blood donors, or (2) using leukocyte filters when administering red blood cells or platelets to these patients (1,6).

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22

Hepatitis in the Renal Allograft Recipient

DAVID ROTH and JOSHUA MILLER
University of Miami School of Medicine, Miami, Florida

I. INTRODUCTION

The success of renal transplantation as a therapy for end-stage renal disease (ESRD) has focused attention on factors that primarily affect long-term patient and graft outcome. Whereas 1-year graft survival has steadily increased during the past 15 years, the half-life of a cadaver kidney has remained relatively unchanged. Cardiovascular events and liver disease have been identified as leading causes of morbidity and mortality in the second post-transplantation decade. Early reports emphasized that together both hepatitis B virus (HBV) and non-A, non-B hepatitis (NANBH) accounted for almost all of the 10% to 25% incidence of chronic liver disease being reported in renal allograft recipients (1–3). Furthermore, significantly increased rates of late mortality were present in the chronic liver disease cohort (2).

Data accumulated in the past 5 years has clearly identified hepatitis C virus (HCV) to be the primary cause of NANBH in the ESRD population, and, as with HBV, adverse outcomes are being associated with HCV infection in renal allograft recipients.

II. HEPATITIS C VIRUS

The remarkable cloning of the hepatitis C virus by Choo and Kuo (4) was directly responsible for unmasking the entity previously referred to as non-A, non-B hepatitis. An assay

detecting anti-HCV antibody was quickly developed (5) and used in surveillance studies to determine the prevalence of infection in the general population (1%–2%). Soon thereafter, ESRD patients were identified as a high-risk group in which anti-HCV positivity was 10 to 20 times that of non-ESRD patients.

Hepatitis C virus infection leads to chronic active hepatitis in approximately 60% of cases, bridging fibrosis and cirrhosis in 20%, end-stage liver disease, and hepatocellular carcinoma (4–8). Several extrahepatic syndromes have also been associated with HCV infection, including essential mixed cryoglobulinemia (9), membranoproliferative glomerulonephritis (10), porphyria cutanea tarda (11), and aplastic anemia (12).

A. Structural and Functional Relationships

Soon after the initial cloning of HCV, the entire viral genome was sequenced and many of its structural and functional relationships were defined (13). Hepatitis C virus is known to be a single-stranded positive-sense RNA virus with similarities to the *Flaviviridae* family.

A structural model of HCV was deduced from its similarity to the *Flaviviridae* family in conjunction with genomic sequencing data (Fig. 1). The entire genome consists of approximately 9500 bases coding for approximately 3000 amino acids. The initial clone was discovered from the NS4 region and the derived protein was designated 5-1-1. The first generation anti-HCV assay was developed from an expanded form of this region of the genome (14).

Because HCV has no DNA intermediate, it cannot integrate into the host genome. Thus, the extremely high rate of persistent infection that follows HCV exposure (60%–

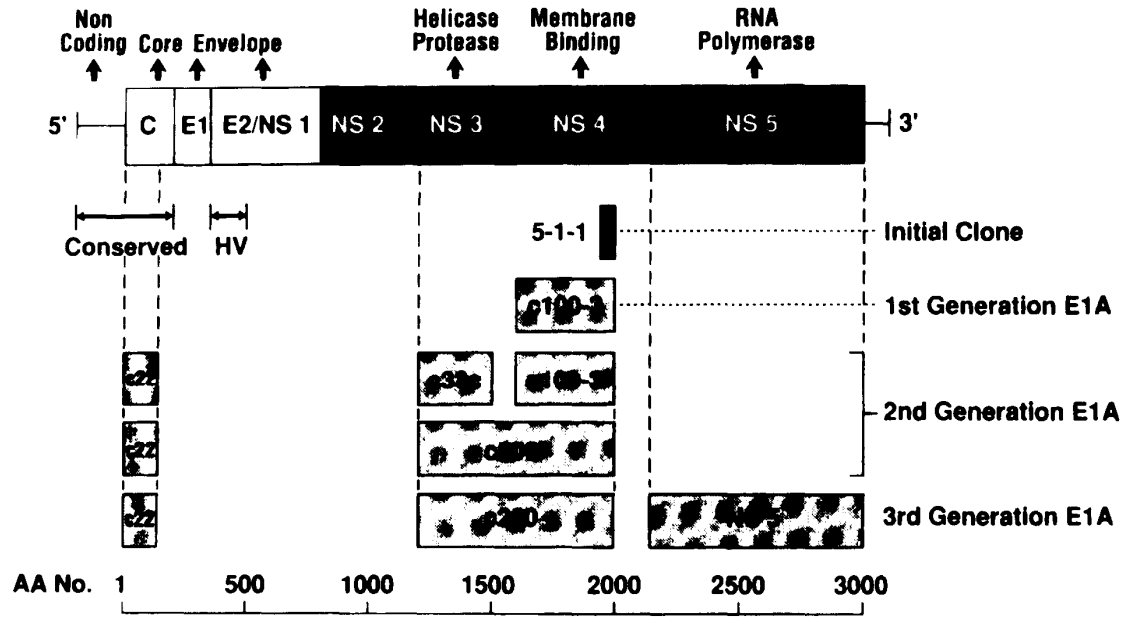

Figure 1 The functional equivalents and major antigens that are used in antibody detection assays are shown. The initial clone discovered was from the NS4 region, and the derived protein was designated 5-1-1. This was expanded to form the c100-3 antigen that served as the basis of the first generation anti-HCV assay. Second generation assays added the c22 core antigen and the c33c antigen from the NS3 region. These antigens increased the sensitivity of the second generation assay by approximately 20% over the first generation test. The third generation assay, pending licensure in the United States, adds an NS5 protein and reconfigures some of the earlier antigens. (From Ref. 14.)

70%) must be explained by a mechanism other than viral integration. It appears that the ability of the virus to mutate rapidly under immune pressure and to exist as a group of related but immunologically distinct variants may explain its high persistence. The term "quasispecies" has been applied to describe the coexistence of multiple HCV mutants, which provides an explanation for the ability of the virus to escape the host immune response. Other mechanisms, including the formation of defective interfering particles that can absorb potentially neutralizing antibodies (15) and an ability to downregulate replication while existing in a quiescent state in the liver, may also account for the uncanny ability of HCV to survive in the host for prolonged periods.

B. Serological and Molecular Biological Assays Detecting Hepatitis C Virus Infection

As shown in Fig. 1, the original clone produced a protein called 5-1-1, which was expanded to form the c100-3 antigen. This served as the basis for the first generation anti-HCV assay (EIA-1) licensed in 1990 (5). It soon became obvious that the EIA-1 had numerous shortcomings. Anti-HCV antibody often did not appear for 12 to 20 weeks after exposure and the sensitivity of the assay in nonimmunocompetent patient groups (e.g., uremic dialysis patients and immunosuppressed transplant recipients) was unacceptably low. Second generation assays were licensed in 1992 and added two epitopes to both the screening enzyme immunoassay (EIA) and the confirmatory recombinant immunoblot assay (RIBA). These assays narrowed the window of seroconversion after exposure to 10 to 15 weeks and improved sensitivity. Third-generation tests, which incorporate an antigen in the NS-5 region, have become available. They provide a slight increase in sensitivity but no significant improvement in specificity. In fact, nonspecificity has plagued each of the assays because of nonspecific reactivation to the 5-1-1 and c100-3 antigens in conditions such as hypergammaglobulinemia, rheumatoid-factor–positive sera, aged sera, and sera from persons recently vaccinated for influenza. The problem of nonspecificity can be partly overcome by confirming positive EIA results with a supplemental assay such as the RIBA.

The measurement of HCV RNA in serum by polymerase chain reaction (PCR) (16) currently is the most sensitive method to detect HCV infection. Hepatitis C virus RNA generally appears in the serum within days of exposure and usually persists despite an absence of associated biochemical abnormalities. However, the reverse transcriptase PCR (RT-PCR) is labor intensive and costly, factors that preclude its widespread application. Technically more accessible is the branched chain DNA (bDNA) assay for HCV RNA (16). This assay uses signal rather than target amplification, is available in kit format, and uses nonradioactive (chemiluminescent) detection. Thus, it is more easily used in the routine clinical laboratory. However, limitation in the ability to detect low-level viremia (<350,000 copies/mL) and to accurately assess high-level HCV RNA in some patient populations restricts widespread use of the bDNA assay, especially in the research setting. It appears that combining bDNA with a reliable RT-PCR assay as the "gold standard" offers the optimal situation to detect viremia and monitor changes in viral load.

C. Transmission of Hepatitis C Virus by Kidney Transplantation

The prevalence of EIA-2 positivity in the United States organ donor population was reported to be 4.2%, a figure three or four times that of the general population (17). In a study encompassing multiple organ procurement organizations, between 2.3% and 8.3% of

donors were EIA-2 positive with 0.8% to 4.2% having confirmed viremia by RT-PCR testing (17). The wide range in the prevalence of HCV infection probably represents geographic variation in the penetration of HCV infection superimposed on patients in whom lifestyle and socioeconomic factors make HCV infection more likely.

The transmission of HCV by solid-organ transplantation has been unequivocally demonstrated (18,19). However, significant variability in transmission rates have been reported from different centers. Pereira et al. (18) reported that 23 of 24 (96%) recipients of an organ from an anti-HCV–positive donor were HCV RNA positive in posttransplant serum. In another study, Roth et al. (19) studied 484 cadaver organ donors and detected RIBA positivity in 6.8%. Liver histology from 24 RIBA-positive donors demonstrated chronic hepatitis in 18 of 24 (75%) specimens, confirming that the majority of seropositive organ donors harbor active disease. Nevertheless, transmission of HCV was only confirmed in 56% of transplants from positive donors into negative recipients (D^+, R^-). This figure was confirmed in another study of HCV transmission in positive donor to negative recipient conditions (20).

Several factors might explain the discrepancy in transmission rates. One possibility may involve differences in organ preservation. Zucker and colleagues (21) demonstrated that pulsatile perfusion of machine-preserved kidneys removed 75% of the viral burden from the kidney in 20 hours and an additional wash and second perfusion increased this amount to more than 99%. Most organ procurement organizations use slush for kidney preservation, whereas centers reporting lower transmission rates (19,20) use pulsatile perfusion. Additional factors that could affect the likelihood of disease transmission include the observation that HCV exists as a group of quasispecies, some of which may be more virulent than others and thus more likely to replicate in a new host. Differences in circulating viral titers and recipient disease susceptibility linked to human leukocyte antigen (HLA) specificities may also be factors contributing to the conflicting transmission rates.

Although significant differences exist in the reported rate of HCV transmission when transplanting a kidney from a positive donor into a negative recipient (D^+, R^-), there remains no doubt that transmission can and does occur. Uncertainty surrounding the consequences of introducing HCV infection into a freshly immunosuppressed recipient has led some centers to decline kidneys harvested from HCV-positive donors (22). Based on 1994 data, exclusion of the estimated 4.2% anti-HCV–positive donors from the 5104 cadaver organ donors harvested that year would represent the potential loss of 428 transplantable kidneys. This is a burdensome situation in a time of profound organ shortage.

As an alternative to discarding potentially usable organs, some researchers have suggested that kidneys from anti-HCV–positive donors be transplanted into anti-HCV–positive recipients. A recent survey of United Network of Organ Sharing (UNOS)-approved United States centers indicated that approximately 25% of them would transplant a kidney from an anti-HCV–positive donor if the recipient was also anti-HCV positive (22). This raises several important issues. First, not all EIA-2–positive donors transmit disease (17). Only those donors determined to be PCR positive in serum transmitted disease with regularity, emphasizing that EIA-2 positivity does not predict transmission and that testing the donor for serum HCV RNA is critical to making a reasoned decision. Second, identifying which potential recipients are truly HCV infected can be difficult. Hepatitis C virus RNA serum titers have been shown to fluctuate in persistent HCV carriers, only to reappear in the circulation at a later date. Moreover, if screening policies rely wholly on the EIA-2, infected patients will be missed because of the low sensitivity of this assay in the immunosuppressed uremic ESRD population (23). Thus, patients presumed not to be infected may in fact harbor intrahepatic virus.

Another critical issue surrounding the transplantation of kidneys from positive donors into HCV-infected recipients is the impact of an additional viral burden on the recipient's liver function. In a chimpanzee model, Okamoto and colleagues (24) studied the effect of challenging an animal infected with HCV genotype 1b with HCV genotype 2a, followed 6 weeks later by an inoculum of genotype 1a. A different pattern of HCV infection emerged in each of three inoculated animals (Fig. 2). In two animals, genotype 1a emerged as the detectable virus in the serum following a period of co-infection with 1b.

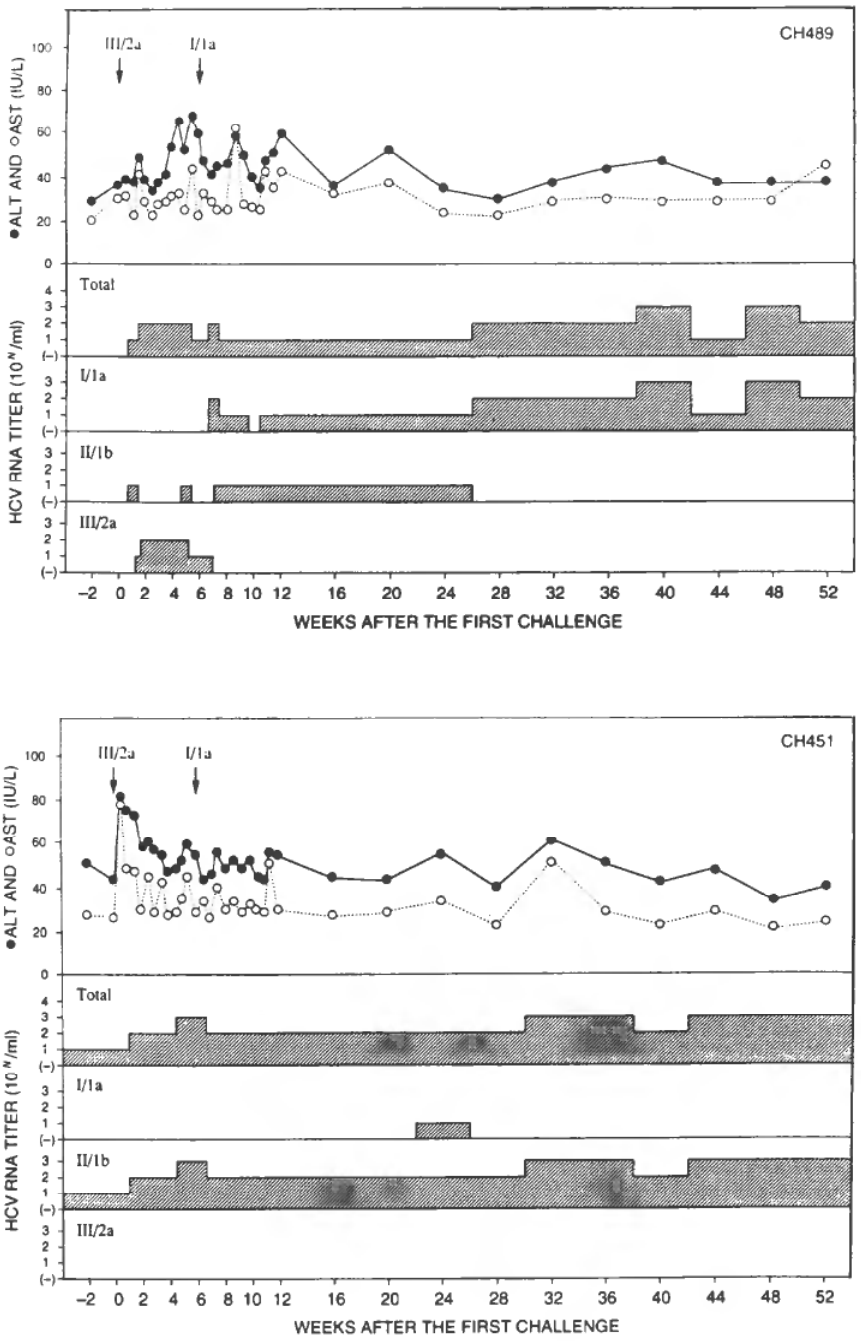

Figure 2 Upper panel: Titers of total HCV RNA and HCV RNA of genotypes I/1a, II/1b, III/2a, as well as ALT and AST levels in a chimpanzee that had carried HCV of genotype II/1b intermittently and was challenged successively 6 weeks apart with HCV of genotypes III/2a and I/1a. Lower panel: In an animal persistently infected with HCV of genotype II/1b, similar challenges with HCV of genotypes III/2a and I/1a resulted in persistent II/1b infection and no superinfection with the challenging genotype. (From Ref. 24.)

Genotype 1b persisted as the only identifiable circulating genotype in the third chimpanzee. These findings were confirmed in a recent report by Widell and colleagues (25) in a group of five kidney recipients. As was seen in the chimpanzee, three patterns of HCV infection emerged after transplantation of a kidney from an HCV-infected donor into a patient with detectable titers of HCV RNA of a different genotype. Persistence of the original genotype, co-infection with both the original recipient genotype and that of the donor, or superinfection with the donor's genotype was demonstrated (Fig. 3). These studies and those of Farci et al. (26) clearly demonstrated sequential susceptibility to HCV infection and the failure of previous or active viral replication to confer immunity.

Data from the clinical arena relevant to this issue are limited. Morales et al. (27) described a group of HCV RNA–positive recipients who received a kidney from EIA-2–positive donors, several of whom were documented to be viremic. At a mean follow-up of almost 2.5 years, there was no excess morbidity or mortality in the D^+, R^+ group compared to a D^-, R^+ cohort. Further reports and longer follow-up are necessary to completely understand the consequences of D^+, R^+ transplantation.

D. The Hepatitis C Virus–Infected Transplant Candidate

Limited data exist pertaining to the appropriate investigation of an HCV-infected patient being seen for pretransplant evaluation. Moreover, available testing does not reliably identify the HCV-infected ESRD patient.

The sensitivity and specificity of the EIA-2 in viremic ESRD patients were reported as 57% and 89%, respectively (18). In a recent survey of 191 consecutive patients being evaluated for kidney transplantation, we also found both the EIA-2 and RIBA-2 to have low sensitivity and specificity (Table 1). As a consequence, the negative predictive value of the EIA-2 and RIBA-2 was 92% and 83%, respectively. Based on these results, our center currently obtains serum for RT-PCR from every candidate being seriously considered for transplantation.

Previous studies have shown that HCV-infected dialysis patients have significant histological changes on liver biopsy. Caramelo et al. (28) detected changes of chronic hepatitis or cirrhosis in greater than 50% of biopsied anti-HCV–positive dialysis patients. Fur-

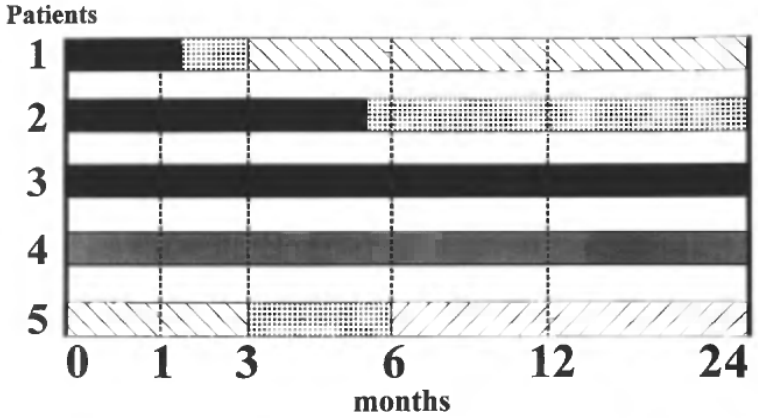

Figure 3 Results of transplantation of a kidney from a donor infected with HCV genotype 3a (patients 1–3) or genotype 1a (patients 4 and 5). Patients 3 and 4 demonstrated persistence of the original recipient genotype without detectable donor HCV. Patients 1, 2, and 5 became co-infected with donor and recipient HCV genotypes, although patients 1 and 5 evolved into superinfection with the donor strain while patient 2 remained co-infected with both HCV genotypes. (From Ref. 25.)

Table 1 Serologic Tests for Hepatitis C Virus Infection in End-Stage Renal Disease Patients

	EIA 2 (%)	RIBA 2 (%)
Sensitivity	75	81
Specificity	92	79
Positive predictive value	73	76
Negative predictive value	92	83

Abbreviations: EIA 2 = second generation ELISA; RIBA 2 = second generation recombinant immunoblot assay.

thermore, serum transaminases in ESRD patients have been shown to be markedly insensitive in identifying patients with significant histological disease (29). For these reasons, our center requires a liver biopsy in all transplant candidates found to be anti-HCV–positive during pretransplant screening.

Data recently collected at our center from prospectively obtained pretransplant liver biopsies in 33 patients demonstrated chronic active hepatitis in 58%, with bridging necrosis present in four of these specimens (Fig. 4). Thirty percent of the patients had minimal to mild changes. Genotyping identified HCV 1a to be the most common (56%), followed by 1b (35%). One patient was co-infected with subtypes 1a and 1b. As seen in Fig. 5, no correlation could be demonstrated between HCV subtype and the extent of histological injury. We were also unable to show an association between histological diagnosis, HCV genotype, and serum alanine aminotransferase levels.

Because long-term prospective studies with longitudinal histological data are not yet available for HCV-infected transplant recipients, it is probably best to follow the guidelines generally applied to the HBV-infected transplant candidate. Patients with active viral replication and advanced histological disease such as chronic active hepatitis with early cirrhosis may be best advised to remain on dialysis.

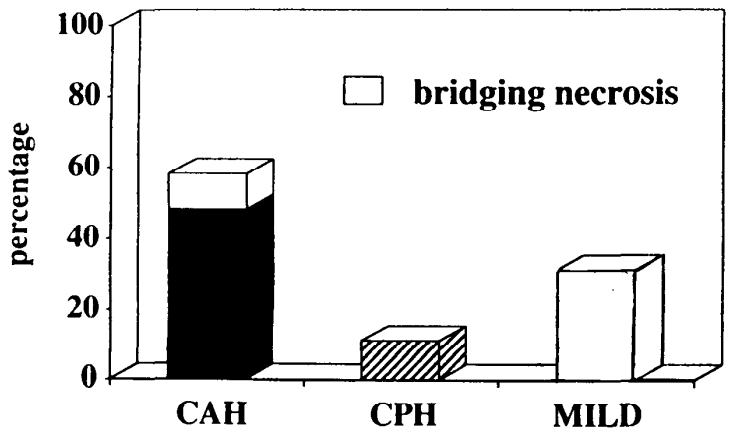

Figure 4 Distribution of histological lesions in pretransplant liver biopsies. Fifty-eight percent of patients had chronic active hepatitis (CAH) while 12% showed chronic persistent hepatitis (CPH), and 30% had minimal changes.

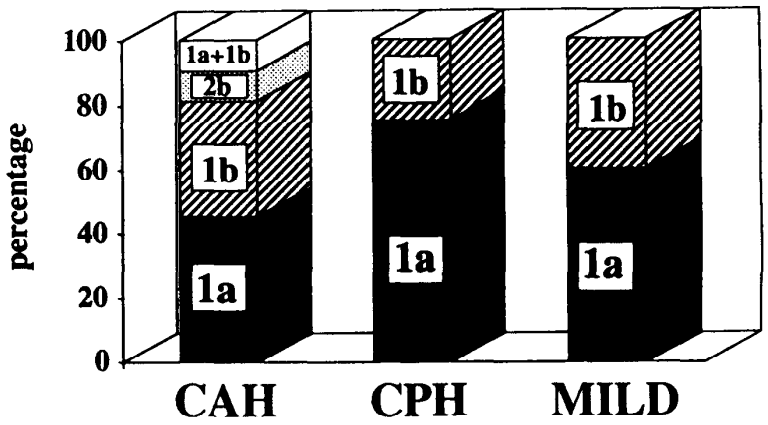

Figure 5 Distribution of HCV genotypes within each histological diagnosis. No correlation was present between HCV genotype and the severity of liver injury *Abbreviations:* CAH = chronic active hepatitis, CPH = chronic persistent hepatitis.

E. Hepatitis C Virus–Infected Renal Allograft Recipient

1. Prevalence of Infection and Liver Histology

The prevalence of anti-HCV and HCV RNA among renal allograft recipients has been reported from a number of centers (30–41). Between 10% and 40% of kidney recipients are anti-HCV positive and the great majority of seropositive patients have circulating HCV RNA (Table 2). These findings are not unexpected considering the high prevalence of anti-HCV among hemodialysis patients (42–46).

The extent of histological liver disease present in the HCV-infected renal transplant population varies widely (33,36–39,47–51) (Table 3). Histological changes at both ends of the spectrum (i.e., minimal changes to cirrhosis) comprise the smallest groups. From 0% to 30% of patients have essentially normal biopsies despite being HCV infected. At the other extreme, cirrhosis is uncommon, with many centers reporting no patients with a histological injury of that magnitude. Most patients have findings of chronic hepatitis.

The correlation between the histological diagnosis and serum transaminase level in

Table 2 Hepatitis C Virus Infection in Renal Allograft Recipients[a]

Author (Reference)	EIA-1	EIA-2	HCV-RNA
Roth (30)	179/596 (30%)	—	—
Ponz (31)	32/67 (48%)	—	—
Morales (32)	66/200 (33%)	—	—
Pol (33)	20/127 (24%)	—	—
Stempel (34)	76/716 (11%)	—	—
Lau (35)	—	18/100 (18%)	18/18 (100%)
Roth (36)	—	109/641 (17%)	39/53 (74%)
Chan (37)	—	19/220 (8.6%)	22/22 (100%)
Goffin (38)	—	22/259 (14.2%)	20/22 (91%)
Orloff (39)	—	76/716 (10.6%)	—
Pereira (40)	—	23/103 (22%)	14/23 (61%)

[a]Data are number positive/number tested.
Source: Ref. 42.

Table 3 Liver Histology in Hepatitis C Virus–Positive Renal Transplant Patients

		Liver Histology				
Author (Reference)	N	Minimal	CPH	CAH	Cirrhosis	Other
Pol (33)	32	6 (19%)	7 (22%)	19 (59%)	0	0
Cassanova (47)	18	2 (11%)	9 (50%)	7 (39%)	0	0
Roth (36)	10	0	4 (40%)	5 (50%)	1 (10%)	0
Chan (37)	13	7 (54%)	1 (8%)	4 (31%)	1 (8%)	0
Orloff (39)	8	3 (38%)	4 (50%)	1 (13%)	0	0
Morales (48)	31	9 (29%)	7 (23%)	11 (35%)	4 (13%)	0
Meshari (49)	14	0	14 (100%)	0	0	0
Glicklich (50)	29	3 (10%)	17 (59%)	4 (14%)	2 (7%)	3 (10%)
Boletis (51)	37	13 (35%)	6 (16%)	11 (30%)	2 (5%)	5 (14%)
Total	192	43 (22%)	69 (36%)	62 (33%)	10 (5%)	8 (4%)

Abbreviations: CPH = chronic persistent hepatitis; CAH = chronic active hepatitis.

the immunosuppressed transplant recipient has been repeatedly shown to be poor (36,37, 51). Moreover, many patients with active viral replication maintain normal serum transaminases. Roth et al. (36) reported 20 of 29 PCR-positive patients to have normal ALT, whereas liver disease was detected in two thirds of the anti-HCV carriers transplanted at another center (31). Thus, between 33% and 52% of anti-HCV–positive transplant recipients in these two reports maintained normal liver function tests, emphasizing the low sensitivity of serum transaminases to detect active viral replication.

Combining the poor correlation between serum transaminases with both the status of viral replication and extent of histological injury, the threshold for biopsy in the HCV-infected renal transplant recipient should be low. This approach is supported by data from Rao et al. (52) demonstrating histological progression to cirrhosis in serial biopsy specimens obtained from transplant patients with chronic active hepatitis. Thus, identifying patients with established chronic hepatitis will help identify patients at risk for cirrhosis and its sequelae (e.g., hepatocellular carcinoma). This would enable screening with ultrasonography or alphafetoprotein levels to be incorporated into the follow-up of such patients. Furthermore, consideration should be given to changes in immunosuppression, such as the discontinuation of azathioprine and lowering of other maintenance immunosuppressive medications.

2. Patient Outcome

The impact of existing HCV infection on outcome parameters in renal allograft recipients has been the subject of considerable investigation. Roth and colleagues (36) studied 109 patients who had a perioperative RIBA-positive serum sample. This represented 17% of the 641 patients transplanted during a 12-year period. Chronic liver disease developed in 34% of the RIBA-positive cohort compared with 18% of a seronegative control group ($p < 0.002$). Amongst those patients still being followed up, HCV RNA was detected in 74% of fresh serum samples. One-half of the uremic patients never demonstrated abnormal serum alanine aminotransferase in more than 5 years of follow-up, emphasizing the low sensitivity of transaminases to identify infected patients. Actuarial patient and graft survival rates at 5 years was 81% and 63%, respectively, for the RIBA-positive cohort and did not differ

from the RIBA-negative group. Furthermore, there were no deaths attributable to subfulminant or chronic liver disease during the period of follow-up encompassed by this analysis.

Equally encouraging results have been reported from other centers. Pol et al. (33) studied 127 HBsAg-negative patients at the Necker Hospital and found 24% to be anti-HCV positive. There was no differences in patient and graft survival between the positive and negative cohorts. Similarly, no difference in outcome between anti-HCV positive and negative recipients has been reported by several other centers (31,34).

In contrast to these studies, two case reports and a recently published series offer a less optimistic outlook. Two heart recipients described in separate case reports died of sequelae of chronic liver failure associated with HCV infection (53,54). Pereira et al. (40) reported a series of D^-, R^+ kidney recipients with a mean follow-up of 45 months. Among recipients with anti-HCV before transplantation, the relative risk (RR) of posttransplantation liver disease was 5.0, the RR of death was 3.3, and death resulting from sepsis carried a RR of 9.9.

Virtually all studies to date addressing this important issue are retrospective in design and lack critical pretransplant histological data, so that the extent of liver disease present before immunosuppression could be factored into the outcome analyses. Roth et al. (55) collected prospective data on 14 HCV-infected recipients who received a kidney from an HCV-negative donor. Pretransplant liver biopsies showed chronic hepatitis in six and mild changes in eight specimens. At a mean follow-up of 11.6 months (range 5–21 months), there were no cases of subfulminant liver failure and no deaths. Viral replication was enhanced as evidenced by an 8- to 10-fold increase in circulating viral titers after transplantation over baseline level (Table 4). Comparison of the chronic hepatitis group with the minimal disease patients yielded no difference in peak alanine aminotransferase or the incidence of rejection but did show significantly more CMV disease (Table 5). Genotypes 1a and 1b were most common; however, no correlation was present between genotype, viral titers, and serum transaminases.

The important studies encompassing the HCV-infected kidney recipient are summarized in Table 6. Although most series have failed to detect a negative impact of HCV infec-

Table 4 Baseline and Posttransplant Circulating Hepatitis C Virus Titers Based on Pretransplant Histology

Posttransplant Interval (days)	HCV Titers[a]		
	Chronic Hepatitis	Mild[b]	*p* Value
Baseline	1.8 ± 2.7	1.3 ± 1.6	NS
<15	2.5 ± 3	3.7 ± 3.4	NS
15–30	4 ± 4.9	7.9 ± 9.6	NS
30–60	2.7 ± 4.2	5.1 ± 8.1	NS
60–120	7.5 ± 6.2	9.2 ± 14.4	NS
120–180	2.6 ± 2.1	9.9 ± 10.4	NS
>180	7.2 ± 7.8[3]	14 ± 19.4[c]	NS

[a]HCV titers are copies x10^6/mL ± standard deviation.
[b]Mild-mild histologic changes.
[c]$p < 0.05$ versus baseline value.
Source: Ref. 55.

Table 5 Clinical Outcomes According to Pretransplant Liver Histology

Variable	Liver Histology[a]		
	Chronic (n = 6)	Mild (n = 8)	*p* Value
Alanine aminotransferase T (U/L)[b]			
Pretransplantation	56 ± 29	39 ± 21	NS
Peak	108 ± 65	158 ± 94	NS
Current	28 ± 10	47 ± 47	NS
Rejection (%)	33	14	NS
Cytomegalovirus (%)[c]	83	14	.03

[a]Determined on pretransplant liver biopsy.
[b]Values are mean ± standard deviation.
[c]Clinically significant disease.
Source: Ref. 55.

Table 6 Posttransplant Hepatitis C Virus Infection

Author (Reference)	N	Follow-Up (mo.)	Impact on Outcome	
			Patient	Graft
Ponz (31)	32	30	None	None
Pol (33)	31	≤180	None	None
Stempel (34)	76	35 ± 20	None	None
Lau (35)	18	50 ± 14	None	None
Roth (36)	109	68 ± 29	None	None
Pereira (40)	23	45	Yes	None

tion on posttransplantation outcome, longer duration of follow-up and prospective studies are needed to fully understand this issue.

3. Glomerular Disease

De novo membranoproliferative glomerulonephritis (MPGN) in the allograft has been reported in HCV-infected kidney recipients (56,57). Roth et al. (56) described five patients with nephrotic-range proteinuria and an immune-complex glomerulonephritis of the allograft. Nephrotic-range proteinuria developed in two of the patients infected with the same HCV genotype within 2 months after transplantation, while the remaining three patients did not become proteinuric until 5 to 10 years after transplantation, despite being infected for many years. Two other patients have been reported with features of MPGN and transplant glomerulopathy (57). Considering the clear association of HCV infection with essential mixed cryoglobulinemia (9) and MPGN of the native kidney (10), it is not surprising to find a similar clinical pattern occurring in the transplanted kidney. Sensitive testing for HCV infection should be included in the evaluation of all transplant recipients in whom significant proteinuria develops. Whether patients whose original kidney disease was an HCV-associated MPGN are at increased risk for recurrent disease after transplantation is not known.

4. Interferon Therapy

In the non-ESRD setting, interferon (IFN) remains the only approved therapy for HCV infection (58). However, only approximately 50% of treated patients clear the virus from the circulation and 50% of those relapse soon after discontinuing treatment. Variables associated with a sustained response appear to include a low serum HCV RNA level before the initiation of therapy and an HCV genotype other than 1b (59). Hepatitis C virus 3a has been shown to be particularly responsive to IFN treatment.

The administration of IFN to renal allograft recipients has been associated with adverse outcomes since the early reports of its use for CMV prophylaxis (60). More recently, several studies have been reported describing IFN use in HCV-infected renal allograft recipients. In one series, acute rejection developed in six of seven patients (61) between 11 days to 8 months after initiating treatment. In another, renal failure developed in 5 of 14 patients. Table 7 summarizes several studies reporting results of IFN treatment of transplant recipients (61–66). The experience thus far has been poor and IFN cannot be recommended for the HCV-infected renal allograft recipient.

The cloning of hepatitis C virus and subsequent development of assays to identify infected patients has opened a new era in current understanding of hepatotropic virus infection amongst renal transplant recipients. Significant changes in organ procurement and allocation, recipient selection, and posttransplant management have all occurred. It is anticipated that further evaluation of these policies will occur as current knowledge of HCV expands and additional therapeutic options become available.

III. HEPATITIS B VIRUS

A. Transmission of Hepatitis B Virus

The transmission of HBV from a hepatitis B surface antigen (HBsAg)-positive kidney donor to a HBsAg-negative recipient has been clearly established (67,68). In the report by Lutwick et al. (67), one of the kidneys harvested from a HBsAg-positive donor was kept in slush while the mate kidney was placed in a pulsatile perfusion apparatus. The recipient of the slush-preserved kidney converted to HBsAg-positive status whereas the recipient of the perfused kidney remained HBsAg negative; HBsAg was detected in the perfusate, a finding reproduced with perfusion of kidneys from HCV-positive donors by Zucker and colleagues (21).

Recently, attention has been focused on the risk of HBV transmission from hepatitis B core antibody positive, HBsAg-negative donors. In one study (69), only 1 of 42 kidney

Table 7 Posttransplant Interferon Therapy

Author (Reference)	Cases	IFN Dose (tiw)	Renal Outcome (No.)	HCV Outcome
Magnone (61)	7	$1.5–5 \times 10^6$	Rej (6)	N/A
Chan (62)	1	3.5×10^6	Rej (1)	PCR (–)
Thervet (63)	13	3×10^6	ARF (2)	No effect
Izopet (64)	15	3×10^6	ARF (5)	No effect
Rostaing (65)	16	3×10^6	ARF (6)	5/16 Cleared
Harihara (66)	3	3×10^6	RF (3)	No effect

Abbreviations: IFN = interferon alpha; Rej = rejection; ARF = acute renal failure; RF = unspecified renal failure; N/A = not available; tiw = three times per week.

recipients converted to HBsAg-positive status following transplantation of a kidney from a HBcAb-positive, hepatitis B surface antibody-negative donor, whereas three of six liver recipients seroconverted to HBsAg-positive status. The single kidney recipient who converted to HBsAg-positive status was HBsAb negative before transplantation. Similar findings were reported in another study (70). Based on these reports, it appears justified to continue to transplant kidneys from HBcAb-positive, HBsAg-negative donors, although every effort should be made for such an organ to be transplanted into an HBsAb-positive recipient.

B. The Hepatitis B Virus–Infected Renal Allograft Recipient

1. Patient and Graft Survival

Liver disease has been recognized as a major contributor to long-term patient morbidity and mortality following successful renal transplantation (2). The contribution of HBV infection to the overall impact of liver disease on long-term posttransplant outcome has been extensively studied but remains controversial. Pirson and colleagues (71) were among the first to report an adverse outcome among HBsAg-positive renal allograft recipients. They observed a fivefold increase in mortality rate secondary to liver disease and significantly reduced patient and graft survival among antigen-positive recipients. Hillis et al. (72) also found that HBsAg-positive renal transplant recipients had a higher mortality rate. Many other investigators, however, have been unable to detect any increase in patient mortality rates in HBsAg-positive recipients (73–77).

One explanation for the varying results reported from different centers may relate to the number of HBsAg-negative, HBV DNA–positive patients present in each cohort of patients. In a study by Degos et al. (78), 62% of HBsAg-positive patients had serum HBV DNA on early testing, but, of those who lacked serum HBV DNA initially, 92% became positive within 12 months after transplantation. Thus, replicative infection is almost universal among immunosuppressed allograft recipients within a short time after transplantation. Among initially HBsAg-negative patients, none had HBV DNA in serum on initial testing, but, in 20% of those tested subsequently, HBV DNA was detected. In 44% of 27 HBsAg-negative patients tested, HBV DNA was detected in initial liver biopsy specimens. This important study confirms the increase in viral replication following the introduction of immunosuppression. Of greater interest is the meaningfulness of the HBsAg-negative, HBV DNA–positive pattern and whether this can help explain the reported differences in patient survival following transplantation in HBsAg-positive and -negative patient cohorts.

Prior studies have classified patients based on HBsAg positivity and negativity, and correlations that emerged distinguished the two groups. Previously reported conflicting observations concerning the impact of HBV on patient and graft outcome may have represented differences in the frequency of HBsAg-negative HBV infection among the various centers. Those centers with a high frequency of HBsAg-negative HBV infection may have failed to detect a difference in outcome between their HBsAg-positive and HBsAg-negative patients.

Differences in allograft survival have been observed in HBsAg-positive and -negative recipients. London et al. (79) found a higher rate of allograft survival among patients with HBsAg positivity before transplantation. Most investigators, however, have been unable to find a difference in graft survival among HBsAg-positive recipients (76,80).

2. Histological Progression

Parfrey and colleagues (1,81,82) prospectively followed up 22 HBsAg-positive patients for a mean of 83 months after transplantation. No patients converted to HBsAg-negative status

and 12 progressed to cirrhosis. Eighty-two percent of the patients whose initial biopsy showed only mild changes or chronic persistent hepatitis progressed to chronic active hepatitis or cirrhosis on serially obtained biopsies. Death attributable to liver disease occurred in one-half of the group. Four of five patients with histological progression had a greater than 10-fold increase in HBV DNA titers. In a study by Pol et al. (83), the course of 122 HBsAg-positive patients was followed up; liver biopsies obtained in the perioperative period demonstrated 80% (98 of 122) to already have chronic liver disease. Moreover, 12 of 16 patients whose initial biopsy was normal progressed to chronic hepatitis (11 of 12) or cirrhosis (1 of 12). Similarly aggressive patterns of progression were observed in patients whose initial biopsy showed chronic persistent or chronic active hepatitis. Progression of histological disease has been reported by others as well (84). Of note, all of the patients in these studies were on azathioprine and steroids as maintenance immunosuppression.

Several more recent studies have examined outcome variables in HBsAg-positive patients on cyclosporine-based immunosuppression. Huang and colleagues (85) studied 20 patients given cyclosporine (CyA) and 13 on azathioprine and steroids only. Progression to cirrhosis was twice as common in the azathioprine group. Furthermore, whereas chronic hepatitis developed in 50% of the CyA patients, greater than three-fourths of the azathioprine cohort progressed to chronic liver disease. Two patients with concomitant delta virus infection progressed to cirrhosis. Rao et al. (86) followed up 26 HBsAg-positive patients with chronic hepatitis on biopsy. Eighteen of 26 (69%) patients died at a mean follow-up of 82 months after transplantation, with 14 of 18 deaths attributable to liver failure. Overall, death rate resulting from hepatic failure (54%) was virtually identical to the mortality rate reported in an earlier study by Parfreys' group (87).

3. Therapy

Interferon-α has been found to be ineffective in the treatment of posttransplantation HBV infection (88). A recent study by Pol et al. (89) evaluated the efficacy of adenine arabinoside 4-monophosphate (ARA-AMP) in HBV-infected allograft recipients. Ten patients received a 28-day course of ARA-AMP. All 10 patients had detectable HBV DNA in serum and 7 of 10 were HBeAg-positive before starting treatment. Hepatitis B virus DNA became undetectable in 4 of 10 patients although two of four subsequently relapsed and 5 of 10 patients had a decrease in HBV DNA titer. Serum transaminases decreased markedly in patients who cleared HBV DNA from the serum. The drug was reported to be well tolerated in 9 of 10 patients. Further testing with antiviral agents will be necessary to identify a safe and highly efficacious therapy.

C. The Hepatitis B Virus–Infected Transplant Candidate

The prognostic value of HBV DNA and/or HBeAg testing has been evaluated in two studies. In an analysis of 42 HBsAg-positive patients (90), 5 of 10 recipients with HBV DNA and/or HBeAg in pretransplant serum samples died during posttransplantation follow-up versus 1 in 15 negative patients. The relative risk of fatal liver disease was eightfold higher in HBV DNA and/or HBeAg-positive recipients. Ten-year patient survival in the positive cohort was 37% versus 67% in negative patients. These findings were not confirmed in a recent study by Nelson et al. (77). In their retrospective analysis of 68 HBsAg-positive recipients, only 1 of 26 HBV DNA, HBeAg-positive patients died during follow-up. Based on the conflicting results of these two studies, the prognostic value of HBV DNA and/or HBeAg in pretransplant serum testing cannot be conclusively determined.

Although histological progression of disease and increased mortality has been ob-

served in HBsAg-positive dialysis patients, viral replication and the aggressiveness of HBV infection appear to be enhanced under the influence of immunosuppression. Taking into account the considerable morbidity and mortality attributable to HBV infection in transplant recipients, HBsAg-positive dialysis patients being considered for transplantation must be carefully evaluated. Initial testing should include studies for HBV DNA, HBeAg, and a liver biopsy. Patients with advanced histological disease and active viral replication (HBV DNA positive) are at high risk to incur significant morbidity from liver disease in the years following transplantation and perhaps should be counseled to remain on dialysis. Patients with more benign histological changes should be apprised of the potential risks of long-term immunosuppression, including progression of histological injury so that an individualized decision can be reached regarding the advisability of transplantation, one that takes into account the patient's age, comorbid illnesses, course on dialysis, and quality-of-life issues.

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ABOUT THE EDITORS

Lorraine C. Racusen is Associate Professor of Pathology at The Johns Hopkins University School of Medicine, Baltimore, Maryland. Her research interests include transplantation and pathophysiology of injury to the renal tubule in clinical acute renal failure, as well as animal models and in vitro cell culture studies of renal tubular injury. The author of over 75 publications, and coeditor of *Solid Organ Transplant Rejection*, Dr. Racusen serves on the editorial board of the journal *Renal Failure* (both titles, Marcel Dekker, Inc.) and is a reviewer for numerous medical journals. She has been coorganizer of the Banff conference on allograft rejection and is a member of the American Heart Association, the American Society of Nephrology, the International Society of Nephrology. and the International Academy of Pathology, among others. The recipient of American Heart Association and Stetler Research Foundation Fellowships, a Mellon Clinician Scientist award, and NIH research grants, Dr. Racusen received the M.D. degree (1975) from the University of Vermont, Burlington.

Kim Solez is Professor of Pathology at the University of Alberta, Faculty of Medicine, Edmonton, Alberta, Canada and President and CEO of Transpath, Inc. The recipient of the Borden Award, Schwentker Award, and a Research Career Development Award from the National Institutes of Health, he is the author or coauthor of over 150 publications, serves on the editorial board of the journal *Renal Failure* and is the coeditor of several books, including *Organ Transplantation: Solid Organ Transplant Rejection;* and *Acute Renal Failure, Second Edition* (all titles, Marcel Dekker, Inc.). Dr. Solez is coorganizer of the Banff conference on allograft rejection. He is a member of numerous professional societies such as the American Society of Nephrology, the American Association of Pathologists, the American Federation for Clinical Research, the International Society of Nephrology, the International Academy of Pathology, the Alberta Society of Laboratory Physicians, the Canadian Medical Association, and the Royal College of Physicians and Surgeons of Canada. Dr. Solez is Chairman of the International Society of Nephrology Commission on Informatics and Director of the cyber Nephrology Center of the National Kidney Foundation. He received the M.D. degree (1972) from the University of Rochester School of Medicine and Dentistry, Rochester, New York.

James F. Burdick is Professor of Surgery and Director of the End Stage Renal Disease Program at The Johns Hopkins University School of Medicine, Baltimore, Maryland. His research focuses on experimental models of transplant rejection and the clinical application of monoclonal antibodies to immunosuppression. The author of over 100 publications on oncology, vascular surgery, and transplantation, he is a member of the American Society of Transplant Surgeons, the American College of Surgeons, the Transplantation Society, the Johns Hopkins Medical Society, the American Surgical Association, and the Chesapeake Transplant Society. Dr. Burdick received the M.D. degree (1968) from Harvard Medical School, Boston, Massachusetts.