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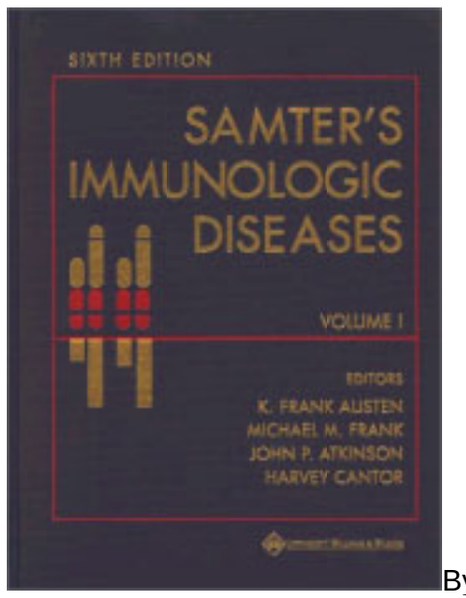

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Samter's Immunologic Diseases

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“Our rate of progress is such that an individual human being, of ordinary length of life, will be called upon to face novel situations which find no parallel in his past. The fixed person, for the fixed duties, who in older societies was such a godsend, in the future will be a public danger.”

Alfred North Whitehead (1861-1947)

“We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.”

T.S. Eliot (1888-1965)

“It might be suggested that, in time, the amount of knowledge needed before a new discovery could be made would become so great as to absorb all the best years of a scientist’s life, so that by the time he reached the frontier of knowledge he would be senile. I suppose this may happen some day, but that day is certainly very distant. In the first place, methods of teaching improve. Plato thought that students in his academy would have to spend ten years learning what was then known of mathematics: nowadays any mathematically minded schoolboy learns much more mathematics in a year.”

Bertrand Russell

“I find the great thing in this world is not so much where we stand as in what direction we are moving.”

Oliver Wendell Holmes

“Of, of course, at the end of a day of experimental work during which I found facts that seemed interesting to me, I let my mind wander into the land of fancy and dreams and let it rove in the garden of illusions which dissipated during the night and had cleared away when I entered my laboratory in the morning. Thus acts the mother after a hard day’s work: at the cradle of her sleeping child, she dreams of his future. A sweet smile lights her face, when she sees the successive stages of his life evolve, of a life that must all be joy, of happiness, of success and triumph. When the sun rises, she resumes her daily task, strict and serious again in contact with the realities.”

From Arthus’ delightful preface to De L’anaphylaxie à l’immunité

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PREFACE

We have just put to rest the sixth edition of *Immunologic Diseases*. The last chapter is in. The four editors and our colleagues at Lippincott, Williams and Wilkins all let out a collective sigh of relief as “it” is now in the production house. This textbook, the initial edition of which was published in 1965, was the first of its type for immunology. In our student, fellowship, and early faculty member days, if we wanted to know the “immunology” of, for example, sarcoidosis or Grave’s disease, this textbook was the “gold standard.” “Samter” was where we went to obtain a comprehensive and authoritative discussion and review of the immunologic underpinnings of a human disease. We often next searched the primary literature, a process facilitated immeasurably by the extensive bibliography that always has been an important feature of this textbook. “Samter” was a mainstay in our efforts to broaden our knowledge base in immunology and an absolute “must” for our small but growing personal library of books on immunology.

This textbook was expertly guided and “coached” by Max Samter for four editions. His thought-provoking and instructive quotes from these four editions added poignancy to the prefaces of the first four editions. We have included several of our favorites at the end of our comments. In the preface to the fifth edition, we honored Max and we want to continue this tradition by restating those words:

“This edition of *Immunologic Diseases* is the second that has not been led by Max Samter. Max, a brilliant clinical allergist and immunologist, was one of the first to anticipate the majestic structure into which basic immunology would develop and to realize that this discipline would have enormous impact on our understanding of clinical illness. The first edition of *Immunological Disease,* published three and a half decades ago with his co-editor Harry Alexander, came to the world at a time when clinician-investigators were just beginning to recognize and group together seemingly disparate diseases with possibly similar immunologic mechanisms. We, the other editors, applaud Max’s extraordinary insight, intelligence, and good humor. We are committed to continue Max’s long tradition of excellence with an edition that vastly expands our understanding of modern immunologic diseases.”

The sixth edition follows the format that has made this book successful. Namely, the book begins with scholarly discussions by noted experts of the basic tenets of immunology. This forms a foundation for the “student” of immunology and provides a background for the more disease- and organ-focused chapters to follow. These latter sections cover in-depth the immunologic aspects of the disease but, in most cases, no longer provide an all-encompassing discussion of a disease. Rather, if the reader desires to learn more about, for example, cardiac involvement in sarcoidosis or SLE, a visit to a cardiology or rheumatology textbook would be appropriate. The purpose of this edition is to primarily focus on the immunologic aspects of these diseases.

In this sixth edition we continue to emphasize the immune system of humans and the immunologic basis of human disease. The bibliographies are still rather extensive but have the added intent of providing a pathway to “finding the right additional information.” Many authors are new and the emphasis in some chapters has changed as might be expected in a fast-moving field such as immunology. Overall, however, this edition is similar to the previous one in content and emphasis. We faced an issue in this edition of how to handle infectious diseases in which, in most cases, the immune system was just doing “its thing.” The major question in infectious diseases for the immunologist is to understand its limitations and compromises in an agent related fashion and to understand how infection contributes to the development of immunologic diseases. Consequently, we have included overviews of how the immune system deals with microbes rather than, in most cases, discussing each disease entity separately.

The advances in immunology over the past decade are astonishing. As one reads the immunologic literature, one cannot but be impressed by our ability to: knock-out or knock-in an immunologic gene of interest; define the actual shape of our proteins (beginning, of course, with wonderful pictures of the major histocompatibility complex protein containing a peptide in the groove); peer into the cell’s interior to explore its signaling pathways; correct oversights such as the almost forgotten innate immune system whose recognition was jumpstarted by the discovery that the Toll-like receptors of the fruit fly had their homologous proteins in humans; and utilize immunologic tenets to create inhibitors of important immune regulators including tumor necrosis factor. These regulators have had amazing success in such diseases as rheumatoid arthritis or inflammatory bowel disease.

One might predict that each key cytokine will play a role in one or more human diseases and that soon we will have a specific inhibitor for each. One goal, therefore, of the clinical immunologist will be to put the right inhibitor or group of inhibitors with the right disease. Moreover, in the preceding example of tumor necrosis factor inhibitors not only is an immunologic protein being inhibited that is a key orchestrator of the inflammatory response, but the agent mediating the inhibition is an immunologic molecule itself – either a humanized monoclonal antibody to tumor necrosis factor or a fusion protein of an antibody and two copies of the tumor necrosis factor receptor. With these agents, the action of tumor necrosis factor is inhibited and the patient’s inflammatory disease becomes less active. As this example illustrates, molecular technology has allowed us to approach immunologic issues with a precision unimagined just a few years ago. Many of our critical reagents and other “tools of the trade” are derived from and based on the technology of molecular biology.

The future of immunology is bright indeed. However, we have much to learn and much to do. We have yet to understand at the most fundamental level the two common scourges of an immune system in disarray – allergy and autoimmunity. We still do not have an adequate idea as to why an individual mounts an immune response to a ubiquitous (in some parts of the world at least) and innocuous substance like ragweed pollen or, in the second situation, synthesizes large quantities of antibodies or T cells that react with self tissue. In many cases we know the structure of the antigen and even what epitope is recognized by an autoantibody or an autoreactive T cell, but we do not know why this self reactive protein or cell developed in the first place or why its production is not turned off. Also, we have yet to adequately harness the great effector activity of the immune system to rid the body of undesirable elements such as malignant cells. While we have had some clinical success, such as with a monoclonal antibody to B-cell and T cell antigens, we have a long way to go in implementing successful strategies to kill most types of tumor cells. Theoretically it seems eminently accomplishable but practically it has been difficult, perhaps because tumor cells are nearly indistinguishable from self except for growing more efficiently. Consequently, which tumor antigens to target and how to target them for destruction are largely unanswered questions.

As we proceed into the new millennium a few cautionary notes are in order. First, the terms “autoimmunity” and “allergy” tend to be employed too generally and to diseases where it is far from certain that, for example, autoimmunity is the problem. Two possible examples of this situation are rheumatoid arthritis and inflammatory bowel disease. In both cases, no specific antigen or effector molecule has been identified. Also, the finding that a major fraction of juvenile rheumatoid arthritis in some parts of the United States is caused by a spirochete (Lyme disease) and that what at one time was called idiopathic mixed cryoglobulinemia is caused by chronic viral infections of the liver should produce a certain level of caution in all of us. Second, of all the major organ systems in the body, none is evolving as rapidly as the immune system. This is not surprising since its role is to respond to swiftly and efficiently mutating microbes. The immune system of the mouse has evolved to deal with mouse pathogens while ours evolved to deal with a different set of pathogens. Thus, advances made in animal models need to be placed into this context. Third, we sometimes tend to neglect the fact that the immune system’s primary job is to handle infectious organisms. Other functions, if any, are quite ancillary. A definitive textbook of immunology will someday be written together with experts in infectious diseases. The design of the book will be such that, for each patient who develops an infection such as pulmonary tuberculosis, the immunologist and infectious disease specialist will analyze the patient’s immune system and then specifically explain why this individual was susceptible and the rest of the family members who were similarly exposed were not. Fourth, the immune system is an organized and interacting “whole” but we immunologists tend to focus on one or another part of the system (trendy immunology, if you will). “Immunology is more than a lymphocyte,” as one author noted.

It is our hope and belief that the basic and clinical immunologic discussions presented in this textbook will be a resource and catalyst for our students of immunology in trying to further understand and apply their tenets to the betterment of humankind. We salute the past and future pioneers in our field of immunology. We recall that our discipline continues to contribute to the field of vaccination – one of the three or four greatest advances in promoting the health of mankind. The reduction in morbidity and mortality from this application of immunologic principles is incalculable. Now we face the daunting task of preventing as well as curing allergic and autoimmune diseases and employing this system to vanquish tumor cells and to enhance immunity against infections.

Finally, we want to thank our assistants, friends and colleagues who made this new edition possible. Our editorial assistants, Alison Angel, Jeanette Clayton, Joanne Miccile, and Lorraine Whiteley carried much of the burden of organizing the day-to-day activities of correspondence, proofreading, reviewing proofs, and vigilance relative to our timetables. Ms. Arlene Stolper Simon spent countless hours editing chapters and reviewing those proofs. We thank the authors who used their vanishing and increasingly valuable time to write these remarkable chapters. One consolation that we each have is that a scientist who writes a chapter always learns the most, even about a field in which he/she is one of the experts. Lastly, we are indebted to our two senior editors at Lippincott, Williams and Williams, Raymond Reter and Jonathan Pine. Their unfailing good humor and optimism about us, our authors, and the book itself is greatly appreciated. By the time this textbook is in print, the genomic sequence will be complete for humans as well for our favorite experimental animal, the mouse. This should provide a fertile data set for immunologists to explore in this millennium. In closing, we hope this textbook meets our goal of making “immunology instructive to the initiated as well as the innocent.”

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1 INNATE IMMUNITY: LINKAGES TO ADAPTIVE RESPONSES

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Innate immunity is a host defense system with an immense repertoire of cellular and humoral components ranging from the skin as a physical barrier to serum proteins that possess highly specific recognition sites for molecular structures common to pathogens. This chapter reviews several major constituents of innate immunity and emphasizes the basic concept recognized by Janeway in 1989, that the innate immune system is critical for turning on the adaptive response (1). Absolutely necessary for effective inflammatory and humoral responses against external pathogens, the innate immune system also seems to guard against autoreactivity and thus protects against autoimmune disease. Innate immunity's involvement during inflammation is discussed, and complement's role in humoral responses and in tolerance is reviewed to illustrate the linkages between the innate and adaptive immune systems.

In general, the innate immune system consists of three major components: (a) phagocytic cells, which eliminate microorganisms by ingesting and degrading them; (b) soluble plasma proteins and glycoproteins that bind microorganisms and target them for phagocytosis (i.e., opsonization) or for attack by the complement system, leading to microbial death by cytolysis; and (c) natural killer (NK) cells, a subset of primitive T cells pivotal for cell-mediated destruction of tumor cells and virally infected cells (Table 1.1). Detailed descriptions of each of these effector arms of innate immunity are provided in later chapters. In contrast to the adaptive immune system with its somatically rearranging, epitope-specific T- and B-cell receptors (TCRs and BCRs), components of the innate immune system do not mutate during the life of the host and do not exhibit memory when confronted with recurrent infections. Instead of somatic memory as defined by the adaptive immune system, the innate immune system has “evolutionary memory” with conserved components that recognize broad classes of molecules from common infectious agents. As a result, genetic deficiencies in various components of the innate immune system can have significant effects on the host's protective immune response.

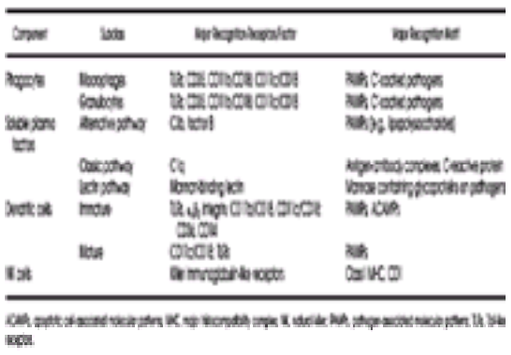

TABLE 1.1. Overview of the Major Components and Receptors of Innate Immunity

ANTIGEN RECOGNITION: IMPORTANCE OF INNATE RECEPTORS

Proper antigen recognition and handling comprise the crux of a normal immune system. Failure to recognize invading pathogens efficiently results in potentially lethal infections, and, conversely, unwarranted recognition of host-derived antigens may lead to autoimmune disease. The innate immune system ensures proper responses based on highly evolved cell-surface receptors and serum proteins that are specific for foreign structures and for self-structures. Many of the system's proteins actually recognize both foreign and host-derived cells and antigens. Because of the inherent risks in this dual recognition, the ability to reconcile mechanisms involved in innate recognition of cells and antigens may lead to novel strategies for both vaccine development and treatment of autoimmune disorders.

Components of the innate response recognize pathogen-associated molecular patterns (referred to as PAMPs) (2). The concept of pattern recognition is a relatively novel formulation of immune recognition. PAMPs include diverse moieties such as lipopolysaccharides (LPS) of gram-negative bacteria, peptidoglycans and lipoteichoic acid of gram-positive bacteria, and mannans of bacteria and fungi (1,3,4). PAMP recognition receptors (PRRs) are similarly diverse. For example, mannan-binding lectin (MBL) is a multimeric serum protein with six carbohydrate recognition domains that bind mannan with relatively high avidity (5). Binding of MBL to pathogen surfaces can activate the complement cascade, resulting in both microbial opsonization to facilitate uptake by phagocytic cells and microbial lysis through complement's membrane attack complex. The mannan receptor is a membrane form of MBL expressed on the surface of mammalian macrophages. Engagement of this receptor directly promotes phagocytosis of the pathogen and subsequent release of proinflammatory chemical mediators (5).

Toll-like receptors (TLRs) are another class of protective PRRs that were described initially in the fruit fly *Drosophila melanogaster* (6). Conserved through evolution, this complex system of receptors responding to distinct ligands includes at least seven *Tlr*-related genes in *Drosophila* and six in humans (7). TLRs are found on mammalian phagocytic cells such as macrophages and on accessory cells such as dendritic cells (DCs) that are important links to adaptive immunity (8). TLRs can bind polypeptide products of proteolytic cascades such as the kinin system, which are often activated during bacterial infection. In addition, TLRs can be activated by secondary innate receptors. For example, mammalian TLR4 is activated indirectly by bacterial LPS after LPS binds the cell-surface receptor CD14 (9). TLR4 activation then triggers activation and translocation of nuclear factor-kB (NF-kB) from the cytoplasm to the nucleus and results in expression of inflammatory cytokines such as tumor necrosis factor-a (TNF-a) and interleukin-1 (IL-1) (9). Currently, intense research is under way to characterize other TLRs and ligands.

The protective power of innate immunity is amplified by a large collection of antimicrobial serum proteins and their cognate cell-surface receptors such as the complement system. When activated, the complement cascade of sequential proteolytic events generates many bioactive peptide fragments (10,11). The C3 component, for example, is enzymatically degraded in a stepwise manner resulting in distinct products identified according to molecular size: C3a, C3b, iC3b, and C3d. For each distinct split product there are one or more cell-surface receptors whose engagement can induce diverse effects on cells of both the innate and adaptive immune systems. Indeed, at least two of these receptors, CD21 and CD35, are discussed later as important links between innate and adaptive immunity (12,13).

IMMUNE SURVEILLANCE

Host cells protect against viral infection or neoplastic transformation by random and continuous sampling of local microenvironments. NK cells are important participants in this immune surveillance (14,15). Technically a subfamily of T lymphocytes, NK cells use “hard wired” recognition receptors rather than somatically rearranged antigen receptors and therefore are considered components of the innate immune system. Both inhibitory and activating receptors, collectively referred to as killer immunoglobulin-like receptors (KIRs), are differentially expressed on NK cells (16,17). NK cell inhibitory receptors recognize conserved regions of the major histocompatibility complex (MHC) class I proteins that are expressed on nearly all host cells (16,18). Host cells expressing these heterodimeric glycoproteins repress NK cell activity. Viral infection or neoplastic transformation can disrupt normal MHC class I expression, and the absence of recognizable MHC class I molecules on host cell surfaces releases the baseline NK cell inhibitory signals, thus permitting NK cell activation. Activating receptors on NK cells include CD2, CD16, and CD69. Coordinate signaling through these receptors allows recognition and potentiates killing of tumor cells or virally infected cells either by direct cytotoxicity or by antibody-dependent cellular cytotoxicity (ADCC) . ADCC is yet another link between innate and adaptive responses during which NK cell cytotoxicity is triggered by the stimulatory Fc receptor FcgRIIIA and by the complement receptor CR3 (CD11b/CD18) after recognition of abnormal cells that are coated with antibody and complement fragments.

The task of continuous immune surveillance by the innate immune system is shared with the alternative pathway of serum complement. Although there are some specific activators of complement, the system exists in a state of perpetual low-level activation through the spontaneous conformational changes or “tickover” of C3 molecules (19). Activated C3 can bind covalently to nearly any surface in its vicinity. C3 attachment to acceptor sites on pathogens triggers rapid amplification of the complement cascade and elimination of those invaders. Host tissues cells are protected from spontaneous complement-mediated injury because host cells express C3 inhibitors. This family of receptors and serum proteins, referred to as regulators of complement activation (RCA) (20), bind activated C3 (C3b) and either block propagation of the complement cascade or cooperate with factor I to degrade the C3 moiety. These regulators include membrane cofactor (CD46), decay-activating factor (CD55), and the Crry receptor (20). Mice lacking the Crry receptor are not viable (21), a finding implying that inadequate inhibition of the complement cascade induces spontaneous injury and death to the fetus.

INFLAMMATION

Inflammation is a major consequence of activating the innate immune system. Classically identified by the four symptoms of heat, rash, edema, and pain, inflammation is the rapid response to an infectious organism that can lead to direct lysis or uptake of pathogens by phagocytic cells within minutes to hours. Serum and cellular components together mediate the phenotype. For example, local activation of the complement system can stimulate mast cell release of proinflammatory cytokines such as TNF-a, chemical mediators such as histamine and a variety of proteases (22). Together, these agents promote dilation and increase permeability of local blood vessels. By upregulating selectins on local endothelial cells, histamine can initiate margination and rolling of circulating leukocytes (23). Additional mediators such as the leukotrienes and platelet-activating factor further enhance the inflammatory response by upregulating integrins and leukocyte influx into the local tissues (24).

Complete characterization of inflammation is not the intent of this introductory chapter. Instead, two animal models of inflammatory processes are presented as specific examples of how inflammation can be both protective and pathogenic to the host. The first example is cecal ligation and puncture (CLP), a model for acute septic peritonitis that includes the combined interaction of serum and cellular components in clearing a bacterial infection. The second example, ischemia reperfusion (IR) injury, is a condition in which the host's innate immune system is provoked to attack self-tissue and can result in death depending on the severity of the inflammatory response.

Acute Septic Peritonitis as a Model for Host Protection

CLP is a mast cell–dependent model of acute septic peritonitis that can progress to fulminant sepsis (25,26). In this surgical model, the cecum is ligated, it is punctured to release bacteria from the intestine, and then the peritoneum is reclosed. Within minutes, bacteria in the peritoneum activate the innate response beginning with mast cell degranulation that, in turn, promotes massive infiltration of neutrophils similar to a ruptured appendix. The innate immune system is essential for host protection because the adaptive response is too slow to be protective in this acute infection model. Nonetheless, mice deficient in serum immunoglobulin M (IgM) have more fulminant sepsis than normal mice undergoing CLP. Reconstitution of IgM-deficient animals with natural IgM from naive unimmunized donors affords some protection against the sepsis syndrome (27). This observation suggests that spontaneously produced natural antibody may be considered a soluble component that is shared between the innate and adaptive immune systems (see later, in the discussion of innate immunity-mediated control of adaptive responses).

Because of its pentameric structure, IgM is effective at binding to pathogens and activating the classical pathway of complement (28,29). Antigen binding induces a conformational change in the antibody that allows C1q binding and initiates the classical complement pathway cascade, with sequential activation of complement components (see chapter 26 for detail on complement pathways). Deficiency in C3 or C4 or in their receptors CD21/CD35 dramatically increases morbidity and mortality in the CLP model (30). The impaired activation of the classical pathway reduces survival by blocking bacteriolysis by the C5-C9 membrane attack complex and by diminishing activation of peritoneal mast cells. In the absence of complement activation, peritoneal mast cells do not recruit neutrophils to the peritoneum, thereby permitting bacteria to escape opsonophagocytosis.

In summary, the CLP model identifies some of the cellular and serum components of innate immunity that are required in protection from severe bacterial infection. Although mast cells express PRRs, costimulatory interactions from IgM natural antibody and the classical pathway of complement are also required for complete mast cell activation. In the absence of IgM, complement or complement receptors (CD21/CD35), neutrophil infiltration and clearance of bacteria from the peritoneum are impaired.

Ischemia Reperfusion Injury: A Model of Pathogenesis

Brief periods of ischemia, which alone do not induce the irreversible cell damage of an infarct, can nonetheless result in severe necrosis after restoration of blood flow. Ischemia appears to alter the expression of surface antigens on local endothelium. These newly exposed antigens can become targets of a vigorous inflammatory response that induces the so-called IR injury. Studies in animal models reveal that IR injury is mediated by many of the same components used in host protection from bacterial infection. Complement proteins, natural IgM, and neutrophils all contribute to inflammation and injury (31,32). Because mice deficient in complement C4 or in IgM are partially protected in a hindlimb model of IR injury (33,34 and 35), this model demonstrates that the protective responses of the innate immune system are also potentially pathogenic.

INNATE IMMUNITY-MEDIATED CONTROL OF ADAPTIVE RESPONSES

One important advance in immunology has been the understanding that the innate immune system regulates adaptive responses. As introduced earlier, the innate system includes “hard-wired” receptors that facilitate either direct or indirect identification of invading pathogens. One example of this phenomenon is the uptake of opsonized microorganisms by phagocytes such as macrophages and DCs, which then process and present microbe-derived peptides to T lymphocytes (36). These antigen-presenting cells further modulate the adaptive response by cytokine release (37). In the absence of costimulation from an antigen-presenting cell, the T-cell response to pathogens would be ineffective and potentially could even lead to anergy (38).

The innate immune system also regulates the development and differentiation of B lymphocytes. Complement, for example, is a positive regulator of B-lymphocyte responses to thymus (T)-dependent antigens (39). Complement-mediated regulation of humoral responses was first noted in 1970, when B lymphocytes were found to bind activated products of the third component of complement (C3) (40). Shortly thereafter, Pepys discovered that mice depleted of serum C3 by treatment with cobra venom factor have diminished antibody responses to T-dependent antigens (41). Building on the Pepys study, investigators subsequently discovered that antibody responses are diminished in animals with naturally occurring deficiencies in complement components C3 as well as C4 and C2 (42,43,44,45,46 and 47) (Fig. 1.1). Humans with deficiencies in C3 and C4 also have diminished responses to T-dependent protein antigens (48). The impaired responsiveness is comparable between animals deficient in classical pathway activators (C4, C2) and C3-deficient animals, a finding suggesting that the effect is mediated through the classical pathway of the complement system. Moreover, the evolutionary conservation of similar phenotypes in species as diverse as guinea pigs, dogs, and humans clearly emphasizes the importance of the classical pathway complement in regulating humoral responses.

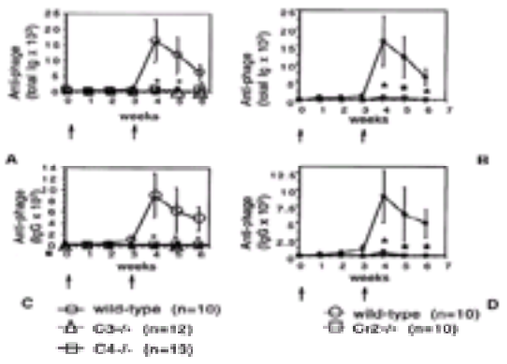

Figure 1.1. Humoral immune responses are impaired in mice deficient in complement C3 or C4 or complement receptors CD21/CD35. Mice (complement-sufficient (WT) or complement-deficient) were immunized at days 0 and 21 with a thymus (T)-dependent antigen (2 × 109 plaque-forming unit bacteriophage) intravenously and total immunoglobulin (Ig) (**A** and **C**) or IgG (**B** and **D**) titers were determined by plaque assay.

Complement's effects on humoral responses are mediated principally through complement receptors CD21 and CD35 found on B lymphocytes and on follicular DCs (FDCs) (49). CD35 is also found on polymorphonuclear cells, macrophages, mast cells, and DCs (49). In mice, the *Cr2* locus encodes both CD35 (CR1) and CD21

(CR2), whereas in humans CD35 and CD21 are encoded by distinct, but closely linked, genes. CD35 and CD21 bind overlapping subsets of proteolytic fragments derived from activated C4 and C3 (see Chapter 26 on the complement system for more details). The C3d and C4d peptide fragments generated during the proteolytic cascade of the classical pathway can covalently attach to antigen (50,51). Complement-coated antigen is more immunogenic than antigen alone. This “adjuvant effect” was demonstrated by two types of experiments. First, by immunizing mice with fusion proteins of hen egg lysozyme (HEL) coupled to multimers of C3d fragments, Dempsey et al. found that addition of three C3d moities were 10,000 times more potent than HEL alone in stimulating an immune response (52).

A second set of experiments used either antibodies directed against CD21/CD35 or a soluble form of the CD21 receptor (sCR2) to compete for uptake of C3d-coupled antigen (53,54,55 and 56). In both examples, humoral responses to T-dependent antigens were impaired. In addition, blocking antibodies against CD21/CD35 were more effective than anti-CD35 alone, a finding suggesting that CD21 may be more important for regulating humoral responses (53).

The finding that CD21 associates with the CD19 and CD81 coreceptors on the surface of human B lymphocytes helped to explain how complement-coupled antigen is more immunogenic than antigen alone (57,58). By engaging the BCR and CD21 simultaneously, the antigen-C3d complex delivers a stronger stimulus than antigen alone (59). Accordingly, because naive B lymphocytes bear low-affinity receptors for antigen, concomitant coreceptor signaling would augment the BCR signal and would thereby lower the threshold of response. Complement receptors may also exert their adjuvant effect by increasing the efficiency of antigen trapping by FDCs (60,61,62 and 63) (Fig. 1.2).

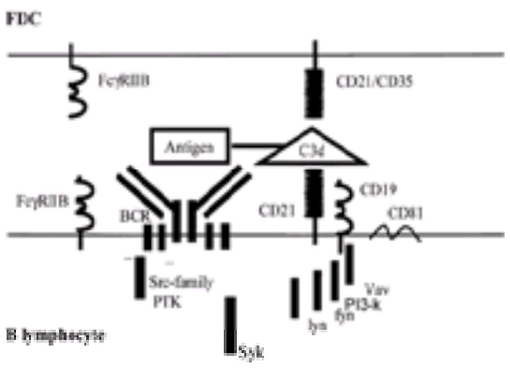

Figure 1.2. Complement recptors CD21 and CD35 are coexpressed primarily on B lymphocytes and follicular dendritic cells (FDCs) in mice. On B lymphocytes, CD21/CD35 form a coreceptor with CD19 and TAPA-1 (CD81). Binding of antigens coupled to C3d coligates the coreceptor with the B-cell antigen receptor (BCR) and results in a lowered threshold of B-lymphocyte activation. CD21/CD35 on FDCs allows efficient antigen trapping within lymphoid follicles.

The availability of *Cr2*$^{-/-}$ mice, deficient in both CD21 and CD35, provides an opportunity to differentiate between the independent contributions of CD21/CD35 expression by B lymphocytes and by FDCs (64,65). Based on the observation that FDCs are resistant to irradiation and are not regenerated from adult bone marrow, Ahearn et al. designed chimeric mice that contained *Cr2*$^{+/+}$ B lymphocytes and *Cr2*$^{-/-}$ FDCs (64). Initially, these chimeric mice respond normally to immunization with T-dependent antigens; however, their long-term memory responses are poor. Therefore, it appears that CD21/CD35 on B lymphocytes is sufficient for generating near-normal antibody titers, but the absence of efficient antigen retention by CD21/CD35 on FDCs may compromise long-term B-lymphocyte memory.

Complement-Regulated B-Lymphocyte Responses

B-lymphocyte maturation and differentiation proceed through an ordered sequence of events that depends on interactions with other cell types and the local microenvironment (66). Moreover, B-lymphocyte responses to antigen vary in each stage of development. For example, cross-linking of the BCR during the immature stage leads to death or anergy (67,68,69,70,71 and 72). By contrast, BCR stimulation of mature B lymphocytes leads to activation. B lymphocytes are tightly regulated at multiple stages, or checkpoints, of development and maturation. The complement system appears to be involved in at least five of these stages (summarized in Fig. 1.3), and complement's influence at these checkpoints further exemplifies the linkage between innate and adaptive immunity.

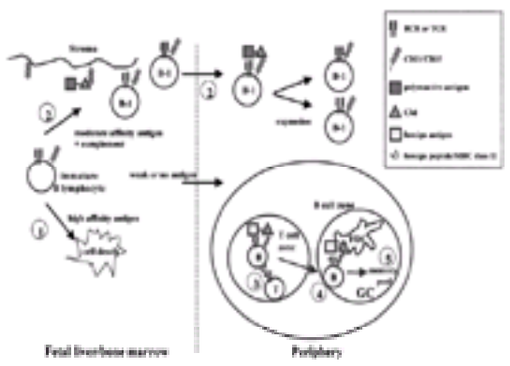

Figure 1.3. Checkpoints in B-lymphocyte development and differentiation in which complement is likely important. At checkpoint 1, immature B lymphocytes recognizing antigen with high affinity are triggered to apoptose. Immature B lymphocytes recognizing antigen with moderate affinity in addition to receiving a signal through CD21/CD35 undergo differentiation into B-1 cells (checkpoint 2). Similarly, signals through CD21/CD35 in the periphery may lead to expansion of B-1 cells during checkpoint 2. Immature B lymphocytes not recognizing antigen or recognizing antigen with low affinity are released into the periphery as B-2 cells, where they survey secondary lymphoid tissues in search of antigen. Their survival after contact with moderate-affinity antigen depends on coreceptor signals through CD21/CD35 during checkpoint 3. Lymphocytes receiving requisite signals continue to differentiate within germinal centers (GCs), where CD21/CD35 is again important for checkpoint 4. B lymphocytes not receiving complement-ligand interactions in GCs die. Successful survival during selection and differentiation in GCs leads to production of long-lived memory B lymphocytes and plasma cells. The life span of these lymphocytes may also depend on continued interaction of antigen deposited on follicular dendritic cells (FDCs) with CD21/CD35 (checkpoint 5)

CHECKPOINT 1: NEGATIVE SELECTION OF IMMATURE SELF-REACTIVE B LYMPHOCYTES

B lymphocytes are regulated during development so those bearing self-reactive antigen receptors are either eliminated or rendered anergic before reaching maturity (73,74). Insight into this negative selection process derives primarily from studies of mice possessing autoreactive Ig transgenes (71,73,74). However, even in these experimental model systems, it remains unclear how self-antigens are presented to the developing lymphocytes. Growing evidence supports a role for the innate immune system in directing negative selection by enhancing interactions between immature lymphocytes and highly conserved self-antigens such as nuclear proteins and DNA (75). This stage can be referred to as the first checkpoint and is discussed further later in this chapter.

CHECKPOINT 2: POSITIVE SELECTION AND EXPANSION OF B-1 CELLS

Natural antibody, in contrast to antibody secreted in response to active immunization, is continuously released by the B-1 subpopulation of lymphocytes (76). Predominantly IgM, natural antibodies tend to be polyreactive with low-affinity binding for antigens such as nuclear proteins, DNA, and phosphatidyl choline that are common structures among both pathogens and host tissues (77,78,79,80 and 81).

Unlike conventional B lymphocytes (i.e., B-2 cells), B-1 cells are found primarily in the peritoneal cavity and reside only at low frequencies in traditional lymphoid tissues (82,83). They rarely undergo class switching or somatic hypermutation in response to antigenic stimulation, a finding suggesting that they do not require germinal centers for further maturation and expansion (84,85 and 86). As a result, antibodies from B-1 cells tend to be encoded by germ line Ig variable region genes that may have been selected through evolution for their ability rapidly to recognize pathogens and to activate the complement cascade. Activation of complement then assists recruitment of cellular components of the innate immune system while at the same time enhancing the humoral responses of the adaptive immune system. As discussed at the end of this chapter, natural antibody may also be important for recognition and handling of self-antigens routinely released from apoptotic cells.

In comparison with conventional B lymphocytes, B-1 cells are more sensitive to signaling through the BCR (87,88,89,90 and 91). As a result, genetic defects altering the BCR's signaling capacity have more dramatic effects on B-1 than on conventional B lymphocytes. For example, mice with defects in critical positive regulators of the BCR signaling pathway such as btk, CD19, vav, or phosphatidylinositol-3-kinase have few, if any, B-1 cells, whereas numbers of B-2 lymphocytes are less severely affected (92,93 and 94). Conversely, defects in negative regulators of BCR signaling (e.g. SHP-1, CD72) leads to larger B-1 cell populations (95,96 and 97). These observations indicate that BCR signaling is critical in the initial positive selection, expansion, or maintenance of B-1 cells. Mice deficient in complement receptors CD21/CD35 also have a reduced frequency of B-1 cells (64), consistent with a role for complement in the selection or maintenance of B-1 cells.

CHECKPOINTS 3 AND 4: PERIPHERAL B-LYMPHOCYTE ACTIVATION AND GERMINAL CENTER RESPONSES

Mature B lymphocytes circulate through the spleen and other lymphoid tissues in continuous search for antigen. Antigen encounter by specific B lymphocytes triggers distinct steps of lymphocyte activation, expansion, and differentiation into memory cells and plasma cells (66). Complement's involvement at multiple steps along this pathway offers a complex example of innate regulation of adaptive responses.

Similar to T cells, B lymphocytes require two signals for full activation (i.e., BCR cross-linking and costimulation) (98,99,100,101,102 and 103). By enhancing localization of antigen to the lymphoid compartment (61,62), complement optimizes BCR cross-linking by antigen, and, as a ligand that signals through the CD21/CD35 receptors, complement further augments BCR activation (104). Complement's covalent attachment to antigen engages the CD21/CD19/CD81 coreceptor complex in concert with BCR stimulation by antigen (Fig. 1.2). If this dual binding generates adequate signaling to reach the activation threshold, then the humoral response continues (checkpoint 3), whereas subthreshold signals delivered through the BCR lead to Fas-dependent apoptosis of the B lymphocyte (105). Evidence supporting the threshold hypothesis came from studies of mice with either a functional or a disrupted *Cr2* locus that were also transgenic for Ig heavy and light chains specific for HEL (106). This model system provided an opportunity to measure the contribution of coreceptor signaling in an immune response against lysozyme. When $Cr2^{+/+}$ HEL Ig transgenic B lymphocytes are exposed to lysozyme, they mount a strong immune response. In contrast, CD21/CD35-deficient ($Cr2^{-/-}$) HEL Ig transgenic B lymphocytes generate a weak immune response because most do not survive within the splenic white pulp. Immunization with high-affinity antigen (i.e., a different form of lysozyme) rescues the transgenic CD21/CD35-deficient lymphocytes. These findings suggest that moderate-affinity antigen plus complement can substitute for high-affinity antigen alone. In other words, coreceptor signaling effectively lowers the threshold for activation and survival of B lymphocytes encountering moderate-affinity antigens, thereby substantiating the threshold hypothesis.

The white pulp of the spleen contains T-cell–rich zones where B lymphocytes are first activated and distinct B-lymphocyte zones that are called follicles (107). Activated B lymphocytes receiving above-threshold signals in combination with costimulation from helper T lymphocytes undergo rapid cell division. Some B-lymphocyte progeny differentiate into antibody-forming cells and move into the red pulp, whereas others enter into lymphoid follicles (108). Activated B lymphocytes in follicles initiate germinal center (GC) reactions that are specialized for further B-lymphocyte differentiation (109,110 and 111). GC immigrants initially mutate their Ig receptor genes by somatic hypermutation and are then subjected to antigen selection within the light zones of GCs (112,113 and 114). Selected B lymphocytes undergo additional rounds of proliferation, Ig class switching, and differentiation into long-lived memory and plasma cells (108,115).

Using the HEL Ig transgene model described earlier, it was unexpectedly discovered that complement ligand is critical for effective B-lymphocyte survival within GCs, irrespective of antigen affinity (106). Transgenic B lymphocytes with CD21/CD35 populate 50% of GCs in mice immunized with high-affinity lysozyme, whereas transgenic B lymphocytes without CD21/CD35 are rarely found in GCs under identical experimental conditions. These results imply that coreceptor signaling does much more than simply enhance BCR signaling, and perhaps it provides a unique B-lymphocyte survival signal at what we refer to as checkpoint 4. A requirement for complement at this checkpoint is novel, and the potential mechanisms remain to be elucidated.

CHECKPOINT 5: COMPLEMENT AND B-LYMPHOCYTE MEMORY

High-affinity antigen-specific memory B lymphocytes are generated at the end of GC reactions. Mechanisms of maintaining B-lymphocyte memory over time are not well understood, but they likely involve continued antigen stimulation (116). FDCs that can store antigen may serve as long-term cellular depots (117,118), and complement receptors, important for retention of complement-decorated antigens by FDC (61,62,65), are good candidates for involvement in the maintenance of long-term memory. If so, then the maintenance of long-term memory B lymphocytes can be considered a fifth checkpoint in which innate immunity impinges on the adaptive immune response.

INNATE IMMUNITY AND AUTOREACTIVITY

Not only does innate immunity help to amplify protective immune responses against invading pathogens, but also it may protect against autoreactivity by modulating recognition of autoantigens (75). For example, cell turnover by apoptosis is a continuous process during hematopoiesis and a common response to environmental stresses such as exposure to ultraviolet radiation. The innate immune system seems to participate in the routine clearance of apoptotic debris in part through recognition motifs referred to as apoptotic cell-associated molecular patterns (ACAMPs) (119,120 and 121). Expressed by phagocytic cells of the innate immune system, receptors specific for different ACAMPs include CD36, CD14, and CD11b/CD18. ACAMPs may be the autoantigen equivalent of PAMPs, but unlike the binding of PAMPs by recognition receptors that potentiate immune responses (see section I), ACAMP receptors may participate in a tolerogenic display of self-antigens to autoreactive lymphocytes.

DCs are antigen-presenting cells that express both PAMP and ACAMP receptors. When an immature DC internalizes a PAMP-bearing pathogen, it undergoes activation and maturation, thus becoming a potent stimulator of T lymphocytes (122). In contrast, on internalization of apoptotic debris, presumably through ACAMP receptors, the immature DCs do not mature and do not potentiate lymphocyte activation (123,124). It is tempting to speculate that ACAMPs induce these cells of the innate immune system to communicate tolerogenic signals to cognate T lymphocytes.

Various soluble components of the innate immune system also contribute to the clearance of self-antigens. Antigen can be “labeled” by covalent coupling to C3b and C4b fragments and by noncovalent interactions with other serum proteins. Two such serum proteins are the acute-phase reactants C-reactive protein (CRP) and serum amyloid P component (SAP), which are highly conserved serum pentraxins whose titers rise during infection and inflammation (125,126). SAP is capable of binding to chromatin released from apoptotic cells and can also bind and activate C1q (127,128). This acute-phase reactant may participate in innate mechanisms that clear the circulation of potentially immunogenic autoantigens, thereby protecting against development of autoimmune disease. This notion derives from animal models in which mice genetically deficient in SAP spontaneously produce anti-DNA autoantibodies and have an increased incidence of renal disease (129) (Table 1.2).

Mouse Genotype	Reference
$SAP^{-/-}$	Bickerstaff et al., 1999 (129)
$C1q^{-/-}$	Botto et al., 1998 (142)
$sIgM^{-/-}$	Boes et al., 2000 (146); Ehrenstein et al., 2000 (145)
$C2^{-/-}xFas^{lpr/lpr}$	Prodeus et al., 1998 (75)
$C4^{-/-}xFas^{lpr/lpr}$	Prodeus et al., 1998 (75)
$TNFR1^{-/-}xFas^{lpr/lpr}$	Zhou et al., 1996 (153)

TABLE 1.2. Deficiencies in Components of Innate Immunity Associated with Lupuslike Disease in Mice

Complement Deficiency and Systemic Lupus Erythematosus

The striking association of complement deficiency with systemic lupus erythematosus (SLE) is compelling, although circumstantial, correlative evidence that innate immunity has significant effects on the adaptive system. Specifically, humans deficient in either complement C1q or C4 almost always develop SLE or lupuslike disease (130,131). These autoimmune disorders are characterized by high-affinity autoantibodies specific for protein and nucleic antigens that are highly conserved among vertebrates and invertebrates including ribonuclear proteins, histones, and dsDNA (132,133 and 134). Apoptotic cell debris is a potential source of lupus autoantigens (135). *In vitro,* experimental induction of apoptosis promotes the translocation, externalization, and release of nuclear antigens including dsDNA in membrane-bound vesicles referred to as apoptotic bodies (136). The identification of direct binding of complement C1q to the apoptotic bodies may implicate a role for complement in

clearance of the released nuclear material (137).

Two general hypotheses have been proposed to explain the increased incidence of SLE among complement-deficient individuals: the clearance hypothesis (138,139) and the tolerance hypothesis (140,141). The former hypothesis is based on complement's known role of binding to foreign antigens and transporting them to the liver and spleen for degradation, thereby facilitating their removal from the circulation. Mice deficient in C1q develop spontaneous lupuslike autoreactivity characterized by rising titers of autoantibodies with progressive glomerulonephritis (142). Mice deficient in SAP are similarly susceptible to lupuslike disease (129). SAP binds chromatin and may be important for its degradation. In humans, SAP also activates classical pathway complement, a finding further supporting the hypothesis that innate immunity promotes efficient clearance of complex nucleoprotein autoantigens released from apoptotic cells (127).

Natural antibody, which binds many self-antigens that are highly conserved among bacteria, yeasts, and viruses (143,144), also appears to be protective against lupus disease, based on recent characterizations of mutant mice whose B lymphocytes express surface IgM but do not secrete soluble IgM. Essentially devoid of natural IgM ($sIgM^{-/-}$), these mice develop autoantibodies and renal changes analogous to the C1q-deficient and SAP-deficient animal models, a finding suggesting that natural IgM may play a protective role in binding and clearance of lupus autoantigens (145,146). One shortcoming of the clearance hypothesis is that it fails to explain adequately how a breakdown in antigen clearance can lead to development of high-affinity autoantibodies.

The tolerance model proposes that innate immunity protects against SLE by delivering lupus autoantigens to sites where immature B lymphocytes are tolerized. For example, efficient targeting of self-antigens to the bone marrow by components of innate immunity could enhance contact between immature autoreactive B lymphocytes and lupus autoantigens. In addition, coreceptor signaling by complement receptors can augment the strength of BCR signaling so self-antigens coated with complement ligand may be more potent tolerogens for immature B lymphocytes than self-antigen alone. Where and how autoreactive B lymphocytes actually encounter self-antigens are unknown, however, although presentation of self-antigens may involve the innate immune system.

One prediction of the tolerance hypothesis is that defects in the induction of B-lymphocyte tolerance will lead to spontaneous development of autoreactivity. For example, mice deficient in proteins involved in the BCR signaling pathway (e.g., CD22, Lyn or SHP-1) spontaneously develop lupuslike disease, presumably because dysregulation of BCR signaling allows self-reactive B lymphocytes to escape tolerance (147,148 and 149). Transgenic mice in which most of their B lymphocytes express an antibody transgene specific for self-antigens, such as dsDNA or MHC glycoprotein, have been instrumental in identifying the distinct stages of B-lymphocyte development and regulation of autoreactivity (70,71). Breeding of the transgenic mice with strains deficient in BCR signaling proteins has demonstrated that the strength of antigen-associated signal transduction dictates whether self-reactive B lymphocytes are eliminated, are anergized, or develop to maturity. For example, by breeding the previously discussed HEL Ig transgenic mouse strain to another strain transgenic for soluble HEL (sHEL), Goodnow et al. (1988) generated a novel animal model of autoreactivity in which HEL Ig transgenic B lymphocytes exist in a nonreactive, or anergic, state (150).

Compared with the HEL Ig single transgenic mice, the life span of mature HEL-specific B lymphocytes in sHEL/HEL Ig double transgenic mice is markedly diminished, a finding suggesting that anergy pares the autoreactive population considerably. Experimental overexpression of the BCR-associated signaling protein CD19 leads to increased signaling on BCR engagement (151). In sHEL/HEL Ig double transgenic mice that overexpress CD19, more HEL Ig transgenic B lymphocytes are eliminated while still immature and those that escape to the periphery are no longer tolerant (152). These findings suggest that enhanced BCR signaling in immature autoreactive cells promotes deletion, whereas enhanced BCR signaling in mature autoreactive cells promotes activation. Similarly, loss of BCR coreceptor proteins CD21/CD35 also disrupts normal tolerizing mechanisms. When the sHEL/HEL Ig double transgenic mice are bred with CD21/CD35-deficient ($Cr2^{-/-}$) mice, increased numbers of mature, nonanergic autoreactive B lymphocytes escape tolerance regulation (75). Not only do these results support the idea that decreased signal transduction through CD21/CD35 permits escape of immature B lymphocytes from central tolerance in the bone marrow, but also they implicate a specific role for complement ligands in normal induction or maintenance of B-lymphocyte tolerance to autoantigens.

In all likelihood, the complement ligands that bind CD21 and CD35 in the induction of anti-HEL tolerance derive from the classical complement pathway because similar autoreactive phenotypes also arise in the absence of C4, but not C3 (75). After bone marrow transplantation from anti-HEL donors into sHEL hosts, anti-HEL B lymphocytes are tolerized reasonably well even when the recipients are deficient in C3. In contrast, the HEL Ig transgenic B lymphocytes that develop in C4-deficient recipients are not properly anergized. Much like in the CD21/CD35-deficient double transgenics, these HEL Ig transgenic B lymphocytes are present at increased frequency and retain the ability to respond to antigen *ex vivo* (75). These observations suggest that the classical pathway mediates CD21/CD35-based signaling for tolerance induction, possibly in the immature B lymphocyte, whereas C3 fragment-mediated signaling is reserved for the activation of mature B lymphocytes.

SUMMARY

The innate immune system includes a diverse and broad range of serum and cellular recognition proteins that, on activation, carry out various effector functions. Not only does the innate system respond immediately to pathogenic infection, but also it initiates and propagates adaptive responses. For example, the classical pathway of complement is likely involved in at least five checkpoints in B-lymphocyte development and differentiation, ranging from negative selection to maintenance of long-term memory. The appreciation that innate immunity protects against autoimmune diseases such as SLE offers further insight into the pathogenesis of this potentially fatal disorder. Continued elucidation of the mechanisms linking innate and adaptive immunity hold promise for more effective vaccine strategies and treatment of autoimmune disorders as well as for a provocative field of research.

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2 ARCHITECTURE OF THE IMMUNE SYSTEM

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Coincident with the development of specific immunity, a series of organs and tissues appeared in jawed vertebrates to provide distinct cellular environments for lymphopoiesis and sites for lymphocyte interaction and response against antigen (1,2 and 3). Thus, the immune system has compartmentalized the generative and response phases of immunity into discrete organs and tissues and interconnected these elements by the blood vasculature and lymphatic channels.

The lymphatic organs are commonly divided into primary, or generative, lymphoid organs and secondary, or peripheral, organs. In the primary lymphoid tissues, organized sets of specialized cells and molecular cues support and direct T- and B-lymphocyte development from hematopoietic stem cell progenitors. This cellular ontogeny includes the generation of antigen-specific receptor molecules, developmental checkpoints to ensure the production of functional receptor molecules, and the removal or functional inactivation of cells bearing receptors that avidly react to self-antigens (4). Secondary lymphoid organs are rich in mature, functional lymphocytes and their accessory cell partners; in secondary lymphoid tissues, lymphocytes, accessory cells, and antigen are brought together to maximize the efficiency of immune responses. The placement of secondary lymphoid tissues in the body and their vascularization direct antigen and antigen-transporting cells through regions of concentrated lymphocyte populations. This parade of antigen and antigen-bearing cells maximizes the numbers of lymphocytes that can respond to infection or immunization and accelerates protective immunity (5,6 and 7).

In humans and rodents, the primary lymphoid organs comprise the bone marrow, the site of B-cell lymphopoiesis, and the thymus, the site of T-cell lymphopoiesis. The major secondary lymphoid tissues include the spleen, the lymph nodes, and Peyer patches. Nonetheless, the tissues of lymphocyte generation vary substantially from species to species. For example, the Peyer patches function in humans and rodents as secondary lymphoid tissues, whereas in sheep, they play a major role in B-cell generation (8). Other species, such as birds, have specialized organs for lymphopoiesis. The bursa of Fabricius is a primary site of B-cell development in all avian species (9,10), and similar developmental activity is present in the rabbit appendix and sacculus rotundus (11,12).

PRIMARY LYMPHOID TISSUES

The generation of mature lymphocytes depends on a common, self-renewing hematopoietic *stem cell* that is present in the bone marrow (13,14). The progeny of this cell undergoes regulated differentiation steps and rounds of proliferation to establish populations of committed precursors and developing lymphocyte forms. This pattern of development is characteristic for both T and B lymphocytes, and the establishment and regulation of precursor cell pools depend on the cellular environments present in the thymus and bone marrow (15,16). The stromal and epithelial cells present in bone marrow and thymus do not merely function as mechanical supports for the hematopoietic cells they surround; rather, they provide essential developmental signals that guide lymphopoiesis.

SECONDARY LYMPHOID TISSUES

As tissues specialized for immune response, most *secondary lymphoid tissues* possess collection systems for antigen and antigen-transporting cells that drain associated nonlymphoid tissue. These collection channels transport antigen into highly organized populations of antigen-presenting cells and T and B lymphocytes (17,18 and 19). All secondary lymphoid tissues make use of specialized vascular structures that enable them to recruit lymphocytes, particularly naive lymphocytes, from the blood (20,21). The secondary lymphoid tissues also contain distinct lymphoid microenvironments, principally zones rich in either T or B lymphocytes (5,6,17,18). These zones also contain accessory cells specific for the appropriate lymphocyte type. Interdigitating dendritic cells (DCs) present in the T-cell–rich areas of secondary lymphoid tissues are specialized for antigen-processing and major histocompatibility complex (MHC)–restricted antigen presentation (22). In contrast, the follicular DCs (FDCs) present in B-cell regions are unable to process antigen but retain it in native form by binding antibody-antigen and complement-antigen complexes by cell surface complement and Fc receptors (23,24).

In various forms, these common elements, collection channels for antigen, specialized blood vasculature, and organized populations of lymphocytes and accessory cells, define types of secondary lymphoid tissues. Lymph nodes are the termini of efferent lymphatic channels that drain the interstitial space of most tissues; lymph nodes collect and localize immune responses to antigen recovered by its associated lymphatics (5,18,19). The spleen filters blood, collecting antigens that have been introduced into the vascular system (17), whereas mucosal-associated lymphoid tissues, such as Peyer patches, are specialized for the collection of antigen across a mucosal epithelium (19,25).

LYMPHATICS

The *lymphatic network* is a system of thin-walled, specialized vasculature that originates as capillary channels in the extracellular space (26,27). Lymphatic capillaries are widely distributed, but are most numerous beneath the skin and the mucosae of the gastrointestinal, respiratory, and genitourinary tracts (28,29). Lymphatic capillaries collect to form larger lymphatic vessels, the efferent lymphatics, which converge and empty into regional lymph nodes. Each *lymph node,* or cluster of lymph nodes, monitors immunologically the region defined by the efferent lymphatic drainage. The efferent lymphatics continue and converge into larger lymphatic vessels, they pass through increasingly central lymph nodes, and finally meet in a single lymphatic vessel, the *thoracic duct.* The thoracic duct empties into one of the great veins supplying blood to the heart that returns the lymph and its cellular components to the blood circulation (28,29).

TISSUES OF PRIMARY LYMPHOGENESIS

Bone Marrow

The *bone marrow* is the major hematopoietic organ in most mammalian species and is necessary for the production of all formed blood elements. Two types of bone marrow, red and yellow, are present in adults. Red bone marrow derives its color from abundant erythrocytes and hemoglobin-containing precursors, whereas yellow bone marrow is colored by the fat cells, or adipocytes, present within it. In adult humans, the proportion of yellow bone marrow can be as high as 70%; red marrow is actively hematopoietic, whereas yellow marrow does not normally produce blood cells, although it can convert to blood-forming tissue under certain conditions (30,31). In newborns and young humans, the majority of bone marrow is red, but in adults, red marrow is common only in the clavicles, vertebrae, sternum, and bones of the pelvis and skull, where it accounts for about 2% of the body mass (30,31).

In the long bones, red marrow occupies the central cavity of the bone shaft. Protruding ridges and spiculae, collectively known as trabeculae, modify the inner surface of long bones. The trabeculae form interconnecting chambers and passageways lined by a single layer of cells, the endosteum (32). This thin cellular sheath contains distinct populations of cells that continuously remodel the bone: osteoblasts that lay down bone and osteoclasts that resorb it (33). In concert, osteoblasts and osteoclasts reform and repair bone structure. In the long bones, blood is supplied by a central nutrient artery, which enters the marrow at midshaft and branches into central, longitudinal arteries (Fig. 2.1). These longitudinal arteries follow the bone's long axis and send out branches into the marrow's periphery. In turn, these smaller arteries ramify further into arterial capillaries and connect to a network of venous sinuses. Venous blood flow from these sinuses is collected by radial veins and exits

the bone marrow through a central longitudinal vein, which runs parallel to the central longitudinal artery.

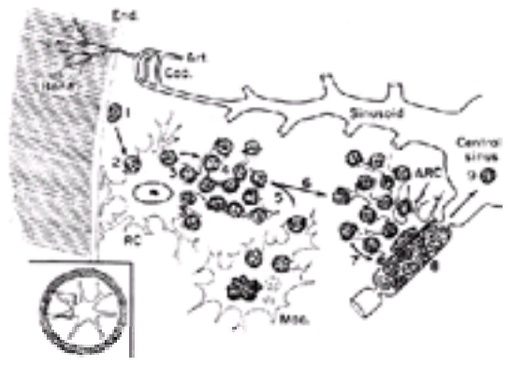

Figure 2.1. The architecture of bone and B lymphopoiesis. **A:** Diagrammatic representation of the vasculature of a long bone showing the pathways of the central (C) and nutrient arteries (N). **B:** Hematopoietic development and differentiation are organized spatially in red bone marrow. Work by D.G. Osmond suggests the placement of early hematopoietic stem cells near the endosteum of the bone marrow *(1).* B-lineage precursors *(2)* differentiate in association with the stromal cell reticulum with individual precursors dividing *(3)* to produce clusters of early B-lineage precursors that retain contact with the stromal cell surface. Successive cell divisions *(4)* of pro-B and pre-B cells result in the central displacement of daughter cells along networks of stromal cell processes. With the rearrangement of the light-chain gene, immature B cells expressing nonfunctional or autoreactive antigen receptors may undergo apoptosis and may be removed by bone marrow macrophages *(5);* maturing B cells bearing membrane immunoglobulin M then follow reticulum cell processes to enter the sinusoids *(6).* After traversing the sinusoid wall *(8)* functional, selected B cells follow along the central sinus to enter the blood vasculature. (From Jacobsen K, Osmond DG. Microenvironmental organization and stromal cell associations of B lymphocyte precursor cells in mouse bone marrow. *Eur J Immunol* 1990;20:2395–2404, with permission.)

The space between the marrow's blood vessels is occupied by irregular cords of tissue containing the bone marrow stroma, a collection of branching, interconnected reticular stromal cells, macrophages, and adipocytes. This reticular framework surrounds the bone's hematopoietic cell populations and functions to support the development of the hematopoietic cell lineages (34,35). In addition to the hematopoietic cells themselves, adipocytes, macrophages, and mast cells are also present in active marrow (34,35). Specific cell lineages and types are generally localized to specific areas within the hematopoietic compartment. Thus, after marrow lympholysis by irradiation (35), B-cell recovery is focused in subosteal areas bearing a distinctive extracellular matrix and rich in reticular stromal cells, megakaryocytes and erythrocytes can be found in approximation to reticular cells within the venous sinuses, and the most mature B lymphocytes are abundant in the centers of the sinusoid lumina (Fig. 2.1).

In mammals, the initial stages of blood formation occur in the hematopoietic tissue, although some of the later stages of cellular maturation may occur within the vasculature (34). To enter the blood circulation, newly formed blood cells pass through the sinus wall. Once there, blood cells, including the lymphocyte precursors, become a part of the blood circulation and may be disseminated by the venous system throughout the body (Fig. 2.1).

Bursa of Fabricius

In all birds, the digestive, urinary, and reproductive tracts open into a common chamber, the cloaca. In the cloaca, a small opening leads to a unique lymphoid follicle, known as the *bursa of Fabricius* (Fig. 2.2). On about the eighth day of embryonic life, lymphocyte precursors migrate from the yoke sack into the bursa, and by the tenth day of development, they extensively proliferate to form mature lymphoid follicles (36,37). The number of lymphocytes in these follicles increases rapidly, partly because of immigration of new lymphocyte precursors, but mainly because of the rapid proliferation of the resident lymphocytes (37).

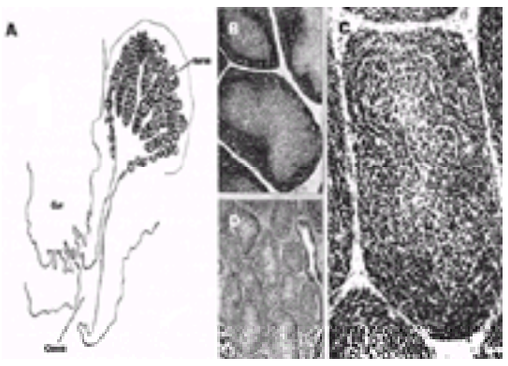

Figure 2.2. The bursa of Fabricius opens into the cloaca **(A)** and contains numerous follicles comprising an associated epithelium and actively dividing B-lymphocytes. Development of the bursa and its lymphoid compartments begins *in ovo* but appears to be driven by microbial flora after hatching. Bursal follicles reach their peak size just prior to sexual maturity and then involute; bursal follicles are virtually absent in mature birds. Within bursal follicles **(B,C)**, B cell–rich areas include peripheral dark zones of dividing lymphocytes that surround a central light zone containing non-dividing cells. Each follicle is delimited by a lacework of connective tissue; after irradiation or administration of nortesterone **(D)**, the follicular capsules become dramatically less cellular due to massive apoptosis. In contrast to the germinal center-like structures of appendix (Fig. 3) or Peyer's patch (Fig. 9), few if any T-lymphocytes (T) are present in the follicles of the bursa of Fabricius even in hatchling or maturing birds. (Original magnification ×100. From Weiss L. *The cells and tissues of the immune system. Structure, functions, interactions*. Englewood Cliffs, NJ: Prentice-Hall, 1972.)

During their residence in the bursa of Fabricius, precursor B cells develop into mature, functional B lymphocytes and acquire novel antigen receptors by a process of gene conversion (9,10,36). These bursal-derived B lymphocytes emigrate from their follicular niches, enter the circulation, and are carried to the peripheral lymphoid organs, primarily the spleen. As the bird becomes sexually mature, the bursa of Fabricius undergoes physiologic involution. In the domestic fowl, bursal activity begins to decline at 7 to 13 weeks after hatching, but bursal involution can be induced by injecting androgenic hormones. Significantly, the injection of such hormones into the eggs during embryonic life arrests bursal development almost completely and results in hatchling birds that are completely deficient of B lymphocytes (37).

Organs that appear to serve homologous functions occur in certain mammalian species, namely, rabbits and sheep. In rabbits, the appendix (Fig. 2.3) and sacculus rotundus have a similar although not absolute role in the development and diversification of mature B cells (38,39). Lymphocyte precursors that enter the fetal and neonatal appendix proliferate, are diversified by immunoglobulin gene conversion or mutation, and mature into responsive, peripheral B lymphocytes (11,12). In the fetal lamb, many of the developmental processes active in the bursa of Fabricius have been observed in Peyer patches, foci of lymphoid tissue that become established along the gut of fetal mammals (8,11,12,39,40 and 41). Within ovine Peyer patches, B-lymphocyte development and genetic diversification by immunoglobulin hypermutation are supported in follicular environments that are strikingly similar (42) to the follicles present in the avian bursa (Fig. 2.2) and rabbit appendix (Fig. 2.3). Mature B cells from Peyer patches emigrate by means of the lymph and blood throughout the fetal lamb and become disseminated into secondary lymphoid tissues.

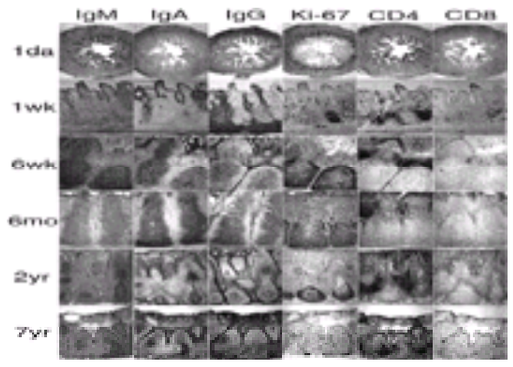

Figure 2.3. The rabbit appendix at various times after birth. The basic structure of the appendix is similar to that of the bursa of Fabricius (Fig. 2.2), with numerous ovoid or trapezoidal follicles containing B lymphocytes (B) in apposition to a supporting follicular epithelium. The development of follicular structure begins after birth and depends on the presence of a gut flora. With time, the follicles enlarge, reaching a maximum in young adult animals and then regressing with advancing age. The appendix contains well-described perifollicular T-cell zones, and $CD4^+$T lymphocytes (T) are scattered about in secondary follicles or germinal centers (GC). These germinal centers contain light and dark zones (LZ, DZ) (original magnification ×100). (From Dasso JF, Obiakor H, Bach H, et al. A morphological and immunohistochemical study of the human and rabbit appendix for comparison with the avian bursa. *Dev Comp Immunol* 2000;24:797–814, with permission.) (See Color Figure 2.3.)

Thymus

During embryonic development, the endoderm of the third and fourth pharyngeal pouches, the ectoderm of the corresponding brachial clefts, and mesenchymal cells from the associated pharyngeal arch develop into the *thymic anlage* (43). Interaction among all three cell types is necessary for complete thymic development, and mutations that block these interactions result in athymia (44). Similarly, ablation of the embryonic neural crest that provides the mesenchymal cells of the pharyngeal arch interferes with thymic development and results in defects similar to those of DiGeorge syndrome (45,46).

In mice and birds precursor cells begin to infiltrate the thymic anlage even before the organ becomes vascularized, and this cell immigration proceeds in ordered waves until early neonatal life (47,48,49 and 50). These immigrants derive from the hematopoietic centers of the embryo, the yolk sac, and the liver. After birth, immigration continues from the bone marrow (51,52). The lymphocyte precursors that enter the nascent thymus interact with the epithelial cells present in the thymic rudiment and induce mutual and specific differentiation. Obvious consequences of these interactions are the organization of the uniformly distributed lymphocyte precursors into specific zones that become the cortex and medulla of the definitive thymus and the induction of vascularization (53). This organization is also associated with the elaboration of specific factors by the thymic epithelium that promote differentiation in the thymic lymphoid population (53,54).

The *human thymus* consists of two lobes, joined at their apex, that extend from the upper end of the sternum to the pericardium (Fig. 2.4). This structure is common in most mammals. Interestingly, in guinea pigs and birds, the thymus consists of reiterated nodules of thymic tissue that follow along the jugular veins of the neck.

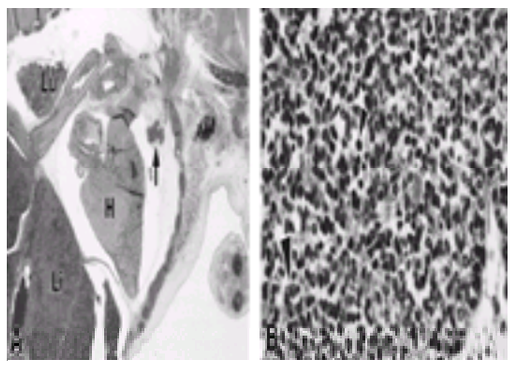

Figure 2.4. Human thymus at 8 weeks, gestation. **A:** The developing thymus is located in the anterior mediastinum overlying the heart (H). The position of the lung (Lu) and liver (Li) are also shown. **B:** At this stage of development, both lymphocyte precursors *(small arrowhead)* and epithelial cells *(large arrowhead)* are present; however, a distinction between cortex and medulla will not become apparent until week 14 of gestation (hematoxylin and eosin stain; original magnification ×3 in **A** and ×132 in **B**). (See Color Figure 2.4.)

The thymus achieves its greatest relative weight at birth; however, the thymus continues to grow until sexual maturation, and it reaches its greatest size just before the onset of puberty. Afterward, the thymus progressively declines in size to less than half of its peak volume. This age-associated decrease in thymic weight is referred to as *physiologic involution.* Involution is accompanied by changes in thymic structure that include large expansions of adipose tissue, diminished thymocyte proliferation, and a lower capacity to produce new T cells. Physiologic involution is controlled hormonally, because castration delays thymic involution, whereas the injection of corticosteroids accelerates it (55,56). Occasionally, transient thymic involution follows acute stress or infection.

The fully developed human thymus is divided into lobules by septa that extend from the thymic capsule into the interior of the organ. Thymic tissue can be divided into two main compartments or spaces: the thymic epithelial space and the perivascular space. The thymic epithelial space can be further subdivided into the subcapsular zone, the cortex, and the medulla. The first of these, the subcapsular zone, contains the most primitive lymphocyte progenitors that arrive from the bone marrow and the least developed populations of thymic lymphoblasts. The thymic cortex lies immediately below the subcapsular zone and is packed with small lymphocytes actively engaged in the generation, expression, and selection of functional T-cell antigen receptor molecules. Finally, the central medulla of the thymus contains lymphocytes undergoing the final stages of thymic selection and maturation. From the medulla, mature T lymphocytes pass through the circulation into the secondary lymphoid tissues and lymphatics.

In children, the cortex is located directly beneath the thymic capsule and contains immature developing lymphocytes (thymocytes) are that densely packed into a lacy meshwork of thymic epithelial cells. These epithelial cells are often obscured by dense masses of thymocytes using conventional histologic stains, but they can be easily detected using labels that are specific for epithelial cells (Fig. 2.5). Most thymocytes, usually at least 90%, are located in the thymic cortex. The medulla is located internal to the cortex and contains a less dense arrangement of more mature thymocytes and thymic epithelium, including characteristic clusters of terminally differentiated thymic epithelial cells called *Hassall bodies* (57,58) (Fig. 2.5). Epithelial cells are more tightly aggregated in the medulla, as compared with the cortex, in which they form stellate figures connected by long cytoplasmic processes (Fig. 2.5). These epithelial components create a reticulum or meshwork that surrounds and supports the thymocytes. The distinction between cortex and medulla becomes evident at week 14 of development in humans, with Hassall bodies first detectable at 15 weeks. The human thymus is considered fully developed by weeks 16 to 20 of gestation.

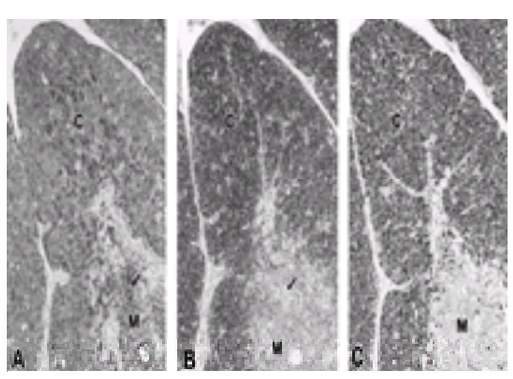

Figure 2.5. Architecture of the pediatric human thymus. Immunoperoxidase staining highlights cell types present in thymic cortex (C) and medulla (M). *Arrows* point to Hassall bodies. Cytokeratin antibodies highlight the meshwork of thymic epithelium **(A),** which is more attenuated in the cortex as compared with the medulla. The immature thymocytes present in the cortex can be uniquely identified as those cells reactive with antibodies specific for CD1a **(B)** and for the Ki-67 nuclear proliferation antigen **(C).** *Brown* indicates positive reaction (original magnification ×33). (See Color Figure 2.5.)

The perivascular space is defined as the area within the thymic capsule that is separated from the thymic epithelial space by a basement membrane and does not contain developing thymocytes (56,57). In human infants, the perivascular space is primarily a potential space, containing blood vessels and small numbers of stromal cells. Branches of the subclavian artery penetrate the thymic capsule and travel within the perivascular space to the medulla, where they subdivide into smaller arteries and arterioles that progressively supply the corticomedullary junction, cortex, and subcapsular region. Venules run parallel to the arterioles in the perivascular space. Eventually, these venules join into medullary veins, which, in turn, empty into the innominate vein.

The thymus exhibits marked changes in architecture and cellular composition with aging. In mice, the thymus appears to grow until sexual maturation, then it progressively decreases in size to less that half of its peak volume. In humans, the thymus size remains approximately the same throughout life; however, thymic output decreases progressively with age (55,56). The decrease in thymic output parallels a decrease in the thymic epithelial space in which thymocyte development occurs. To maintain organ size, the perivascular space increases in the human thymus correspondingly, filling up with adipose tissue and variable numbers of peripheral lymphocytes (Fig. 2.6). However, despite this thymic atrophy, foci of immature thymocytes and molecular evidence for ongoing thymocyte development have been observed in humans in the eighth decade of life (59,60,61 and 62).

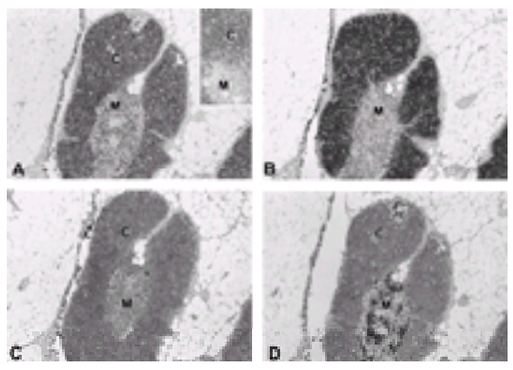

Figure 2.6. Architecture of the adult human thymus. Age-related thymic atrophy results in an increase in adipose tissue within the perivascular space (seen on the **left** and the **right** of each panel) concomitant with a decrease in the thymic epithelial space that is involved in production of new thymocytes. The architecture of the remaining cortex (C) and medulla (M) is relatively unaltered. Immunoperoxidase staining identifies the phenotype of cells present in the cortex and medulla. **A:** CD3 stain (T cells); the **inset** shows $CD8^+$ cells (immature cortical and a subset of medullary thymocytes). **B:** CD1a (immature cortical thymocytes and medullary dendritic cells).**C:** CD68 (macrophages). **D:** CD20 (B cells). *Brown* indicates positive reaction (original magnification ×16; ×40 for the **inset**). (See Color Figure 2.6.)

In addition to age-related thymic involution, the thymus may also undergo involution from hormonal or other factors. Castration delays thymic involution in rodents, whereas injection of corticosteroids accelerates it (63,64). Occasionally, thymic involution follows acute stress or infection (65,66). This acute involution is transient, and normal thymic architecture is restored on recovery.

Thymectomies of neonatal mice by J.F.A.P. Miller demonstrated that the thymus is the primary site for the development of mature T lymphocytes (67). The thymus houses early committed T-cell progenitors and functions to develop this pool of cells into functional, mature T lymphocytes.

ORGANS OF THE IMMUNE RESPONSE

Spleen

The largest definitive lymphoid tissue of higher vertebrates is the *spleen;* it contains some 25% of the body's lymphocytes (17,68). The spleen is not supplied with efferent lymphatic channels but is joined to the vasculature by a single splenic artery and vein. These blood vessels enter the smooth capsule of the spleen at an indentation known as the *hilum.* Blood percolating through the splenic sinuses is filtered through a complex reticulum of phagocytic cells that remove immune complexes, particulate matter, and damaged erythrocytes (17,68,69). This blood flow represents some 5% of cardiac output in humans (70), and it effectively supplies the spleen with antigen and antigen-transporting cells.

Structurally, the spleen is divided into three distinct compartments: the red pulp, the white pulp, and a marginal zone that separates the two (Fig. 2.7). The immunologic compartment of the spleen is predominately located within the white pulp, which accounts for 15% to 20% of the splenic parenchyma (17,69). The white pulp is the secondary lymphoid tissue of the spleen, and like all other secondary lymphoid tissues, it is segregated into T- and B-cell zones that are organized around the vasculature that supplies antigen, accessory cells, and lymphocytes.

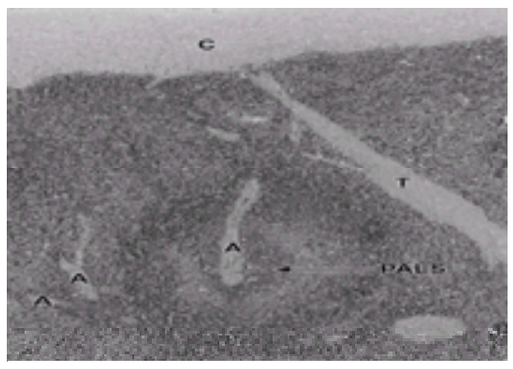

Figure 2.7. Architecture of the human spleen. The spleen is surrounded by a fibrous capsule (C) that invaginates to form trabeculae (T). The white pulp is arranged concentrically around arterioles (A) to form the periarteriolar lymphoid sheath (PALS) (hematoxylin and eosin stain; original magnification ×16). (See Color Figure 2.7.)

After the splenic artery penetrates the spleen's capsule at the hilum, it ramifies into numerous branches that run along invaginations of the splenic capsule, called *trabeculae* (Fig. 2.7). The trabecular arteries further branch to produce central arterioles, which finally give off still finer vessels, the penicillary tufts that end in arterial capillaries. Blood is discharged from the arterial capillaries into the venous sinuses of the red pulp, which expand or contract in response to the volume of blood passing through them. The walls of these sinuses are perforated with the openings of venous capillaries that eventually coalesce into veins of the splenic pulp. These merge into trabecular veins and return filtered blood to the circulation through the splenic vein (17,69,70 and 71).

Lymphatic vessels do not drain into the spleen. However, the spleen contains efferent lymphatic vessels that carry lymph out of the organ. These efferent lymphatics originate in the white pulp, where they lie in approximation to the central arterioles and near the trabeculae.

The red pulp contains extensive blood sinuses that are lined with macrophages; these sinuses are organized into tissue cords (68,70). The red pulp contains most of

the erythrocytes present in the spleen as well as the other formed elements of the blood, and it also contains large numbers of plasmacytes.

The white pulp is arranged concentrically around the central arteries and arterioles (Fig. 2.7). The innermost cylinder of lymphocytes that surrounds the central arteriole is the *periarteriolar lymphoid sheath* (PALS). The PALS contains mostly T lymphocytes, DCs, and small populations of macrophages (71). Lymphoid follicles, areas rich in B lymphocytes, are joined to the PALS, either surrounding it or forming eccentric columns to one side. Whereas most follicular cells are B lymphocytes, the lymphoid follicle also contains characteristic clusters of FDCs and scattered populations of macrophages. In naive animals, the white pulp constitutes approximately 15% of the splenic volume; however, infection or repeated immunizations can double the white pulp volume. This increase is largely due to expansion of the B-cell–rich lymphoid follicles and the development of intense areas of antigen-driven B-cell proliferation and differentiation, known as *germinal centers*. Follicles containing active germinal centers are known as *secondary follicles* (71).

A marginal sinus surrounds the PALS and follicle and separates the splenic white pulp from the red pulp (Fig. 2.7). The area between the white and red pulps is referred to as the *marginal zone;* it constitutes a distinct microenvironment that is populated by distinct macrophage and lymphocyte populations and is important for humoral responses to certain thymus-independent antigens (72,73).

Lymph Nodes

The structure of *lymph nodes* is (Fig. 2.8) similar to that of the spleen in that each lymph node is contained by connective tissue capsule that sends many trabecular projections into the node's interior (5,18,19,74). The intratrabecular areas are occupied by a network of reticular cells and by free cells, mostly lymphocytes that are lodged in the mesh. The external regions of the lymph node, or cortex, contain more lymphocytes than its interior or central medulla. Within the lymph node's cortex, lymphocytes are organized into B-cell–rich follicles and perifollicular zones of T cells. T cells also extend in approximation to the B-cell zones to form pericortical regions.

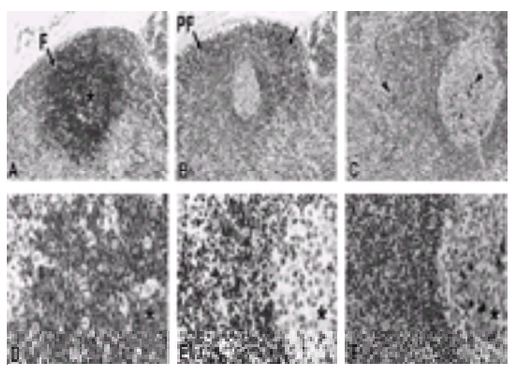

Figure 2.8. A–F: Architecture of the human lymph node. Lymphocytes within lymph nodes are organized into B-cell–rich follicles (F in **A**) and T-cell–rich perifollicular zones (PF in **B**). *Arrowheads* point out representative macrophages in **C.** Germinal centers are denoted by an *asterisk* in **A** and **D–F.** Immunoperoxidase stains identify specific cell types present in various lymph node regions. **A, D:** CD20 (B cells); **B, E:** CD3 (T cells); **C, F:** CD68 (macrophages). *Brown* indicates a positive reaction (original magnification ×33 for **A–B,** ×66 for **C,** and ×132 for **D–F.**) (See Color Figure 2.8.)

The interior of the lymph node contains channels or sinuses with walls made of reticular cells. In contrast to the blood sinuses of the spleen, the sinuses of lymph nodes contain lymph rather than blood. These sinuses are formed by the channels of afferent and efferent lymphatic vessels that enter and drain the lymph node. Afferent lymphatic vessels bring lymph and lymph cells into the large subcapsular sinus of lymph nodes. This drains into many cortical or trabecular sinuses that often are formed parallel to the penetrating trabeculae of the capsule. These merge into lymphatic cords that are drained by the efferent lymphatic vessels that exit the lymph node at the lymph node hilum. Thus, lymph nodes act as cellular filters for the lymph that flow through them, entering through the afferent vessels and leaving through the efferent vessels (18,19,28,29,74).

Characteristically, multiple afferent lymphatic vessels empty into the lymph node at a site opposite the hilum. The hilum serves as the entry point for lymph node arteries and the exit for lymph node veins and the efferent lymphatics.

Blood supply to the lymph node is similar to that of the spleen. Arterial blood penetrates each lymph node at a hilum, ramifies into trabecular projections and forms baskets of capillary loops within the region of the follicular cortex. In the lymph node, the capillary baskets surround the B-cell areas, and the postcapillary venules are lined by a unique high cuboidal endothelium; venules lined with an endothelium having this special morphology are known as *high-endothelium venules* (HEVs) (20,21). Postcapillary blood is collected by veins, which return to the hilus alongside the penetrating arterial blood supply.

Within the lymph node, afferent lymphatics open into the *subcapsular or marginal sinus,* an opening that separates the fibrous capsule of the lymph node from the underlying organized lymphoid tissue (Fig. 2.8). Lymphocytes may be found in the subcapsular sinus, but macrophages, DCs, and the reticular stromal cells of the lymph node are the most common cell types present. The lymph and its cellular components that enter the subcapsular sinus then pass through the densely packed lymphocytes present in the lymph node cortex by way of radial sinuses before reaching the central part of the lymph node or medulla. The medulla contains branching partitions or cords of stromal cells and fibers, separated by medullary sinuses rich in macrophages and plasma cells. The sinus coalesces to form the efferent lymphatic channel that drains the lymph node. Thus, lymph-born antigens derived from distant sites filter through the lymph node cortex and medulla. Lymphocytes, particularly naive lymphocytes, are also continuously passing through the cortex. Naive lymphocytes enter lymph nodes through the HEVs that form basketlike structures interposed between the B- and T-cell zones of the cortex. HEVs display particular adhesion molecules that interact with the homing receptors on lymphocyte surfaces and serve to recruit circulating lymphocytes (20,21).

The lymph node cortex can be broadly divided into two structural and functional regions populated by T- or B-lymphocytes. The B-cell zones, or follicles, not only contain B-lymphocytes but also FDCs, specialized macrophages, and oftentimes small numbers of $CD4^+$ T lymphocytes. As in the spleen, follicles present in lymph nodes are divided histologically into two categories: primary- and secondary follicles. Primary lymphoid follicles are composed of small resting B-cells and an associated network of sparse FDC processes, whereas secondary follicles contain an inner region of rapidly proliferating B-cells associated with a dense FDC reticulum. This germinal center is surrounded by a compressed rim of resting B-cells known as the mantle zone. The number of follicles present in a lymph node and the ratio of primary and secondary follicles define immunologic activity in the lymph node (5,18,19). Resting lymph nodes, those with little or no active immunity, often contain few primary follicles, whereas lymph nodes that house active immune responses may be nearly filled with secondary follicles (71,75). The pericortical regions of lymph nodes contain T lymphocytes and large numbers of DCs that are specialized for the effective presentation of antigen T cells (5,18,19,22,75). This T-cell–rich pericortex extends into the lymph node and separates the individual B-cell follicles.

Peyer Patches: Gut-Associated Lymphoid Tissue

Secondary lymphoid tissues within mucosal organs are usually situated within the *lamina propria,* a well-vascularized sheath of connective tissue immediately below the mucosal epithelium (19,25,76). These *gut-associated lymphoid tissues* (GALT) contain B-cell follicles that are surrounded by a region of T cells much like the pericortex of lymph nodes (Fig. 2.9). Vascular trees containing HEVs enter this T-cell zone and provide a mechanism for lymphocyte recruitment. In the absence of efferent lymphatics, antigen enters the Peyer patches through an overlying mucosal epithelium that is specialized for the uptake and transport of antigen (77). These specialized epithelial cells, M cells, are capable of transporting antigens ranging in size from soluble proteins to intact microorganisms from the mucosal lumen to the underlying GALT (77,78). Plexuses of lymphatic capillaries surround Peyer patches and serve to transport lymphocytes, accessory cells, and antigen to draining lymph nodes (19,25,76,77 and 78).

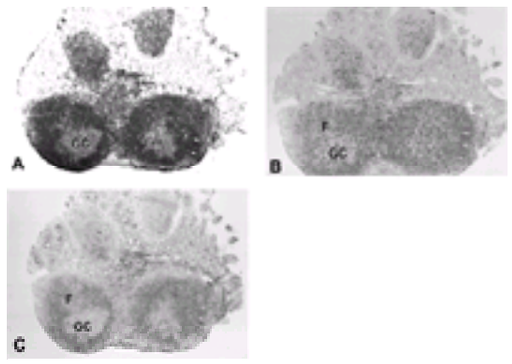

Figure 2.9. A–C: Architecture of Peyer patch. Peyer patches consist of organized aggregates of B-cell follicles (F) with germinal centers (GC) and intrafollicular zones of T cells that are present within the lamina propria of small bowel. Immunoperoxidase stains identify the locations of B cells (**A,** CD45RAB/B220 stain), helper-T cells (**B,** CD4/GK1.5 stain), and macrophages (**C,** F4/80 stain) (original magnification ×16). (See Color Figure 2.9.)

B and T lymphocytes are distributed with the Peyer patches as they would be within a lymph node that is undergoing an active immune response; that is, B cells are organized into active, secondary follicles that are separated by interfollicular zones of T lymphocytes. In contrast to lymph nodes, in normal mice Peyer patches do not exhibit a resting structure; germinal centers are always present because of incessant activation by antigens of the gut flora (19,25,76).

Like the lymph nodes that punctuate the lymphatic vasculature, Peyer patches form late in fetal development by a process that depends on lymphotoxin-a (LT-a) (79,80 and 81). During the formation of the Peyer patch GALT, invagination of lymphatic vessels is followed by the ordered immigration of DCs and lymphocytes and their subsequent resolution into follicular and interfollicular zones (80,81). This organogenesis is remarkable in that it relies on signals that are later used to effect immunity (5,80,81).

Structural Organizers of Secondary Lymphoid Tissues

The detailed architectures of the spleen, lymph nodes, and Peyer patches are unique, but they all share fundamental features that characterize secondary lymphoid tissues. Generally, T and B lymphocytes are segregated, with areas of T-cell predominance containing few B cells and substantial numbers of DCs. B-cell areas of secondary lymphoid tissues contain primary follicles that represent the sites where antigen-activated B cells that have received T-cell help expand, mature, and initiate the germinal center reaction before becoming antibody-forming cells and entering the memory cell pool.

Organizing immunocytes within the secondary lymphoid tissues is thought to increase the efficiency of immune responses by organizing B and T lymphocytes in association with their accessory cells and by providing a framework that permits the rapid circulation of naive cells through a space where antigen become concentrated. Indeed, the efficiency of organized secondary lymphoid tissue structure has been demonstrated experimentally (5).

What are the signals that organize T and B lymphocytes into discrete zones within the secondary lymphoid tissues? The membrane form of LT-a is now recognized as providing crucial signals required for the biogenesis of secondary lymphoid tissues and the organization of primary B-cell follicles and T-cell zones within these structures (5,82,83,84 and 85). Tumor necrosis factor (TNF) mediates independent signals for primary B-cell follicle structure and can cooperate with membrane-associated LT contributing to other aspects of secondary lymphoid tissue development (84,85). Some of the developmental actions of LT become fixed in the architecture of secondary lymphoid tissues, whereas others require continuing expression of LT to be sustained. For example, once formed, the lymphatic modifications that house the lymph nodes are stable, whereas withdrawal of LT-expressing B lymphocytes results in the loss of FDC networks and primary B-cell follicles over a period of days to weeks (86,87). Significantly, B cells have been identified as the lineage that delivers the LT signal for the formation and maintenance of the FDC network (5,84,85). Thus, B cells use LT as an inductive signal for the formation of lymphoid tissue, structures that, in turn, are required for the B cell to express its immune function most clearly (5). The actions of LT and TNF in signaling the formation of secondary lymphoid tissues are mediated primarily through the LT receptor LT-bR and the TNF receptor TNFR-I, but participation by additional receptors is likely (86,87 and 88). Experiments in which mixtures of lymphoid cells, purified cell populations, or bone marrow are transferred from one strain of mice to another have defined rules that govern the compartmentalization of lymphocytes within the spleen (5,79,86,87 and 88).

TRANSIENT STRUCTURES OF THE IMMUNE RESPONSE

Germinal Center

The *germinal center reaction* (Fig. 2.10) is prominent in primary and secondary responses to thymus-dependant antigen and represents cellular processes leading to the generation of high-affinity memory B cells and long-lived plasmacytes. (71,75,90,91 and 92). It is within germinal centers that antigen-specific B cells acquire mutations in the V(D)J region of rearranged immunoglobulin genes (91,92). In conjunction with affinity-dependent selection, this process of somatic hypermutation is necessary for the affinity maturation of serum antibody (90,92,93).

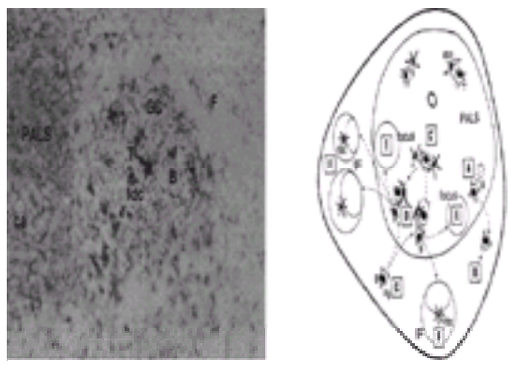

Figure 2.10. Germinal center reaction in murine spleen. Immunization with most thymus-dependent antigens initiates the germinal center (GC) reaction. GCs form within the B-cell follicle (F) by the expansion of antigen-specific T- and B-cell immigrants (T, B) from the periarteriolar lymphoid sheath (PALS). Both lymphocyte types proliferate in the reticulum of follicular dendritic cells (fdc) forming a distinct histologic structure containing light and dark zones (LZ, DZ). Original magnification ×200. Thus, the primary humoral immune response begins with antigen-dependent activation of lymphocytes [C, C']. Activated T- and B-lymphocytes then meet along the border of the PALS and follicle [D] where cognate interactions drive proliferation in both lymphocyte compartments. Some daughter B-lymphocytes remain adjacent to the T-cell area and differentiate into foci of antibody-secreting plasmacytes [E]. Other, clonally related, B-cells migrate along with specific T-cells back into the follicle and lodge in the processes of fdc where the GC reaction begins. Modified from ref. 71. (See left panel of figure in Color Figure 2.10.)

Germinal centers form within the primary follicles of secondary lymphoid tissues, creating secondary follicles (71,75,89); similar histologic structures also form at sites of chronic inflammation, such as in the synovium of patients with rheumatoid arthritis (94,95). In primary immune responses, activated T lymphocytes appear in T-cell–rich areas shortly after immunization and are subsequently met by antigen-specific B cells that migrate from the follicle to the edge of the T-cell zone (96,97). Interaction between these antigen-specific lymphocytes leads to CD154-dependent B-cell proliferation and the subsequent migration of both T and B lymphocytes back into the follicle (71,75,96). In mice, antigen-specific lymphoblasts begin to fill the FDC reticulum within 3 to 5 days after primary immunization (71,75,96,97,98,99 and 100). These dividing cells displace small, nondividing follicular B-cells to create a zone of compressed cells, the follicular mantle zone (75,96). Once established, the nascent germinal center polarizes to form two distinct zones: a dark zone that is adjacent to the T-cell region and contains rapidly dividing centroblasts and a light zone that contains nondividing centrocytes, FDCs, and virtually all the $CD4^+$ cells present in the germinal center (71,75,96,97 and 98).

Hypermutation in germinal center B cells is initiated approximately 1 week after the primary response begins (91,92,101), generally coincident with the polarization of germinal centers into light and dark zones (71,75). Mutations accumulate at a steady rate for at least 2 to 3 weeks by the incorporation of frequent nucleotide substitutions and, less commonly, short deletions or insertions (91,100,101). This process results in clonal genealogies that recapitulate repeated rounds of mutation,

selection, and proliferation (91). The mechanism of V(D)J hypermutation is unknown, but it introduces a distinctive pattern of nucleotide misincorporations. Characteristically, hypermutation favors transition mutations and exhibits biased nucleotide exchanges that reflect both antigen-driven selection and the intrinsic properties of the hypermutation process (102,103).

The germinal center reaction is normally dependent on the presence and activity of $CD4^+$ helper-T cells (96,97,99); however, surprisingly low numbers of helper cells can support the formation of histologically typical germinal centers (104,105). Indeed, reconstitution experiments in congenitally athymic nude mice demonstrate that fewer T cells are necessary to reconstitute the germinal center reaction than to generate foci of antibody-forming cells (105) present in the bridging channels of the spleen (75,96). The nearly normal kinetics and magnitude of the germinal center reaction in the presence of limiting numbers of T lymphocytes suggest that cognate T-B interactions may not be required to sustain follicular activation or proliferation. This germinal center–like reaction may even generate increased numbers of immunoglobulin M (IgM)-positive antigen-specific precursor cells and may prime for enhanced IgM responses on subsequent rechallenge (104,105). However, IgM®IgG isotype switching is poorly supported by limited numbers of T cells, and the extent of immunoglobulin V(D)J hypermutation is directly proportional to the numbers of helper-T cells available during the response (105).

The cellular and molecular signals that direct and control germinal center formation are not well understood. Genetic defects and the administration of antibodies or fusion proteins that disrupt collaboration between antigen-specific T and B lymphocytes block or reduce antibody production and the germinal center reaction (91,93,99,106,107 and 108). Mice that cannot express MHC class II molecules exhibit no or few germinal centers in response to protein antigens, presumably because they cannot support the generation of $CD4^+$ T lymphocytes that drive B-cell activation (96,98 and 99). In the absence of complement, germinal center responses are strongly suppressed by diminishing the ability of FDC to trap antigen-antibody complexes on their surface or diminishing signals necessary for B-cell survival in the germinal center (109,110 and 111). Administration of passive antibody specific for CD154 or CD86 at the initiation of the germinal center reaction has little or no effect on early serum antibody titers but significantly alters the germinal center response (93). Treatment with anti-CD154 antibody abrogates the germinal center reaction within 48 hours, a finding demonstrating that the germinal center reaction is maintained by CD154-dependent processes. This disruption does not result in the death of germinal center B cells, but it causes their migration from the follicles (93,99). In contrast to the disruption of germinal centers by anti-CD154 antibody, administration of antibody specific for CD86 does not disrupt germinal center architecture. Instead, transient blockades of CD86 function impair germinal center development by retarding local B-cell proliferation, diminishing frequencies of V(D)J point mutations, and impairing humoral immune memory (15,93,112). These effects are specific to the germinal center B-cell compartment, because passive anti-CD86 antibody has no effect on early antibody levels or T-cell priming (93,112).

Although the germinal center reaction is thymus dependent, several thymus-independent antigens (113,114 and 115) elicit the formation of germinal centers or germinal center–like reactions within splenic follicles. The kinetics of the germinal center–like reaction that is induced by thymus-independent antigens is initially similar to that seen in primary, thymus-dependent responses but ends sooner (114,115). In the case of the germinal center–like structures formed in response to haptenated lipopolysaccharide, the follicular reaction was not judged to be as intense as that observed in carrier-primed animals (114). However, responses to thymus-independent bacterial vaccines are often substantial, with some 60% to 100% of follicles containing antigen-specific germinal centers (105). Studies demonstrate that some inflammatory signals elicit the emigration of lymphocyte populations from the bone marrow into secondary lymphoid tissues (116,117 and 118). Immature B-cell emigrants share many phenotypic characteristics of germinal center B cells and may be capable of organizing into histologic structures resembling germinal centers.

Extrafollicular Plasmacytic Foci

In many (71,75,96), but not all (119), primary antibody responses, discrete collections, or foci, of antigen-specific *lymphoblasts* and *plasmacytes* form in secondary lymphoid tissues (Fig. 2.11). When present, plasmacyte foci represent the initial site of antibody production and the major source of early serum antibody. In murine spleen, plasmacytic foci are located adjacent to the PALS, in extrafollicular bridging channels that communicate directly to the marginal sinus and red pulp (75,119). These foci are formed before the nascent germinal center, and genetic analysis of B lymphocytes in neighboring foci and germinal centers indicates that both populations are founded by common precursors (120,121). Plasmacyte foci are transient and generally lost by the second week of primary responses (71,75,120,121,122); in secondary responses, foci are uncommon, and plasmacytes are most abundant in the splenic red pulp and medullary cords of lymph nodes (71,75,119). The formation of plasmacytic foci can be blocked by the administration of passive antibodies that inhibit cognate interaction between T and B lymphocytes; however; once formed, these foci are resistant to such inhibition, a finding suggesting independence from continuing T-cell help (93,122). Despite this independence, IgM®IgG class switching occurs in plasmacytic foci (121,123,124) although V(D)J hypermutation does not (91,121,122).

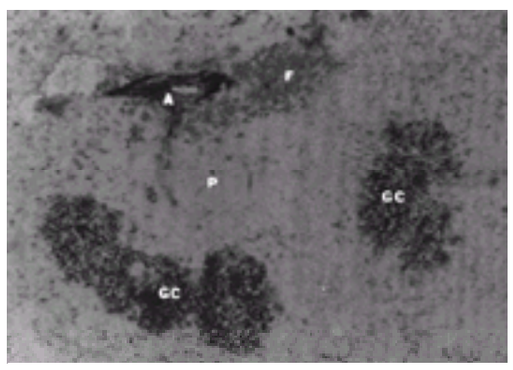

Figure 2.11. Plasmacytic foci and germinal centers in murine spleen. Following a period of proliferation near the periphery of the splenic T-cell zone, the periarteriolar lymphoid sheath (P), antigen-specific B lymphocytes differentiate into plasmacytes (F) and remain in close approximation to the T-cell zone. These large foci of AFC produce the earliest serum antibody and the plasmacytes within them may undergo IgM®IgG class switching but do not support V(D)J hypermutation. Original magnification ×200. From ref. 119. (See Color Figure 2.11.)

Inflammatory Lymphoid Tissue

Chronic inflammation is frequently associated with the development of lymphocytic follicles at sites that do not normally support secondary lymphoid tissue, including the thyroid and salivary gland, nasal sinus mucosa, eustachian tube, and joint synovium (94,95,125,126). The development of MALT (mucosa-associated lymphoid tissue) lymphomas offers the best studied example of naturally acquired, ectopic lymphoid tissue. The stomach is the most common site for these MALT lymphomas, even though normal gastric mucosa has no associated lymphoid tissue (127). However, chronic inflammation from gastric pathogens can induce MALT *de novo,* a process almost pathognomonic for *Helicobacter pylori* infection (127,128). This so-called acquired MALT histologically resembles normal Peyer patch with active germinal centers, a perifollicular T-cell zone, and vascularization by HEVs. Remarkably, T- and B-cell populations present in MALT associated with *H. pylori* gastritis are specifically activated by *Helicobacter* antigens, and the elimination of infection by antibiotics commonly results in the eventual loss of the acquired MALT (127,128 and 129).

The triggering signal for the neogenesis of lymphoid tissue is mediated by LT-a. Tissue-specific expression of transgenic LT-a modulates the local induction of adhesion molecules and chemokines, which, in turn, direct immunocyte recruitment and vascularization (130,132).

Infection and chronic inflammation can elicit the formation of lymphoid tissue and active germinal centers at sites where such tissues are not normally present (94,95,125,126,127 and 128). It is plausible that this process of tissue neogenesis is analogous to the developmentally regulated formation of lymph nodes and Peyer patches (79,80 and 81). The genetic instability that is typical of germinal center B cells (133) may initiate the oncogenic changes that result in MALT lymphoma (134,135,136 and 137).

CURRENT ISSUES IN LYMPHOID ARCHITECTURE

The immune system is an evolutionary adaptation that has radiated to all jawed vertebrates. Remarkably, signals that determine immune effector function also seem to be responsible for the organogenesis of many peripheral lymphoid tissues, especially lymph nodes and Peyer's patches. It is now generally thought that developmentally regulated inflammatory signals initiate the formation and organization of these organs, just as environmentally induced inflammation can generate acquired lymphoid structures. Kratz et al. have called this process *lymphoid neogenesis* (132). Identification and an understanding of the redundant signals that both organize immune structure and mediate immunity represent remarkable possibilities for the integration of molecular and cellular biology.

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3 MAJOR HISTOCOMPATIBILITY GENE COMPLEX: STRUCTURE AND FUNCTION

Hidde Ploegh, Ph.D.

MAJOR HISTOCOMPATIBILITY COMPLEX AS A GENETIC REGION

The *major histocompatibility complex* major histocompatibility gene complex (MHC) is a genetic region that encodes a set of glycoproteins essential for immune recognition. Initially discovered in mice as the genetic locus that controls the rejection or acceptance of grafts from a genetically distinct individual of the same species, the products encoded by the MHC are now known to serve as essential intermediaries in conveying antigenic information to antigen-specific receptors on T lymphocytes.

The discovery of the MHC in mice was followed by the definition of a similar genetic complex in humans, followed in short order by the characterization of homologous gene clusters in other mammals, birds, amphibians and fish. The complete nucleotide sequence of a 2.3-Mb region encompassing the entire human MHC has now been published, as has that of the chicken. The sequence of the mouse is close to completion, and it is only a matter of time before the genomic sequence of model organisms such as *Rerio danio* (zebrafish) will have been added to the list. The comparative analysis of vertebrate MHCs illustrates both structural conservation of MHC genes, indicative of conserved function, and extreme divergence and evolutionary plasticity in the number of genes contained within it: whereas the human MHC encompasses some 200 genes, that of the chicken is a minimalist version possessing only 17 functional genes. The range of products encoded by the human MHC not only includes the structural genes for the MHC product *sensu stricto,* but also provides the information for proteins that function as accessories to MHC products. Not all the MHC-encoded genes have obvious immunologic functions, but many of them do. Specifically examples of such accessory genes found in the MHC include the following: genes that encode subunits of the proteasome, the proteolytic organelle involved in cytosolic proteolysis; genes that encode the transporter required for delivery of peptides to MHC molecules; a gene that encodes a chaperone involved in providing class I molecules with antigenic peptide; and a gene that encodes a chaperone involved in providing class II molecules with its peptide cargo.

The increase in information on the genetics of the MHC was made possible by large-scale population and family studies. In addition, the detailed analysis of inbred strains of mice and their suitability for *in vivo* experimentation made essential contributions to the understanding of both MHC structure and function. The introduction of modern molecular biologic tools allowed the definition of the extremely high degree of polymorphism of the genes encoded in the MHC. This polymorphism is at the heart of the immune system's ability to confront many different pathogens: each polymorph variant of an MHC product is capable of presenting a unique set of antigens to specific receptors on T lymphocytes. The genetics of MHC products is straightforward: these products are expressed codominantly and are inherited in strictly mendelian fashion. Notwithstanding their extensive polymorphism, no unusual mechanisms appear to give rise to new allelic variants. The fixation of the large number of MHC alleles in the population must surely relate to the biologic function of the products they encode, a function that, to a first approximation, is strictly immunologic.

The identity of the polymorph variants expressed in a given individual is conveniently determined using antibody-based methods, and for routine human leukocyte antigen (HLA) typing, such methods continue to be used (Table 3.1). A more refined analysis relies on the use of the polymerase chain reaction, which allows definition of MHC polymorphisms with single nucleotide precision. The complete coding sequences of the most commonly occurring MHC alleles are now a matter of record. An international database maintains and expands on the available sequences and ensures adherence to a rational nomenclature for newly identified alleles. As far as the HLA system is concerned, the immunologically relevant loci are the so-called class I and class II loci. The structure of the class I products is described later; for class I molecules, the so-called heavy chain is encoded in the MHC. The human MHC contains the following class I loci (Fig. 3.1): HLA-A (195 alleles known), HLA-B locus (399 alleles), HLA-C locus (94 alleles), HLA-E (5 alleles), HLA-F (1 allele), and HLA-G (14 alleles). Class II loci encode two subunit proteins, in which the genes for both subunits are MHC encoded. These genes are as follows: DRa (2 alleles), DRb1 (269 alleles), and DRb2 (63 alleles); DQa1 (20 alleles) and DQb1 (45 alleles); and DPa1(19 alleles) and DPb1 (92 alleles).

Protein	Residues	Peptide Sequence
HTLV Tax	(11–19)	LLFGYPVYV
HIV Pol	(476–484)	ILKEPVHGV
Flu matrix	(58–66)	GILGFVFTL
TRP2	(288–296)	SLDDYNHLV
TRP2	(180–188)	SVYDFFVWL
HCMV pp65	(495–503)	NLVPMVATV
p53	(65–73)	RMPEAAPPV
MART-1	(27–35)	AAGIGILTV
gp100	(280–288)	YLEPGPVTA
HIV Gag	(77–85)	SLYNTVATL

[a] Alignment of ten cellular and virus-derived nonamer peptides presented by HLA-A2 reveals anchor binding motifs. Small hydrophobic amino acids are preferred by this allele at at the second and ninth residues.

TABLE 3.1. Definition of Anchor Residues for the HLA-A2 Molecule as Inferred from an Alignment of Ten Peptide Sequences that Bind to HLA-A2[a]

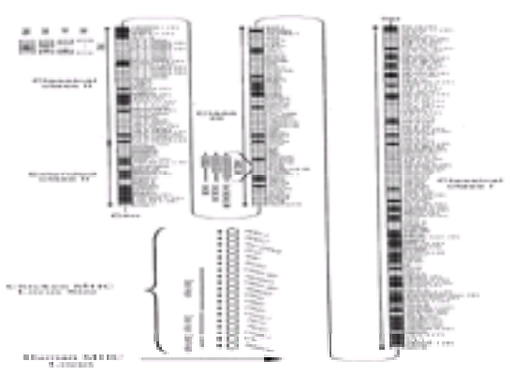

Figure 3.1. Structure and gene content of the human and chicken major histocompatibility complex (MHC). (*Nature* 1999;401:923–925, with permission.)

The DO and DM genes encode proteins that assist the class II products in their function, as described in Chapter 6. They are themselves MHC genes and are structurally related to class II products. Their polymorphism is limited: eight alleles for DOa and b, and four and six allelic forms, respectively, for DMa and DMb. In addition to these MHC-encoded membrane glycoproteins, the MHC contains the genes for components of the complement system, also referred to as class III MHC genes. A database of all HLA alleles whose nomenclature has been approved may be found on *http://www.ebi.ac.uk/imgt/hla/index.html.* The sequence for chromosome 6 itself may be found at *http://www.sanger.ac.uk/HGP/Chr6//.* The complement system is discussed in Chapter 2.

FUNCTION OF MAJOR HISTOCOMPATIBILITY COMPLEX PRODUCTS

Pathogens such as viruses usurp the cellular synthetic apparatus for the synthesis of their nucleic acids and use the host protein synthetic apparatus to produce viral polypeptides. These reactions are confined to the cytoplasmic compartment. As long as the viral genome and the products encoded by it are not displayed at the cell surface, the infected cell remains invisible to the immune system. By the same token, bacterial pathogens that are specialized to invade the mammalian cell and to multiply intracellularly are not immediately accessible to cells and molecules of the immune system. The cytoplasm—the inside of the cell—and endocytic compartments—topologically equivalent to the outside of the cell—represent two topologically distinct sites that can sustain survival and multiplication of pathogens. A successful defense against these pathogens demands that both types of compartment can be sampled. It is now clear that the MHC products evolved to perform such surveillance. The function of MHC products, in essence, is the conversion of antigenic proteins into entities recognizable by T lymphocytes. This is perhaps the most remarkable aspect of the function of MHC products: their ability to present short fragments of proteins to antigen-specific receptors on T lymphocytes. Whereas surface immunoglobulins on B lymphocytes can recognize antigen without the intervention of any third-party molecules, the antigen-specific receptors of T cells generally do not recognize intact antigen, but rather short peptides, generated by proteolysis from proteins in both the cytoplasmic and extracellular compartments. The antigen receptors on CD4 and CD8 T lymphocytes, for the most part, obligately require the engagement of MHC-encoded products loaded with fragments derived from antigenic proteins. The conversion of an intact protein into an entity that can be recognized by T lymphocytes is called *antigen processing,* and the combined result of this proteolytic conversion together with display of the resultant MHC peptide combination at the cell surface is referred to as *antigen presentation.* In the following discussion, the structure of MHC products is described, as are the cell biologic processes that underlie the conversion of an intact protein into a peptide suitable for display by MHC products.

STRUCTURE OF THE MAJOR HISTOCOMPATIBILITY COMPLEX–ENCODED PRODUCTS

MHC products are generally classified as class I and class II. Both types of protein are membrane glycoproteins (Fig. 3.2 and Fig. 3.3). Class I molecules are composed of an MHC-encoded heavy chain of approximately 45 kd. The heavy chain associates noncovalently with the light chain, b_2-microglobulin. MHC class I products are encoded by the HLA-A, HLA-B, and HLA-C loci in humans and by the H-2K, H-D, and H-L loci in the mouse. These class I molecules are also referred to as classic class I products. This classification distinguishes them from the so-called nonclassic or class Ib products. MHC class II products are composed of two MHC-encoded subunits, the a and b chain, of approximately 35 and 30 kd, respectively. In humans, class II products are encoded by the HLA-DR, HLA-DQ, and HLA-DP loci, and in the mouse, they are encoded by the I-A and I-E loci. MHC products consist of a large extracellular domain, transmembrane segments that anchor the MHC products in the lipid bilayer, and a short cytoplasmic tail. The cytoplasmic tails contain information that may allow these products to be delivered to distinct intracellular sites as well as allow retrieval from the cell surface. The most important portion of the MHC product is its extracellular domain, through which antigen is presented and the T-cell receptor is engaged. The three-dimensional structure of the luminal domain of numerous MHC class I and class II products has now been determined, and it shows their structures to be remarkably similar. The membrane-proximal domains of the luminal portion are immunoglobulin G (Ig)–like domains that occur in paired fashion. The Ig-like domains support a platform of an eight-stranded b sheet on top of which two long a-helical segments are found. The combination of the b sheet (“floor”) and the helices forms the peptide binding crevice. The mode of peptide binding is such that a contiguous surface is formed, composed of residues contributed by both peptide and MHC product. The T-cell receptor for antigen recognizes this surface and interacts with side chains of the MHC product as well as with residues contributed by bound peptide. The MHC-encoded subunits display polymorphism: residues that show allelic variation are found in and around the peptide binding pocket. Thus, polymorphisms directly affect the architecture of the peptide binding cleft and determine which peptides are bound by a particular MHC product. At the same time, polymorphic residues—the amino acid substitutions that distinguish the different allelic products—outside the peptide binding area proper also contact the T-cell receptor for antigen. The manner in which T lymphocytes are selected (Chapter 5) and the structure of the T-cell receptor itself (Chapter 4) ensure that only the host's own MHC products can be used in a productive manner for antigen presentation to T cells, a phenomenon known as *MHC restriction.* The ability of T-cell receptors to recognize antigenic peptides only when presented in the context of an appropriate MHC molecule explains the central position occupied by MHC products not only in the recognition and elimination by cytotoxic T lymphocytes of cells infected with a virus, but also the ability of T lymphocytes to interact with B lymphocytes to provide the help essential for B cell activation and differentiation.

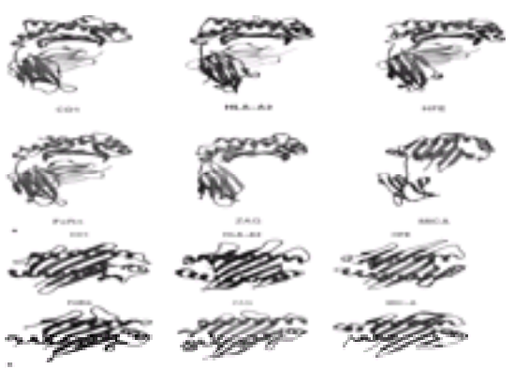

Figure 3.2. A: Structure of major histocompatibility complex (MHC) class I products and related structures. The HLA-A2 structure is representative of the so-called classic class I products, involved in presentation of peptide to T-cell receptors. The CD1 molecule presents lipid antigens. The HFE product interacts with and modifies internalization of the transferrin receptor. The neonatal Fc receptor (FcRn) is involved in transepithelial transport of immunoglobulins in the gut of the newborn. The ZAG protein is a zinc-binding serum protein. The MIC-A product is encoded by a gene linked to the MHC (Fig. 3.1) and is probably involved in antitumor immunity and eradication of cells subjected to stress. Note the presence of the common light chain, b_2-microglobulin, in CD1, HLA-A2, HFE, and FcRn. **B**: As in **A,** but structures viewed from above. Note the presence of the peptide in the cleft of the HLA-A2 molecule. (See Color Figure 3.2.)

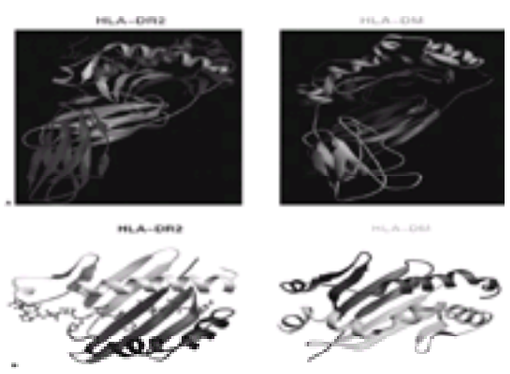

Figure 3.3. A: Comparison of the structure of the class II molecule HLA-DR2 and of the DM molecule, a catalyst involved in the peptide loading of major histocompatibility complex (MHC) class II molecules. **B**: As in **A,** viewed from above. (See Color Figure 3.3.)

MODE OF PEPTIDE INTERACTION WITH MAJOR HISTOCOMPATIBILITY COMPLEX PRODUCTS

The atomic structure of MHC products has allowed a detailed description of the interactions with their peptide cargo. Class I molecules possess a peptide binding groove that accommodates peptides in the size range of eight to ten residues. Exceptionally, slightly longer peptides may be accommodated. The architecture of the class I peptide binding groove provides for conserved features that accommodate the N and C terminus of the peptide. Accommodation of the peptide termini is essential to confer stable binding. Occlusions at either end of the peptide binding pocket and the need to accommodate the peptide termini explain the strict limits imposed on peptide size by the class I peptide binding cleft. In addition, several pockets provide docking sites for amino acid side chain in the peptide. The structure of

these pockets indicates a pronounced preference for particular amino acids, such that only peptides possessing that particular amino acid can occupy this pocket and provide the interactions required for stable binding. These positions are referred to as *anchor residues.* Usually, class I molecules require at least one so-called primary anchor residue, which is nearly invariant. In addition, secondary anchor positions may be defined, at which several amino acid side chains can be accommodated with equal efficiency.

Often, the nature of the C-terminal residue is that of an anchor residue: murine class I molecules invariably require an aliphatic amino acid (Leu, Ile, Met, Val) at the C terminus. This mode of interaction results in so-called peptide binding motifs that are unique for the different allelic class I products. In the mouse, the H-2Kb locus product prefers a Tyr or Phe residue at position 5, whereas the H-2Db product prefers an Asn residue at position 5. For many human class I molecules, the preferred binding motif is known and is a useful parameter to predict which peptides from a given polypeptide chain may be presented by that MHC class I product. These motifs may be determined experimentally by extracting peptides from purified MHC class I molecules and by subjecting the eluted peptides to sequence analysis by Edman degradation. At the so-called anchor positions, most class I–bound peptides possess the preferred anchor residue, whereas the other positions are either entirely variable or show only some restriction in the side chains allowed at that position, a trait that manifests itself as the occurrence of many different amino acids at a given cycle of Edman degradation.

More recent studies employ tandem mass spectrometry to acquire sequence information for MHC bound peptides. By demanding only a few truly fixed parameters to allow stable binding, a single MHC class I allelic product can accommodate a substantial number of different peptides. For an individual heterozygous at the HLA-A, HLA-B, and HLA-C loci, six different class I products are expressed, and this number apparently suffices to cover the spectrum of antigenic peptides that must be displayed in the defense against the wide range of pathogens that confronts the species. The interaction of peptides with MHC class I products is dominated by contacts between the peptide side chains and the MHC product.

MHC class II products have an overall structure similar to that of class I molecules. Their peptide binding groove operates on different principles, however. First, there is no requirement for tight binding of the peptide termini, and the architecture of the cleft is such that it lacks the occlusions found at both ends of the class I peptide binding cleft. This means that no fixed length characterizes a class II ligand. Furthermore, whereas anchor residues can be defined for class II molecules much like for class I molecules, many of the contacts between MHC class II products and peptide involve main chain atoms of the peptide that are contacted by amino acid side chains of the MHC product. Chapter 6 provides a detailed account of class II–peptide interactions. The website *http://wehih.wehi.edu.au/mhcpep//* maintains a current database of MHC-bound peptides for both class I and class II products.

ANTIGEN PROCESSING AND PRESENTATION

An obvious prerequisite for class I–restricted antigen presentation is the presence of the MHC class I molecules themselves. The class I molecules follow a biosynthetic pathway typical of a type I transmembrane protein: signal sequence-dependent insertion into the endoplasmic reticulum (ER), cotranslational attachment of the N-linked glycans, and delivery to the cell surface by the secretory pathway. All nucleated cells can synthesize MHC class I molecules, but wide ranges in the levels of expression are seen. Professional antigen-presenting cells and lymphocytes are strongly positive for class I molecules. Hepatocytes and fibroblasts express modest levels, and cells of the central nervous system have few, if any, MHC class I products. Exposure to the appropriate cytokines such as interferon-g (IFN-g) can dramatically increase expression of MHC class I molecules on cells that would otherwise express only modest levels of MHC class I molecules.

It is useful to divide antigen processing and presentation into a series of discrete steps that are reasonably well defined biochemically and cell biologically. This classification applies to the pathways used by class I and class II molecules alike. The basic concept is the conversion of a protein antigen into a MHC-bound peptide. This conversion requires (a) tagging of the protein antigen for degradation, (b) proteolysis, (c) delivery of the resultant peptides to the compartment of peptide loading, (d) delivery of peptide to the MHC product, (e) peptide binding, and (f) display of the peptide-MHC complex at the cell surface.

MHC Class I–Restricted Antigen Presentation: Acquisition of Antigen and Tagging the Antigen for Destruction

Antigens destined for presentation by MHC class I molecules reside predominantly in the cytoplasm, where protein synthesis occurs. Cytoplasmic proteins are tagged for destruction through the ubiquitin-proteasome pathway. This pathway comprises the activation of ubiquitin, a conserved 76-residue polypeptide, and its transfer to the protein to be destroyed. Ubiquitin is attached through its C terminus in isopeptide linkage to an appropriate lysine residue in the target protein. The signals that invite ubiquitin conjugation are not completely understood, but they may include defective translation (premature termination, misincorporation of amino acids), oxidative or proteolytic damage, and more specific modifications such as phosphorylation.

The initial attachment of a single ubiquitin moiety is followed by the addition of further ubiquitin moieties, again in isopeptide linkage to the preceding ubiquitin. A minimum of four ubiquitin molecules is required to allow the tagged protein to dock with the proteasome, the main protease responsible for cytosolic proteolysis. To date, no specific inhibitors that allow intervention in the ubiquitin conjugation reaction have been identified.

Proteolysis

Proteolysis of ubiquitin-tagged proteins is carried out by the proteasome. This complex consists of a catalytically active core particle, built from four rings of seven subunits each. The outermost rings contain seven distinct a subunits; the innermost rings of b subunits contain seven distinct subunits, only three of which are catalytically active. The core particle is equipped at either end with a so-called cap complex or a 19S particle.

The cap complex contains some 20-odd polypeptides, required for ubiquitin recognition, ubiquitin removal, and adenosine triphosphate (ATP)–dependent polypeptide unfolding to allow entry of the substrate into the catalytically active region of the core particle. Proteasomal proteolysis is usually highly processive: the degradation of a 400-residue protein may take as little as 3 to 4 minutes, with release of few or no intermediates.

The final hydrolysis products are short peptides in the range of three to ten residues. Remarkably, in cells exposed to IFN-g, the three catalytically active subunits are replaced by three IFN-g–inducible b subunits: LMP2 and LMP7, both of which are encoded in the MHC, and the MECL1 subunit. These three subunits, having replaced the constitutively expressed X, Y, and Z subunits, convert the proteasome into the so-called *immunoproteasome.* Furthermore, the 11S or PA28 regulator complex is likewise IFN-g inducible. These combined replacements rearrange the proteasome such that the products generated are better suited for presentation by class I MHC products. Whether the b subunit replacements result in altered cleavage specificity remains controversial. Investigators agree, however, that these replacements improve overall class I–restricted antigen presentation. Crystallographic evidence suggests that the regulator may promote the release of partial digestion products from the proteasome, thus favoring the generation of peptides in the size range appropriate for binding to class I molecules.

The catalytic mechanism employed by the proteasome is unusual, in that the active site nucleophile is an N-terminal Thr residue. Proteasome inhibitors of pronounced specificity have been described, among which are the natural products lactacystin and epoxomycin. Furthermore, modified tripeptides have been described that are potent proteasome inhibitors. These compounds can be used on living cells and are often used to implicate the proteasome in the proteolytic process under study. Sensitivity of T-cell recognition to treatment of the antigen-presenting cell with a proteasome inhibitor is used as a criterion to implicate the proteasome in the process, although the usefulness of these inhibitors is no better than the extent to which their specificity has been characterized. Peptides generated by proteasomal proteolysis may be further processed, both in the cytosol itself and after translocation into the ER. Most of this trimming appears to be restricted to the N terminus of the peptide. The enzyme leucine aminopeptidase is yet another example of an IFN-g–inducible protease implicated in further processing of proteasomally generated peptides.

Delivery of Peptides to the Compartment of Peptide Loading

Peptides generated by proteasomal proteolysis still reside in the cytosol. The biosynthesis of type I membrane proteins such as MHC class I molecules involves their cotranslational insertion into the lumen of the ER. Nascent class I heavy chains are directed to the ER in a conventional signal sequence-dependent manner. As the nascent chain is elongated, its signal sequence is removed by signal peptidase, and the N-linked glycans are attached on emergence of the consensus N-linked glycan attachment site (residue 86 in human class I molecules) in the ER. For a limited number of class I molecules, these signal peptides can provide peptide ligands for MHC class I molecules. This mode of presentation is of particular relevance for the display of the nonclassic class I molecule HLA-E, which is a potent ligand for receptors on natural killer (NK) cells. Folding of the class I heavy chain is assisted by ER-resident chaperones such as calnexin and calreticulin, both of which exploit the N-linked glycan on the class I heavy chain for recognition. Assembly with the light chain b_2-microglobulin occurs shortly after completion of the heavy chain. These reactions complete the construction of the folded class I molecule. How do the class I molecules acquire peptides? The MHC encodes two genes that specify an ATP binding cassette containing transporter, the TAP1 and TAP2 genes. The TAP1 and TAP2 polypeptides associate and form an ATP-dependent peptide transporter. This transporter translocates peptides into the lumen of the ER at the expense of ATP hydrolysis. The substrate preference of the peptide transporter is well matched with that of the binding specificity of the class I molecules it serves. In particular in the mouse, the TAP complex prefers peptides with the same C termini as those preferred by the class I molecules themselves: Leu, Ile, Val, Met. The human TAP complex, like the class I molecules it serves, is more relaxed in its substrate preference, as far as the C terminus is concerned. The length preference of the TAP complex is likewise well matched with that of the class I molecules: TAP preferentially translocates peptides in the 5- to 15-residue size range. Cells deficient in the TAP complex are defective in the assembly of stable class I molecules and fail to display class I molecules at the cell surface in normal amounts: their level of expression is approximately 5% of that seen in wild-type cells. On delivery of peptides to the lumen of the

ER, further proteolysis may trim the newly translocated peptides. The available evidence favors trimming predominantly at the N terminus, whereas the C-terminal cleavage appears dictated predominantly by the proteasome.

Delivery of Peptide to the MHC Product

The TAP complex is associated with a polypeptide called tapasin, a polypeptide that can also be coimmunoprecipitated with the MHC class I molecules themselves. The gene that encodes tapasin maps to the MHC. Although it was first assumed that the tapasin would simply promote close proximity of the class I molecule awaiting peptide to the TAP complex that delivers peptides, it now appears that tapasin retains “empty” MHC class I molecules in the ER. The possibility exists that the proximity of the class I molecule to the TAP complex, as brought about by the tapasin linker protein, promotes delivery of peptides to the class I molecule. A special case is peptides derived from signal sequences: after removal by signal peptidase of the signal sequence itself, further cleavage of the signal sequence is catalyzed by signal peptide peptidase. Usually, the N-terminal cleavage fragment is released toward the cytosol. Presentation of such fragments requires reimport into the ER through TAP. The C-terminal fragment of the signal peptide is probably released into the lumen of the ER.

Peptide Binding

No further accessory proteins appear to be required to achieve peptide loading of the class I molecule. Although the “empty” class I molecule bound to tapasin is assumed to have a conformation subtly distinct from that of a peptide-occupied class I molecule, no crystal structure of an “empty” class I molecule confirms this notion. Class I molecules not occupied with peptide show increased thermolability and have a strong tendency to dissociate even before they leave the ER. These molecules are destroyed by the normal pathway of ER quality control.

Display of the Peptide-MHC Complex at the Cell Surface

Peptide-loaded class I molecules are released from the ER and enter the constitutive secretory pathway. The so-called high mannose-type oligosaccharide, transferred onto the nascent chain, is converted into so-called complex type oligosaccharides by Golgi-resident glycosyl transferases. Mature class I molecules contain the typical complex-type oligosaccharides, including sialic acids. Once present at the cell surface, the class I peptide complex is available for recognition by $CD8^+$ T cells or for the delivery of inhibitory signals to NK cells. Once the synthesis of a pathogen-derived polypeptide ceases, the half-life of class I molecules at the cell surface determines the persistence of the relevant peptide-MHC complex.

MHC CLASS II–RESTRICTED ANTIGEN PRESENTATION

At this juncture, it is instructive briefly to compare the class I and class II pathways of MHC-restricted antigen presentation and to analyze the same key steps used to dissect class I–restricted presentation. More details on the class II pathway may be found in Chapter 6. MHC class II molecules follow an early biosynthetic sequence conceptually similar to that of class I molecules, with several important distinctions. Assembly of the class II heterodimer in the ER involves a third, non–MHC-encoded polypeptide referred to as invariant chain, because there appears to be only a single gene that encodes this nonpolymorphic protein. The invariant chain fulfills several important roles. First, it trimerizes to generate a scaffold onto which the class II molecules assemble (Table 3.2). Second, the invariant chain (Ii) contains a segment that occupies the class I peptide binding cleft. Because the association of Ii occurs early in the biosynthetic pathway of class II molecules, Ii temporarily occupies the peptide binding cleft of the class II molecule and thus prevents confusion about whether TAP-delivered peptides must choose between class I and class II molecules: the class II peptide binding pocket is not available to ER-resident peptides. The invariant chain, finally, contains in its cytoplasmic tail targeting information that directs the Ii–class II complex from the Golgi apparatus into the secretory pathway. Although most class II molecules are delivered directly into the endocytic pathway, the small numbers of class II–Ii complexes that escape sorting and move directly to the cell surface are internalized from the cell surface by internalization signals, again contained in the cytoplasmic tail of Ii. The delivery of class II molecules to the endocytic pathway is the major departure in the biosynthetic pathway that distinguishes class II molecules from the route followed by class I molecules. This distinction determines the sets of proteins sampled by either MHC product for presentation to T-cell receptors.

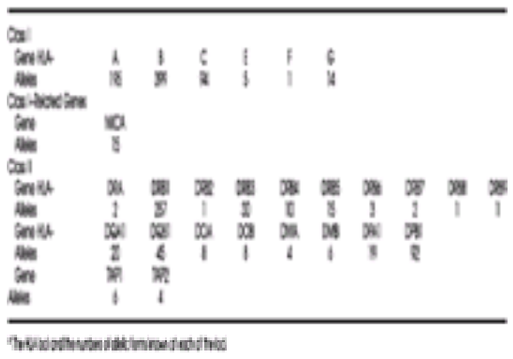

TABLE 3.2. Human Leukocyte Antigen (HLA) Genes and Their Extent of Polymorphism[a]

Class II: Acquisition of Antigen and Tagging Antigen for Destruction

Proteins destined for class II–restricted presentation are acquired by endocytosis, either in receptor-mediated fashion or by less specific processes such as pinocytosis or phagocytosis. The conditions that prevail in the endocytic pathway expose internalized proteins to an increasingly harsh regimen of low pH, reducing conditions, and high concentrations of proteolytic enzymes. No specific event appears to tag proteins for destruction. Unless they are specifically designed to survive in the endocytic pathway, most proteins are destroyed by lysosomal proteolysis.

Class II: Proteolyis

Proteolysis itself is carried out by the abundant proteases that reside throughout the endocytic pathway. Large collections of aspartyl and cysteinyl proteases degrade polypeptides into smaller peptides, including the size range appropriate for binding to class II molecules. The invariant chain itself is likewise attacked by proteases in the endocytic pathway. What remains once proteolysis of Ii is complete is a small remnant, referred to as CLIP (class II–associated invariant peptide), which remains firmly wedged in the peptide binding cleft of the class II molecule.

Delivery of Peptides to the Compartment of Peptide Loading and Delivery of Peptide to MHC Class II Molecules

The binding portion of class II molecules is located in the same topologic compartment as are the internalized proteins. In addition, the machinery required for degradation (proteases, reducing agents, low pH environment) is in the same compartment as the class II molecules themselves. Therefore, no specialized delivery functions are required to allow the class II molecules to meet peptides that can bind to them. There appear to be no specialized functions required for delivery of peptides to the MHC product itself.

Peptide Binding

The act of peptide binding to class II molecules require dislodging the Ii remnant, CLIP, from the peptide binding cleft. This is accomplished through interaction of the class II CLIP complex with the DM molecule. DM exerts true catalytic function, in that it promotes the exchange of CLIP for antigenic peptide. DM has no role in detaining class II molecules that are not occupied with peptide, because cells deficient in DM continue to display class II molecules at the cell surface. However, because of their inability to exchange the Ii remnant for antigenic peptide, such DM-deficient cells cannot present antigen effectively through class II molecules.

Display of Class II–Peptide Molecules at the Cell Surface

Unlike class I molecules, the delivery of class Ii peptide complexes to the cell surface appears to be a tightly regulated process, especially in professional antigen-presenting cells such as dendritic cells. Externalization of class II peptide complexes is poorly understood, but it is likely to be controlled by exposure of class II positive cells to the appropriate cytokine milieu. Again, this appears to be different from the situation encountered for class I molecules. Although it is regulated, class

I–restricted presentation encompasses more of a constitutive element than seen for class II–restricted presentation. This brief synopsis, expanded on in Chapter 6, highlights the similarities and differences between the two major pathways of antigen processing.

CD1 MOLECULES

One aspect of antigen presentation involves gene products not encoded by the MHC, yet their function and properties are best considered along with MHC class I and class II presentation pathways. Humans have a family of class I–related proteins collectively referred to as CD1 antigens (Fig. 3.4). Four subtly distinct forms of CD1 exist: CD1a, CD1b, CD1c, and CD1d. The genes that encode these products are not located in the MHC, yet the structure of the protein products is similar to that of a class I product. The CD1 molecules, like their classic MHC encoded counterparts, present antigen to T cells. However, both the nature of the antigen and the mode of antigen acquisition set them apart: CD1 products present lipid antigens to T cells. The CD1d product appears capable of presenting classes of lipids structurally distinct from those presented by the CD1a, CD1b, and CD1c molecules. The CD1 protein is a two-chain structure, the light chain being identical to b_2-microglobulin. The peptide binding pocket prominently present in classic class I molecules has been modified such that the CD1 molecules accommodate the hydrophobic acyl chains of lipids and their derivatives, with hydrophilic head groups protruding from the lipid binding cleft to engage T-cell receptors. Prominent classes of lipids presented are glycosylceramides, inositides, and mycobacterially derived lipids. These lipids are acquired in endocytic compartments where microbes are exposed to all manner of hydrolases, some of which must be capable of liberating the lipid antigens. The mechanism of transfer of these lipid molecules onto the CD1 products has not been clarified.

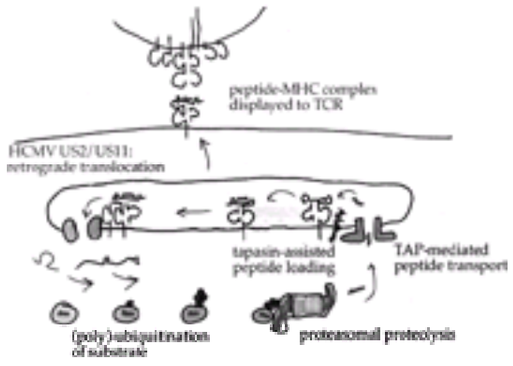

Figure 3.4. Overview of the major histocompatibility complex (MHC) class I–restricted pathway of antigen presentation. For details, see the text. For a detailed discussion of the various strategies used by viral pathogens for immune evasion, see Tortorella et al.

Three distinct routes of antigen presentation have been defined: the class I pathway focused predominantly on peptides generated in the cytosol; the class II pathway, which samples predominantly endocytic compartments for peptides; and the CD1 pathway, which is responsible for presenting lipidlike molecules generated in endocytic compartments. The different isoforms of CD1 appear to target selectively to different stations of the endocytic pathway, mostly through signals carried in their cytoplasmic tail. In this manner, the different endocytic compartments can be examined for the presence of microbially derived lipid antigens.

REGULATION OF ANTIGEN PRESENTATION

Most of the components of the antigen-processing machinery are active constitutively in the cell types that express them. The class II pathway and the CD1 pathway are restricted mostly to professional antigen-presenting cells; the class I pathway is found on most, if not all, nucleated cells. This machinery can be tuned on exposure to cytokines. The T-helper cell (Th1)–type cytokine IFN-g is one of the most potent inducers of both class I and class II pathways. Not only is the level of MHC products and their accessories stimulated in IFN-g–exposed cells, but also it appears that the details of class II trafficking, in particular in dendritic cells, are subject to drastic changes, coincident with differentiation or maturation of the antigen-presenting cell. Generally, immature dendritic cells are more prone to internalize antigen as well as class II molecules than are mature dendritic cells. This aspect is further explored in Chapter 6. Antiinflammatory cytokines such as interleukin-10 have the opposite effect on class II trafficking: interleukin-10 tends to counteract the effects of IFN-g. Although it is outside the scope of this chapter, the antigen presentation pathways have been targeted by pathogens. Because antigen presentation is among the earliest processes that alert the organism to the presence of a pathogen, these pathogens have acquired countermeasures that allow them effectively to elude many aspects of the pathways described earlier. The large DNA viruses (poxviruses, herpesviruses) are notorious for the diverse array of gene products that interfere with cytokine signaling and antigen presentation, respectively.

In the case of autoimmune disease, or predisposition to infectious disease, the antigen presentation–MHC axis occupies a central place. No T cell can recognize an antigen unless it is properly presented by an MHC product. For many autoimmune diseases described elsewhere in this volume, the association of disease with certain alleles of the MHC (particularly for class II products) is evident. In many cases, it is not known which antigens are presented, although a plausible case has been made for myelin basic protein and other myelin-derived peptides in the case of multiple sclerosis. Such peptides must be presented efficiently for an autoimmune T cell to be able to mount an attack. At the same time, the involvement of MHC products in the earliest stages of T-cell development helps to explain both the positively shaping forces exerted by MHC proteins complexed with self-peptides and the elimination of potentially self-reactive T cells from the newly generated repertoire (Chapter 5). Clearly, these selective events impinge on the available repertoire of T cells within a given individual and must be taken into account in all considerations of autoimmunity, whether arising spontaneously or precipitated by an infectious event.

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4 T-CELL ANTIGEN RECEPTORS

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In the last decade and a half there has been tremendous progress in identifying the molecules and genes that govern T-cell recognition, and more recently, we have seen the first concrete information on their biochemistry and structure. Although T-cell receptors for antigen (TCRs) share many similarities, both structural and genetic, with B-cell antigen receptors (immunoglobulins), they also possess a number of unique features related to the roles that particular types of T cells play in the immune system.

For classically defined helper and cytotoxic T cells, the most important of these differences was first suggested by the experiments of Zinkernagel and Doherty (1,2), who showed that viral antigen recognition by cytotoxic T cells was possible only with a certain major histocompatibility complex (MHC) haplotype on the infected cell. Evidence for this phenomenon of “MHC-restricted recognition” also was demonstrated for helper T cells (3,4). We now know that this type of T-cell recognition involves the recognition of fragments of antigens (e.g., peptides) bound to specific MHC molecules (see Introduction and Chapter 20). Because all antigens must eventually be degraded, this form of T-cell recognition seems very complementary to that of B cells, as pathogens can escape recognition by obscuring an antibody-binding site or using “decoy” molecules.

TCRs occur as either of two distinct heterodimers, ab or gd, both of which are expressed with the nonpolymorphic CD3 polypeptides g, d, e, z, and in some cases, the RNA splicing variant of z, h, or Fe chains. The CD3 polypeptides, especially z and its variants, are critical for intracellular signaling (5). The ab TCR heterodimer–expressing cells predominate in most lymphoid compartments (90% to 95%) of humans and mice, and they are responsible for the classic helper or cytotoxic T-cell responses. In most cases, the ab TCR ligand is a peptide antigen bound to a class I or class II MHC molecule. T cells bearing gd TCR are less numerous than the ab type in most cellular compartments of humans and mice. However, they make up a substantial fraction of T lymphocytes in cows, sheep, and chickens (6). Recent work on the structural characteristics and specificity if gd TCRs suggests that they may be much more like immunoglobulins than like ab TCRs in their antigen-recognition properties. In particular, they do not seem to require MHCs or other molecules to present antigens, but instead appear to recognize antigens directly (6). Although it is not yet clear what role they play in the immune response, this is a very active area of current research, and many interesting leads are being pursued.

T-CELL RECEPTOR/CD3 POLYPEPTIDES

The search for the molecules responsible for T-cell recognition first focused on deriving antisera or monoclonal antibodies (mAbs) specific for molecules on T-cell surfaces. Ultimately, a number of groups identified “clonotypic” sera (7) or mAbs (8,9,10,11 and 12). Most of these antibodies were able to block antigen-specific responses by the T cells they were raised against or, when coated on a surface, could activate the T cells they are specific for. They immunoprecipitate 85,000 to 90,000 MW disulfide-bonded heterodimers consisting of two 40,000 to 50,000 MW glycosylated subunits (a and b). Peptide-mapping studies showed that there was a striking degree of polymorphism between heterodimers isolated from T cells with different specificities, thus indicating an antigen-specific polymorphism akin to that of immunoglobulins (13,14).

Work in parallel to these serologic studies exploited the small differences (~2%) observed between B- and T-cell gene expression (15) and isolated both a mouse (16,17) and a human (18) T-cell–specific gene that had antibody-like V-, J-, and C-region sequences and could rearrange in T lymphocytes (16). This molecule was then identified as TCRb by partial sequence analysis of immunoprecipitated materials (19). Subsequent work rapidly identified two other candidate TCR cDNAs identified as TCRa (20,21) and TCRg (22). It was quickly established that all antigen-specific helper or cytotoxic T-cells expressed TCRab heterodimers. Where the TCRg fit in remained a puzzle until work by Brenner et al. (23) showed that it was expressed on a small (5% to 10%) subset of peripheral T cells together with another polypeptide, TCRd. The structure of TCRd remained unknown until a new TCR locus was discovered within the TCRa locus, between V_a and J_a (24). Antisera raised against portions of this gene showed that it was TCRd (25,26 and 27). Formal proof that the TCR a and b subunits were sufficient to transfer antigen/MHC recognition from one T cell to another came from gene-transfection experiments (28,29), and equivalent experiments also have been done with gd TCRs (30).

As shown in Fig. 4.1, all TCR polypeptides have a similar primary structure, with distinct variable (V), diversity (D) in the case of TCR b and d, joining (J), and constant (C) regions exactly analogous to their immunoglobulin counterparts. They also share many of the amino acid residues thought to be important for the characteristic variable and constant domains of immunoglobulins (31). The C_b region is particularly homologous, sharing 40% of its amino acid sequences with C_K and C_l (17,18). The TCR polypeptides all contain a single C-region domain (versus up to four for immunoglobulins) followed by a connecting peptide or hinge region, usually containing the cysteine for the disulfide linkage found joining the two chains of the heterodimer [some human TCRgd isoforms lack this cysteine and consequently are not disulfide linked (32)]. *N*-linked glycosylation sites vary from two to four for each polypeptide, and are indicated in the figure. C-terminal to the connecting peptide sequences are the hydrophobic transmembrane regions, which have no similarity to those of *IgH* genes, but instead have one or two positively charged residues that appear to be important for interaction with the CD3 molecules and T-cell signaling (33), perhaps through interaction with the acidic residues found in all CD3 transmembrane regions.

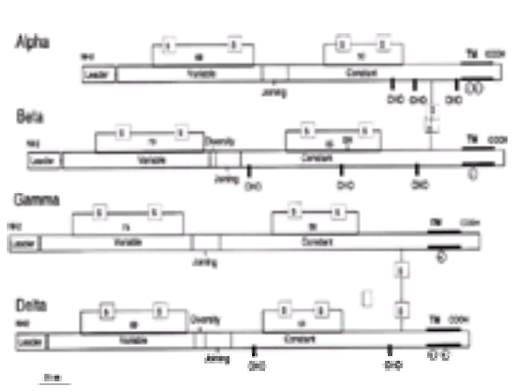

Figure 4.1. T-cell receptor (TCR) polypeptides. Schematic diagram of typical TCR polypeptides. Disulfide bonds, N-linked carbohydrate attachment sites (CHO), and transmembrane charges are indicated for TCRa, -b, -g, and -d.

T-CELL RECEPTOR STRUCTURE

Similarities to and Differences from Immunologlobins

As discussed earlier, the sequences of TCR polypepides show many similarities to those of immunoglobulins and thus it has long been suggested that both heterodimers would be antibody-like in structure (31,34,35). These similarities include the number and spacing of specific cysteine residues within domains, which in antibodies form intrachain disulfide bonds. Also conserved are many of the inter- and intradomain contact residues, and secondary structure predictions are largely consistent with an immunoglobulin (Ig)-like “b barrel” structure. This consists of three to four anti-parallel b strands on one side of the barrel facing a similar number on the other side, with a disulfide bridge (usually) connecting the two b “sheets” (sets of b strands in the same plane). All Ig variable and constant region domains have this structure, with variations in the number of b strands in variable region domains (by convention including V, D, and J sequences) compared with constant domains (34).

Efforts to derive x-ray crystal structures of TCR heterodimers and fragments of heterodimers has been very difficult, but in the last 5 years, a number of TCR fragments and heterodimer structures have been solved, particularly from the laboratory of Mariuzza et al. (36,37), who solved the structure of a V_b C_b polypeptide and a V_a fragment. In general, these domains all are very Ig-like, with the classic b-barrel structure in evidence in all three domains. At each end of the barrel in each V-region domain, there are four loops between the b sheets, three of which form the complementary determining regions (CDRs) of immunoglobulins. The fourth loop, between the D and E strands, has been implicated in superantigen binding. The six CDR loops from the two variable domains form the antigen-binding surface of immunoglobulins, and as we discuss shortly, TCRs as well. Whereas the V_b domain depicted in Fig. 4.2 follows the canonic V-domain b sheet structure, V_a differs significantly in that one of the sheets has been translocated to the other half of the barrel (as schematized in Fig. 4.2). This acts to remove a bulge in the side of the V_a domain, and it has been suggested that this would allow dimers of TCRs or perhaps higher-order structures to assemble (38). More recently, Garcia et al. (39) were able to solve the structure of the C_a in the context of a complete heterodimer, and it has a remarkable variation of the classic Ig-like domain (39). Here there is only one half of the classic b barrel, that is, one set (or “sheet”) of b strands, whereas the rest of the somewhat truncated domain exhibits random coils. This type of structure is unprecedented in the Ig superfamily and seems likely that it will be true for C_d as well, as it has many sequence similarities to C_a (24). The functional significance of such a variant structure in unknown, but it has been suggested that this incompletely formed Ig-like domain may be responsible for the observed lability of TCRa, and this may allow greater flexibility in the regulation of its expression. Another possible explanation is that this alteration may be designed to accommodate one or more of the CD3 molecules (39). Interestingly, the recently solved structure of a V_d domain (40) shows a somewhat more V_H-like pattern, consistent with CDR3 length analyses and its apparent ligand-recognition properties (see later).

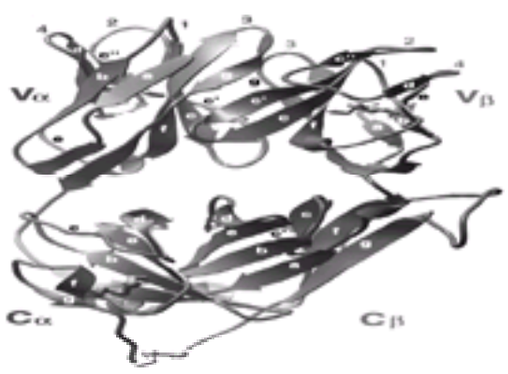

Figure 4.2. T-cell receptor (TCR) structure. Ribbon diagram of the structure of an ab TCR heterodimer (extracellular portion). b Strands are indicated in *letters*, and complementarity-determining region (CDR) loops in *numbers*. The *yellow spheres* depict disulfide bonds within the V and C domains and joining the a and b chains. (From Garcia KC, Decagon M, Stanfield RL, et al. An ab T-cell receptor at 2.5Å and its orientation in the TCR-MHC complex. *Science* 1996;274:209–219, with permission). (See Color Figure 4.2.)

With respect to complete heterodimer structures, there are now data from five ab heterodimers (39,41,42,43 and 44), and they largely resemble an Fab fragment of an antibody. Whereas many features of these structures are shared with their antibody counterparts, several unusual features are emerging that may be significant. These include

1. In one structure (39), four of seven *N*-linked sugars diffracted to high resolution, indicating that they are not free to move very much and thus are likely to play a structural role, particularly in C_a/C_b interactions.
2. There is much more contact between V_b and somewhat more between V_a and C_a than in the equivalent regions of antibodies.
3. The geometry of the interaction of V_a and V_b more closely resembles that of the C_H3 domains of antibodies than .... V_HV_L.
4. Between the CDR3 loops of V_a and V_b, there is a pocket that can [and does in at least one case (41)] accommodate a large side chain from the peptide bound to an MHC.

Another key question is whether any conformational change occurs in the TCR on ligand binding. Conformational changes in the TCR or in the CD3 polypeptides in particular may hold important clues as to the mechanism of signal transduction across the membrane after TCR engagement. Recent studies also have indicated that TCR/peptide/MHC complexes have an inherent ability to self-associate (46), and thus it would be very interesting to know the structure of such a cluster and how it may relate to early signaling events.

Also associated with both ab and gd TCR heterodimers are the CD3 polypeptides, which are not covalently linked but coprecipitate with TCRs under certain conditions (as reviewed in 5,47). These are CD3g, -d, -e, and -z. The z chain is usually disulfide-bond homodimer; but certain other forms are also found, such as z-h or z-FceRI (5).

With respect to overall stoichiometry, current evidence favors at least two TCR heterodimers per CD3 cluster. This is based on a number of findings, particularly the work of Terhorst et al. (as reviewed in ref. 5) and more recently that of Fernandez-Miguel et al. (45), who showed that in T cells that have two transgenic TCRb chains, antibodies to one V_b can immunoprecipitate the other. It also was found that they are often close enough to allow fluorescence energy transfer, meaning that the two TCRb chains in a cluster are within 50 Å of each other (45).

T-CELL RECEPTOR GENES

As shown in Fig. 4.3, *TCR* gene segment organization is similar to that of immunoglobulins, and the same recombination machinery seems responsible for joining separate *V* and *D* segments to a particular *J* and *C*. This was initially indicated by the fact that the characteristic seven- and nine-nucleotide conserved sequences adjacent to the V, D, and J regions with the 12- or 23-nucleotide spacing between them, first described for immunoglobulin genes, also are present in *TCR*s (48,49). The most conclusive evidence of this common rearrangement mechanism has been shown recently by the fact that both a naturally occurring recombination-deficient mouse strain [severe combined immune deficiency, SCID (50)] and mice engineered to lack recombinase activating genes (RAGs) 1 (51) or 2 (52) are unable to rearrange either *TCR* or immunoglobulin gene segments properly. As with immunoglobulins, if the V-region and J-region gene segments are in the same transcriptional orientation, the intervening DNA is deleted during recombination. DNA circles of such material can be observed in the thymus (53,54), the principal site of TCR recombination (see later). In the case of *TCR*b (55) and *TCR*d (56), there is a single V region 3' to the C in the opposite transcriptional orientation to J and C. Thus rearrangement to these gene segments occurs through an inversion. Variable points of joining are seen along the *V, D,* and *J* gene segments as well as random nucleotide addition (N regions) in postnatal TCRs (57). The addition of several nucleotides in an inverted repeat pattern, referred to a P element insertion, at the V/J junction of the TCRg chains, also has been observed (58).

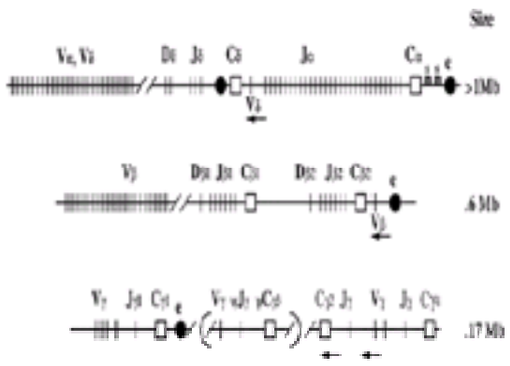

Figure 4.3. T-cell receptor (TCR) genes. Outline of murine TCR loci.

Organization of the T-cell Receptor Loci

In humans and in mice, there is a single a-chain C-region gene composed of four exons encoding (a) the constant region domain, (b) 16 amino acids including the cysteine that forms the interchain disulfide bond, (c) the transmembrane and intracytoplasmic domains, and (d) the 3' untranslated region (Fig. 4.3) (59,60 and 61). The entire ad locus in humans has now been mapped (62) and spans about 1.1 MB. The mouse TCR locus appears to be similar in size. There are 50 different J-region gene segments upstream of the C region (Fig. 4.3) in the murine locus (63). At least eight of the J-region gene elements are nonfunctional because of in-frame stop codons or rearrangement and splicing signals that are likely to be defective. A similar number of a-chain J regions are present in the human locus (61,62). This very large number of a-chain J regions compared with the immunoglobulin loci may indicate that the functional diversity contributed by the J segment of the TCR (which constitutes a major portion of the CDR3 loop) makes a special contribution to antigen recognition (see later).

Both the murine and human C_d, J_d, and two D_d gene segments are located between the V_a and J_a gene segments (24,64,65,66 and 67). In the murine system, there are two J_d and two D_d gene segments on the 5' side of C_d (24,64), and the C_d gene is approximately 75 kb upstream of the C_d gene, but only approximately 8 kb upstream of the most 5' known J_a gene segments. The human organization is similar, with three D_a gene segments and two J_d (67). Surprisingly, in both species, all of the D elements can be used in one rearranged gene rather than alternating, as is the case with TCRb or IgH. That is, in mice one frequently finds V_d, D_1, D_2, J_d rearrangements (57), and in humans, V_d, D_1, D_2, D_3, and J_d (67). This greatly increases the junctional or CDR3 diversity that is available, especially because of the potential for N-region addition in between each gene segment. This property makes TCRd the most diverse of any of the antigens receptors known, with approximately 10^{12} to 10^{13} different amino acid sequences in a relatively small (10 to 15aa) region (57).

The location of D_d, J_d, and C_d genes between V_a and J_a gene segments raises the possibility that TCRd and a could share the same pool of V-gene segments. Although there is some overlap in V-gene use, in the murine system, four of the commonly used V_d genes (V_d1, V_d2, V_d4, V_d5) are very different from known V_a sequences, and they have not been found to associate with C_a (68). The other four V_d gene families overlap with or are identical to V_a subfamilies (V_d3, V_d6, V_d7, and V_d8 with V_a6, V_a7, V_a4, and V_a11, respectively).

The mechanisms that account for the preferential use of certain gene segments to produce d versus a chain are not known. Whereas some V_d genes are located closer to the D_d and J_d fragments than V_a genes (such as V_d1), other V_d genes (such as V_d6) are rarely deleted by V_a J_a rearrangements and thus seem likely to be located 5' of many V_a gene segments (57).

An implicit characteristic of the a/d gene locus is that a rearrangement of V_a to J_a deletes the entire D/J/C core of the d-chain locus. In many ab T cells, the a-chain locus is rearranged on both chromosomes, and thus no TCRd could be made. In most cases this is due to V_a®J_a rearrangement, but evidence suggesting an intermediate step in the deletion of TCRd has been reported (69). This involves rearrangements of an element termed TEA to a pseudo-J_a 3' of C_d. The rearrangement of TEA to this pseudo J_a would eliminate the d-chain locus in ab T cells. Recent gene targeting of the TEA element resulted in normal levels of ab and gd T cells, but use of the most J_a cells was severely restricted (70), suggesting that its function has more to do with governing the accessibility of the most 5'$_{Ja}$ cells for recombination.

The entire human 685-kb b-chain gene locus has been completely sequenced (71), and the organization is shown in Fig. 4.3. One interesting feature is the tandem nature of J_b-C_b in the *TCR*$_b$ locus (). This arrangement is preserved in all higher vertebrate species that have been characterized (mouse, human, chicken, frog). The two C_b coding sequences are identical in the mouse and nearly so in humans and other species. Thus it is unlikely that they represent two functionally distinct forms of C_b. However, the J_b clusters have unique sequences and thus this may be a mechanism for increasing the number of J_b gene segments. Together with the large number of J_a gene segments, there is far more combinatorial diversity ($J_a \times J_b = 50 \times 12 = 600$) provided by J regions in ab TCRs than in immunoglobulins.

Most of the V regions are located upstream of the joining and constant regions, and in the same transcriptional orientation as the D and J gene element and rearrange to D_bJ_b gene by deletion. Similar to the case of V_d5, a single V_b gene, V_b14, is located 3' to C regions and in the opposite transcriptional orientation; thus rearrangements involving V_b14 occur through inversion (55).

The organization of the mouse and human g-chain loci have been thoroughly defined (72,73,74,75,76,77 and 78). The human g genes span about 150 kb (77) and are organized in a fashion similar to that of the b-chain locus with two J_gC_g regions. An array of V_g regions in which at least six of the V regions are pseudogenes (filled in), are located 5' to these J_gC_g clusters, and each of the V genes is potentially capable of rearranging to any of the five J regions. The sequences of the two human C_g regions are very similar overall and differ significantly in only the second exon. In C_g2 this exon is duplicated 2 to 3 times, and the cysteine that forms in the interchain disulfide bond is absent. Thus C_g2-bearing human T cells have an extra large g chain (55,000 MW) that is not disulfide bonded to its d-chain partner (74).

The organization of the murine g-chain genes (Fig. 4.3) is very different from that of the human genes, in that there are three separate rearranging loci (73,74) and spans about 205 kb (78). Of four murine C_g genes, C_g3 is apparently a pseudogene in BALB/c mice, and the J_g3 C_g3 region is deleted in several mouse strains including C57 Bl/10. C_g1 and C_g2 are very similar in coding sequences. The major difference between these two genes is in the five-amino acid deletion in the C_g2 gene, which is located in the C II exon at the amino acid terminal of the cysteine residue used for the disulfide formation with the d chain. The C_g4 gene differs significantly in sequences from the other C_g genes (in 66% overall amino acid identity). In addition, the C_g4 sequences contains a 17-amino acid insertion (compared with C_g1) in the C II exon located at similar position to the five-amino acid deletion of the C_g2 gene (G. Kershard, S.M. Hedrick, unpublished results).

Each of the C_g genes is associated with a single J_g gene segment. The sequences of J_g1 and J_g2 are identical at the amino acid level, whereas J_g4 differs from J_g1 and J_g2 at nine of 19 amino acid residues.

Chromosomal Locations of T-cell Receptor Genes and Translocations Associated with Disease

The chromosomal locations of the different *TCR* loci have been delineated in both mouse and humans, and the results are summarized in Table 4.1 (as reviewed in ref. 79). One significant factor in cancers of hematopoietic cells is chromosomal translocations, which result in the activation of genes that are normally turned off or the inactivation of genes that are normally turned on. Thus B- or T-lymphocyte neoplasia is frequently associated with inter- or intrachromosomal rearrangements of Ig or TCR loci, and in some cases, both (80,81).

	Mouse Chromosome	Human Chromosome
TCR-α	14	14(q11-q12
TCR-δ	14	14(q11-q12
IgH	12	14(qter)
TCR-β	6	7(q35)
CD4	6	12
CD8	6	2(p11)
Thx	6	2(p12)
TCR-γ	13	7(p14)
CD3-γ	9	11(q23)
CD3-δ	9	11(q23)
CD3-ε	9	11(q23)
CD3-ζ	1	1
Thy-1	9	11(q23)
Igλ	16	22(q11.2)
MHC	17	6(p21)
Pre-T-α	17	6

TCR, T-cell receptor; Ig, immunoglobulin; MHC, major histocompatibility complex.

TABLE 4.1. Locations of Receptors

These translocations seemed to mediated by the V(D)J recombinase machinery, indicating the inherent danger and need for tight regulation of this pathway. Such rearrangements are particularly common in the a/d locus, perhaps because this locus spans the longest developmental window in terms of gene expression, with *TCR*d being the first and *TCR*a the last gene to rearrange during T-cell ontogeny (as discussed in more detail later). In addition, the a/d locus is in excess of 1 Mb in size, and this provides a larger target, for rearrangement than does either TCRb or TCRg. Interestingly, in humans, *TCR*a/d is on the same chromosome as the *IgH* locus, and V_H®J_a rearrangements (by inversion) have been observed in some human tumor material (82,83). The functional significance of this is not known.

Particularly frequent is the chromosome 8–14 translocation [t(8;14) (q24;q11)], which joins the a/d locus to the c-*myc* gene, analogous to the C-*myc*®IgH translocation in many mouse myeloma tumors and in Burkitt lymphomas in humans (80,81). In one cell line, a rearrangement occurs between the J_a-region coding sequences, and a region 3' of c-*myc* (84). In both B- and T-cell malignancies, the translocation of c-*myc* into IgH or TCRa/b appears to increase the expression of c-*myc*, and may be a major factor in the unregulated cell growth that characterizes cancerous cells. Other putative protooncogenes that have been found translocated into the TCRa/b locus are the LIM domain containing transcription factors Ttg-1 (85), and Ttg-2 (86,87), which are involved in neural development; the helix/loop/helix proteins Lyl-1 (88,89) and Scl (90,91), which are involved in early hematopoietic development; and the homeobox gene Hox 11 (92), which is normally active in the liver. How these particular translocations contribute to malignancy is unknown, but it presumably causes aberrations in gene expression that contribute to cell growth or escape from normal regulation. In T-cell leukemia patients infected with the human T-cell lymphoma virus (HTLV)-I, there are large numbers of similar translocations, and here it is thought that HTLV-I itself is not directly leukemogenic, but acts by causing aberrant rearrangements in the T cell it infects, some of which become malignant.

Another disorder that exhibits frequent TCR and Ig locus translocations is ataxia telangiectasia (AT), an autosomal recessive disorder characterized by ataxia, vascular telangiectasis, immunodeficiency, increased incidence of neoplasia, and an increased sensitivity to ionizing radiation. Peripheral blood lymphocytes (PBL) cells from patients with AT have an especially high frequency of translocations involving chromosomes 7 and 14 (93). These sites correspond to the TCRg, -b, and -a loci, and the Ig heavy-chain locus (94,95). Thus it appears as though one of the characteristics of AT patients is a relatively error-prone rearrangement process that indiscriminately recombines genes that have the TCR and Ig rearrangement signals.

BIOCHEMISTRY OF ab T-CELL RECEPTOR/LIGAND INTERACTIONS

Although it has long been established that T cells recognize a peptide in association with an MHC molecule, it was many years before a formal biochemical demonstration was possible that this was due to TCR binding to a peptide/MHC complex. Part of the difficulty in obtaining measurements of this type has been the intrinsically membrane-bound nature of MHC and TCR molecules. Another major problem is that the affinities are relatively low, with K_d values of 10^{-4} to 10^{-7} *M*, too unstable to measure by conventional means.

Ultimately these technical problems were resolved, and the first measurements of TCR affinities for peptide/MHC complexes were made by Matsui et al. (95) and Weber et al. (96). They obtained K_d values of ~50 μ*M* and ~10 μ*M*, respectively. Subsequent work, particularly using surface plasmon resonance instruments with their remarkable sensitivity to weak macromolecular interactions (97,98,99,100,101,102,103 and 104), has allowed rapid progress in this area and extended the range of affinities observed to 300- 0.1 μ*M*. This has thus far been the method of choice for measuring the kinetics of TCR binding to its ligands [excepting the very highest affinity TCRs (105)]. Kinetic measurements show that while the on-rates of TCR binding to peptide/MHC molecules vary from very slow (1,000 msec) to moderately fast (200,000 msec), their off-rates fall in a relatively narrow range (0.5 to 0.01 per second) or a t½ of 12 to 30 seconds at 25°C, similar to other membrane-bound receptors that recognize membrane molecules on other cells (106,107). In the case of the class I MHC–restricted TCR, 2C, this relatively fast off-rate is significantly stabilized if soluble CD8 is introduced (108). This same effect of CD8 stabilization had previously been seen by Luescher et al. (109) in their unique cell-based TCR-labeling assay. Similar BIAcore experiments using soluble CD4 have not produced comparable results (J. Hampl, J. Boniface, unpublished data) Whereas all of the BIAcore measurements cited earlier were performed at 25°C because of instrument limitations, the off-rates are generally much faster (from 10 to 20 times) at 37°C (110,111).

Altered Peptide Ligands

To what extent are we now able to predict a T-cell response based on the binding characteristic of its TCR to a ligand? One of the most intriguing discoveries concerning T-cell reactivity in recent years has been the phenomenon of altered peptide ligands. These are single amino acid variants of antigenic peptides that either change the nature or degree of the T-cell response (partial agonists) or prevent a response to a normally stimulating ligand (antagonists) (112,113). Discussions concerning the mechanism of these “altered peptide” responses have centered around whether they are due to some conformational phenomenon involving TCRs and/or CD3 molecules or to affinity or kinetic characteristics. With the data in hand, we can now say that most, but not all (see especially ref. 104), T-cell responses correlate very well with the binding characteristics of their TCRs (as reviewed in ref. 114).

How might the relatively small differences in the binding characteristics of the ligands cause such different T-cell signaling outcomes as agonism or antagonism? As McKeithan (115) and Rabinowitz et al. (116) noted, any multistep system such as T-cell recognition has an inherent ability to amplify small differences in signals that are received on the cell surface to much larger differences at the end of the pathway, in this case gene transcription in the nucleus. Thus antagonism may occur at one threshold and an agonist response at another. Alternatively, an antagonist ligand may traverse the activation pathway just far enough to use up some critical substrate, as proposed by Lyons et al. (102). Yet another possibility also suggested is that some antagonists may act even earlier, by blocking TCR clustering at the cell surface (46).

Role of CD4 and CD8

What is the role of CD4 and CD8 with respect to the T-cell response to agonist and antagonist peptides? In the case of a T-helper cell response, the presence of CD4 greatly augments the amount of cytokine produced and, in some cases, determines whether there is a response at all (as reviewed in ref. 117). Much of the effect of CD4 seems to come from the recruitment of lck to the TCR/CD3 complexes. In addition there is a significant positive effect even with CD4 molecules that are unable to bind lck, and thus there appears to be an affect on TCR/ligand interaction as well. CD8 also greatly augments the response of class I MHC–specific T cells (113), and, as discussed earlier, CD8 stabilizes TCR/peptide/MHC complexes. Overall it seems likely that each of these coreceptor molecules has two roles: to stabilize TCR/ligand interactions physically and to aid in signaling by recruiting lck. Data show, consistent with this, that the presence of CD4 can convert an antagonist peptide into a weak agonist (118,119). Work by Hampl et al. (120) puts a new twist on this by showing that whereas weak agonist peptides are made almost as potent as the best peptides by the presence of CD4, little or no effect is seen on antagonism.

These results suggest that CD4 engagement is not automatic and simultaneous with TCR binding, but rather is recruited later into preexisting TCR/peptide/MHC complexes (or oligomers), as suggested by Germain (121,122). In some cases, antagonist/MHC complexes will be stable long enough for CD4 to have an effect, in which case, the T cell could be stimulated, but in the antagonism experiments of Hampl et al. (120), the TCR/ligand association does not last long enough for CD4 to affect the outcome. It also is interesting that subsequent studies of Krummel et al. (123) showed that whereas CD3 and CD4 colocalize at the initiation of T-cell recognition, in most cases, CD4 migrates away soon after the first calcium elevation (e.g., after the initiation of signaling, but long before the T cell is fully committed to action). Thus CD4 seems to play a very transient role in the recognition process.

TOPOLOGY AND CELL BIOLOGY OF T-CELL RECEPTOR/PEPTIDE/MHC INTERACTIONS

An analysis of TCR sequence diversity has shown that the vast majority of amino acid variation resides in the region between the V- and J-region gene segments, which corresponds to the CDR3 regions of antibodies (124). This has led to models in which the CDR3 loops of V_a and V_b make the principal contacts with the

antigenic peptide bound to the MHC (38,124,125). Support for such a model has come from many studies in which it has been shown that the CDR3 sequences of TCRs are important predictors of specificity (as reviewed in ref. 124), as well as the elegant mutagenesis studies of Hedrick et al. (126,127), who showed that a single CDR3 point mutation could alter the specificity of a TCR (126) and also that a CDR3 “transplant” could confer the specificity of the donor TCR onto the recipient (127). In addition, a new approach to TCR/ligand interactions was developed by Jorgensen et al. (128,129), who made single amino acid changes at positions that affect T-cell recognition but not MHC binding in a given peptide. These variant peptides are then used to immunize mice that express either the a or b chain of a TCR that recognizes the original peptide, and the responding T cells are analyzed. The results from this study and work in another system by Sant'Angelo et al. (130) are very similar, in that every mutation at a TCR-sensitive residue triggered a change in the CD3 sequence of V_a, V_b, or both, and in some cases, changed the V_a or V_b gene segment as well. One of the more striking examples of a CDR3/peptide interaction occurred in the cytochrome *c* system, where a Lys®Glu change in the central TCR determinant on the peptide triggered a Glu®Lys charge reversal in the V_a CDR3 loop, arguing for a direct Lys®Glu contact between the two molecules (129).

Structural Analyses

Most definitive is the work of Garcia et al. (39) and Garboczi et al. (41), who nearly simultaneously solved the crystal structures of two different TCR/peptide/class I MHC complexes. As illustrated in Fig. 4.4, these studies show a TCR binding surface much like an antibody fitting down between the two opposite “high points” of the class I MHC a helices, in a roughly diagonal configuration. In these structures and subsequent ones of the CDR3, loops are centrally located over the peptide, but the V_a CDR1 and the V_b CDR1 are also in a position to contact the N-terminal and C-terminal peptide residues, respectively. Such a contact between V_a CDR1 and an N-terminal residue was seen in the Garboczi structure (41), whereas that of Garcia et al. (39) has insufficient resolution at this point. Although more structures are needed, the results are sufficiently consistent with the results of the peptide-immunization and mutation experiments cited previously to indicate that all TCR/peptide/MHC complexes will have roughly the same topology. An extensive mutagenesis/modeling study also arrived at this same orientation (130a). This oriented recognition constitutes a major departure from antibody/antigen interactions and may derive from the need to intercalate CD4/CD8 molecules during activation. The recent structure of a class I MHC/CD8 complex is a first step in understanding how this might be accomplished (131).

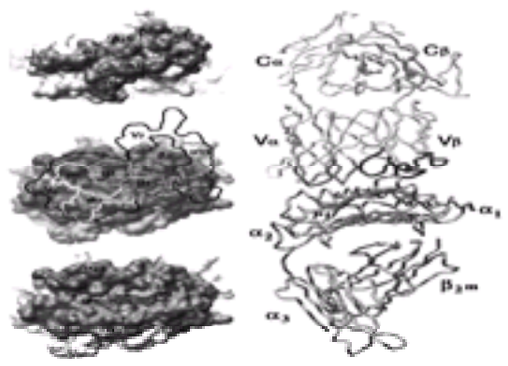

Figure 4.4. T-cell receptor (TCR)/peptide/major histocompatibility complex (MHC). The structure of an ab TCR binding to peptide/MHC. (From Garcia KC, Decagon M, Stanfield RL, et al. An ab T-cell receptor at 2.5Å and its orientation in the TCR-MHC complex. *Science* 1996;274:209–219, with permission.) (See Color Figure 4.4.)

Formation of an “Immunologic Synapse”

Although it has long been known that cross-linking surface immunoglobulin molecules on B lymphocytes or TCR/CD3 molecules on T lymphocytes can trigger activation, it has not been appreciated until recently just how these artificial stimuli relate to the normal process of T-cell activation that begins with TCR engagement of the appropriate ligand. That there is an inherent tendency of TCRs to cluster with peptide/MHC ligands was shown by Reich et al. (46) with pure molecules in solution, and it was soon afterward shown by Kupfer et al. (131a) that the key surface molecules in a T-cell/B-cell couple distributed themselves in a characteristic “bull's-eye” pattern, which they referred to as a supramolecular activation complex, or SMAC, and others have termed an “immunologic synapse.” In these “synapses,” the TCR on the T cell occupies the central region, directly opposite a cluster of the appropriate MHC on the B cell that it is recognizing. The outer ring is composed of leukocyte function–associated antigen (LFA)-1 on the T-cell side and its ligand intercellular adhesion molecule (ICAM)-1 on the B-cell side. Various other surface and signaling molecules distribute themselves into these two areas or segregate into other patterns (A. Kupfer, personal communication). The dynamics of synapse formation have now been directly visualized by using the T cell/artificial membrane systems of Dustin et al. (134) or the green fluorescent protein (GFP)-tagged CD3 system of Krummel et al. (123) involving T-cell/B-cell couples. Figure 4.5 shows a time course of synapse formation from Grakoui et al. (133). This shows a “bulls-eye”-type pattern in which the MHC is gathered into the center, whereas the ICAM-1 (an accessory molecule) tends to be on the outside. Parallel studies on T cells have shown that there is a parallel accumulation of TCR and LFA-1 (the ligand for ICAM-1) at the interface. Another important contributor to synapse formation appears to be costimulatory signals, which, as shown by Wülfing et al. (135) and Viola et al. (136), trigger a membrane cytoskeletal movement towards the T/B interface. This movement may involve membrane rafts (136) and appears dependent on myosin motors (135). It is triggered only by a combination of TCR and costimulates receptor engagement (either CD28 or LFA-1), and it is likely that the resulting movement of cytoskeletally attached membrane molecules, such as TCR/CD3, drive the formation of synapses (C. Wülfing et al., unpublished data). Although the formation of this type of structure is not absolutely necessary for some T-cell stimulation to occur *in vitro*, it may be *in vivo*, especially as antigen stimulation without costimulation generally produces no response or an aborted one in an organism. As to why the different surface molecules segregate into different regions of the synapse, this is not known, but it may enhance signaling reactions or may just reflect the different sizes of the various receptors and their ligands (137,138).

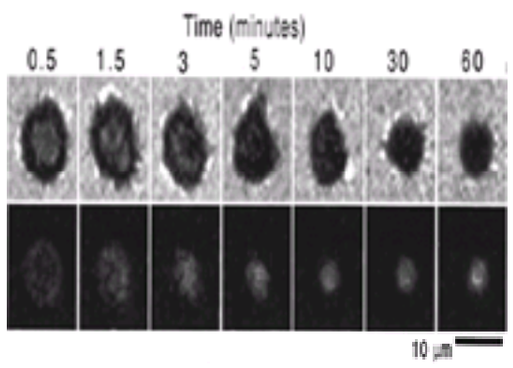

Figure 4.5. Formation of an immunologic synapse. An antigen-specific T cell **(A)** contacts an artificial lipid bilayer containing labeled major histocompatibility complex (MHC; *green*) and intercellular adhesion molecule (ICAM-1; *red*). Within minutes, it has recognized the antigen contained within the MHC and gathered the MHC and ICAM-1 into the distinctive “bulls-eye” pattern. (From Grakoui A, Bromley SK, Sumen C, et al. The immunological synapse: a molecular machine controlling T-cell activation. *Science* 1999;285:221–227, with permission.) (See Color Figure 4.5.)

ab T-CELL RECEPTOR AND SUPERANTIGENS

One of the most interesting and unexpected areas to emerge from the study of ab T-cell reactivities is the discovery of the “superantigen.” Whereas a particular antigenic peptide might be recognized by only one in 100,000 or fewer T cells in a naive organism, a given superantigen might stimulate 1% to 20% of the T cells (as reviewed in ref. 139,140 and 141). As is discussed in more detail later, the physical basis for this is that the superantigen binds to a V_b domain of the TCR on T cells while simultaneously binding to an MHC class II molecule on an antigen-presenting cell (although not in the peptide-binding groove). This allows a single superantigen, such as SEA, to stimulate virtually every murine T cell bearing V_b1, -3, -10, -11, -12, or -17 (~15% of all ab T cells), regardless of what V_a it is paired with or what CDR3 sequence is expressed. Clearly this is a unique class of T-cell stimulatory molecule.

Widespread Use by Pathogens

The first indication of a superantigen effect was the discovery of Kappler et al. (142) of a mouse strain–specific deletion of T cells expressing a specific V_b. This showed

that the effect was due to certain alleles of the minor histocompatibility loci known as mls, which had the abilities to stimulate specific V_b-bearing T cells and caused the elimination of those T cells in mouse strains that carried that particular allele (143,144,145,146 and 147). Intensive efforts to clone these loci revealed that they were endogenous retroviruses of the mouse mammary tumor virus family (148,149,150,151 and 152), and various family members bind different V_b domains. Meanwhile, Janeway et al. (153) showed that *Staphylococcus* enterotoxins could polyclonally activate naive T cells in a V_b-specific manner without a requirement for antigen processing. Unlike the MMTV proteins, which are type II membrane proteins, the enterotoxins are secreted. Subsequently, proteins having superantigenic properties have been isolated from other bacteria [*Yersinia pseudotuberculosis* (154,155), *Streptococcus* (156), and *Mycoplasma* (157,158)]. There also is evidence of superantigen-like activities in other mammalian viruses such as rabies (157), Epstein-Barr virus (160), cytomegalovirus (161), and also in the parasite *Plasmodium falciparum* (malaria) (162) and *Toxoplasma gondii* (163), but the actual molecules have not yet been identified. Because so many pathogenic or parasitic organisms possess these molecules, apparently by convergent evolution, there must be some selective advantage, but in most cases, there is no conclusive evidence as to what this might be. The one exception is the MMTV superantigens, in which it has been shown that polyclonal T-cell stimulation allows the virus to infect much more efficiently the B lymphocytes that are activated by the T cells (164,165). This may be a special case, however, and most authors have suggested that superantigens primarily serve to confuse and occupy the immune system while the pathogen escapes specific targeting and elimination. Large doses of superantigens also have been implicated in various “shock” syndromes, such as food poisoning or “toxic shock” (139), but this is probably not their everyday purpose, as it would violate the general rule that the host and parasite should coexist.

It also has been suggested that superantigens might be involved in triggering autoimmune diseases. Here the hypothesis is that a large number of some V_b-bearing T cells are activated by a pathogenic superantigen, and that subsequently, self-reactive T cells within those activated cells are stimulated by a particular tissue antigen. Although a superantigen-like predominance of particular V_b-bearing cells has been reported in rheumatoid arthritis (166,167) and Kawasaki disease (168), other investigators have not been able to confirm these reports.

The biochemistry of superantigen binding to TCRs and MHC is similar to that of TCR/peptide/MHC interactions (99,169), but the topology is quite different. In particular, it has been found that Mls-1a presentation to T cells is most affected by mutations on the “outside” surface of the V_b domain (amino acids 22, 70, 71), which do not affect peptide/MHC recognition (70,171). In contrast, CDR grafting experiments by Patten et al. (172) have implicated CDR1 and CDR2 of V_b3 in bacterial superantigen reactivity. Similarly, other investigators have found differences in the way different superantigens bind to TCRs, but none has found any involvement of CDR3 sequences.

Recently, the x-ray crystal structures of the superantigens SEC2 and SEC3 complexed with a mouse TCRb chain have been reported (173). Similar to the Patten et al. data (172), these bacterial superantigens bind to CDR1 and CDR2 and somewhat to the outside of the V_b domain. When modeled on the superantigen/MHC structures, the resulting TCR/SAg/MHC complex would displace the TCR somewhat (but not entirely) away from the MHC binding groove (173), thus making the interaction largely insensitive to the TCR/peptide specificity, as suggested by Jorgensen et al. (129). Because this is only the first report of the structure of TCR/SAg complex, it is entirely possible that other molecules will interact differently. Indeed, as the mls antigens are structurally very different from the bacterial SAgs and in their mode of MHC binding. Therefore, their mode of binding to V_b domains is likely to be unique as well.

Why do superantigens only interact with the b chain? One possibility is that the b chain offers the only accessible “face” of the TCR, perhaps because CD4 blocks the V_a side (as suggested by antibody-blocking studies). A second more exotic possibility is that superantigens have usurped a physiologically important mechanism for T-cell activation that is used only rarely. For example, immature thymocytes express only low levels of the TCR and yet are remarkably sensitive to antigen. Perhaps there exists a cellular protein that links the TCRb chain with an MHC molecule as an adjunct to normal binding and recognition (174).

gd T CELLS

Characteristics of gd T Cells

Although ab T cells were originally defined on the basis of functional characteristics, such as providing T-cell “help” or initiating cytotoxicity, gd TCR-bearing cells were not discovered through any biologic assay, but instead first came to light through the accidental discovery of a TCRg cDNA clone. This led to the development of an antiserum with which Brenner et al. (32) discovered a unique subset of T cells, composing 5% to 10% of peripheral T cells in humans and mice. It has been 14 years since this discovery, and we are still trying to understand what these cells recognize and how they function within the immune system. There has been substantial progress, but these questions are still largely unresolved. We review here some of the salient characteristics of these enigmatic cells.

gd T cells are the first to appear in the fetal thymus, fully 2 days before ab T cells in the mouse, but in later weeks, ab T cells quickly predominate. In both mouse and human adults, gd T cells represent only a small fraction of thymocytes (175) and lymphocytes in all of the secondary lymphoid organs. However, they are found in larger numbers in the mucous membranes of a variety of tissues such as the skin (176), small intestine (177), female reproductive tract (178), and lung (179).

One population of gd T cells that has been studied intensively are the $CD4^-CD8^-$ gd T lymphocytes, which have a dendritic morphology and are embedded in the epidermis of the skin (176,180). These cells have been termed dendritic epidermal cells or DECs. Curiously, 90% of these cells express a TCR with identical Vg and Vd sequences (180).

As to what these DEC cells “see,” recent experiments have shown that they can respond to mouse keratinocytes or to an extract of keratinocytes added directly to the DECs (182). The nature of the determinant recognized is unknown, but it is significant that it is a self-protein, albeit one that is made only by “stressed” cells, supporting the proposal that this type of gd cells may serve as a kind of sentinel (184). The gd T cells found in the female reproductive epithelia and tongues of mice preferentially express V_g4 and V_d1 (178). In the BALB/c strain of mice, most of the TCRd sequences are the same (183), but others are diverse, and this phenomenon has not been seen in other strains.

Another population of gd T cells that has been studied extensively is resident in the epithelium of the small intestine (177). The gut intraepithelial lymphocytes (IELs) consist of a population of ab T cells and a population of gd T cells. They are phenotypically $CD4^-CD8^-$ or $CD4^-CD8^+$. Unlike $CD8^+$ab T cells, the CD8 molecules on gd IELs express the CD8a chain and no CD8b (184). IELgd TCRs use different V_g and V_d chains, and the CDR3 regions of both the g and d chains show significant diversity in both length and sequence, suggesting that they can “see” a wide variety of ligands.

Earlier studies showed that gd T cells can secrete a variety of lymphokines and mount cytolytic responses, and therefore have the potential to function as ab T cells. Their preferential localization in the epithelium also suggested that they may be responsible for a “first line of defense” (reviewed in 184). This hypothesis is supported by the increase of gd T lymphocytes seen early in infections by some bacteria and a virulent Sendai virus strain, before ab T-cell responses are observed (185,186). However, in other infection models, gd T cells accumulate within the inflammatory lesions late in the infection after the virus has been cleared (reviewed in 187), suggesting that they may be responding to cells that are damaged and/or stressed by the infection. Consistent with this is the demonstration that some gd T cells can kill virus-infected cells *in vitro* but that the recognition is not virus specific (188).

More recently, mice deficient in ab or gd T cells have been used to dissect the roles of these cells in the immune defense against intracellular pathogens (bacteria, protozoa, and viruses) (189,190,191 and 192). These T-cell deficiencies were induced either by the administration of an mAb against ab or gd T lymphocytes or by disruption of a TCR gene through homologous recombination. It was found the role(s) that gd T cells play is dependent on the types of infection. In models such as bacille Calmette-Guérin (BCG) and *Salmonella* infections, ab but not gd T cells are essential in controlling the infection. In other cases such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, gd T cells are able to compensate for the absence of ab T cells. Interestingly, in *Listeria* and *Vermiformis* infections, a lack of gd T cells does not change the pathogen load but instead results in a different pathology in the infected tissue (189,190,191 and 192). This has led to the suggestion that gd T cells may somehow regulate immune and nonimmune cells to maintain host tissue integrity (193). This possibility is supported by data showing that certain gd T cells can produce keratinocyte growth factor and chemokines (182), as well as regulate the development of epithelial cells (194) and influence ab T-cell responses (195,196,197 and 198). It also is compatible with the analysis of gd T-cell recognition requirements, in that these cells can mediate cellular immune functions without a requirement for antigen-processing and specialized antigen-presenting cells (reviewed in ref. 6). Therefore they have the capacity to initiate immune responses by recognizing other lymphoid cells or damaged cell/tissues directly. Whereas all these experiments point to a unique role for gd T cells in the immune system, gd T-cell specificity and their exact effector functions in any pathologic situation remains undefined. It is interesting to note that the function of gd T cells has been studied mainly in mouse and humans, yet they are significantly more abundant in birds and artiodactyls (175). This may be because they encompass other functions in these organisms or that species of this type have a greater need for gd T cells.

Antigen Recognition by gd T Cells Does Not Require Antigen Processing

During the past few years, a number of studies have shown that gd T cells have profound differences in their antigen-recognition requirements compared with ab T cells. Some gd T cells also seem to recognize entirely different types of antigens. More specifically, these experiments show that the many gd T cells do not recognize processed and presented proteins but rather intact ones, and that in some cases, they can recognize nonprotein entities (as reviewed in ref. 6). Examples of intact

protein recognition include the mouse class II MHC molecules IE^k by the T-cell clone LBK5 (199); the recognition of the mouse nonclassic MHC class I molecules T10 and T22 by the T cell clone G8 (204,205); and the recognition of a herpes simplex virus glycoprotein, gI, by the T-cell clone TgI4.4 (201). It is noteworthy that only the last of these is a foreign antigen. It also may be significant that epitope mapping with mutant IE molecules shows that amino acid residues in the a helices of the IEa and IEb chains that affect ab T-cell recognition do not affect gd stimulation (199).

Interestingly, T10 and the closely related T22 molecule (94% identity) were identified as the ligand of another gd KN6 (202,203). Whereas G8 was generated by immunizing BALB/c nude mice with B10.BR spleen cells, KN6 was derived from a double-negative C57BL/6 thymocyte. Thus these nonclassic class I molecules can be considered to be “natural” ligands for gd T cells. Neither is capable of binding peptides, as indicated by early biochemical studies (205) and most definitively by x-ray crystallographic analysis (206). It also was found recently that T10/T22 is inducibly expressed on activated lymphocytes (204), suggesting that gd T cells specific for it may serve autoregulatory or even suppressive functions (204).

gd T Cells Also Can Be Stimulated by Nonpeptide Antigens

gd T cells from healthy human peripheral blood and from patients with tuberculoid leprosy or rheumatoid arthritis were found to respond to heat-killed *Mycobacteria*. The major T-cell stimulatory components in the former are not the mycobacterial heat shock proteins, but instead have been identified to be phosphate-containing, nonpeptide molecules (207,208,209 and 210). Although the consensus is that “phosphate” is a necessary component, compounds identified from various laboratories with different mycobacteria-responsive clones appear to be distinctive in their structures (211). The nonphosphate moieties include unusual carbohydrate and phosphate moieties; a 5'-triphosphorylated thymidine or uridine substituted at its g-phosphate by an as yet to be characterized low-molecular-weight structure; isopentenyl pyrophosphate and related prenyl pyrophosphate derivatives, synthetic alkenyl and prenyl derivatives of phosphate, pyrophosphate as well as g-monoethyl derivatives of nucleoside, and deoxynucleoside triphosphates (210). Although the relative biologic importance of these compounds remains to be determined, it is clear that a major class of stimulants are phosphate-containing nonpeptides. It also is clear that multiple phosphate-containing compounds are able to stimulate different clones with different efficacies.

An important finding is that all of these compounds can be found in both microbial and mammalian cells. Constant et al. (208) proposed that the mammalian TTP-X and UTP-X conjugate may be involved in a “salvage pathway” in DNA and RNA synthesis, and thus could be involved in a metabolic pathway related to DNA or RNA synthesis, such as cell proliferation. Such a molecule would fit with the stress antigen or “conserved primitive stimulus” expected for gd T-cell ligands (184,211). Tanaka et al. (210) proposed that a link in the recognition of both microbial pathogens and hematopoietic tumor cells by these gd T cells is provided by the common set of prenyl pyrophosphate intermediates, isopentenyl and related prenyl pyrophosphate derivatives. These compounds are present in normal mammalian cells as precursors in lipid metabolism for the synthesis of farnesyl pyrophosphate. In mammalian cells, farnesyl addition has been proposed to be a critical modification for the membrane association of the ras protein and is required for its transforming activity. gd T-cells with this specificity accumulate in lesions caused by mycobacterial infections in humans (212,213) and are able to respond to virally and bacterially infected cells. Together with the data cited previously, the original sentinel hypothesis of Allison and Havren (211) may well be generalizable to all or most of the T cells carrying this receptor, and thus it may have a unique function with respect to recognizing gd cells response to a class of antigens shared by a number of pathogens and transformed, damaged, or stressed cells.

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5 T-CELL BIOLOGY AND THE THYMUS

Jonathan Sprent, M.D., Ph.D., and Charles D. Surh, Ph.D.

Like B cells, T cells are mobile cells that patrol the body in a highly disciplined fashion. In the absence of exogenous antigen, naive T cells remain in interphase and percolate through defined regions of the lymphoid tissues. They move constantly from blood to the spleen and lymph nodes and then back to blood through the lymphatic vessels. In the presence of exogenous antigen, however, antigen-reactive T cells cease their blood to lymph recirculation and become temporarily trapped in the lymphoid tissues, where they undergo extensive proliferation and differentiation into activated effector cells. These cells then move back into the circulation and disseminate throughout the body and mediate their various effector functions to eliminate the antigen. Most newly generated effector cells die rapidly, but a few of the responding cells survive to become long-lived memory cells.

This chapter provides a brief overview of the normal patterns of T-cell migration and the changes in migration that occur when T cells encounter antigen and differentiate into effector and memory cells. The decisive influence of the major histocompatibility complex (MHC) in controlling T-cell specificity and function is discussed, followed by a description of how the T-cell repertoire is selected in the thymus during early ontogeny.

SUBSETS OF T CELLS

T cells can be divided into subsets based on the expression of surface markers and antigen-specific receptors.

Lineages of T Cells

Typical T cells express antigen-specific heterodimers composed of a and b chains, each chain containing variable (V) and constant (C) regions (1,2,3,4 and 5). In the extrathymic environment, these ab T-cell receptors (TCRs) are found on two broad subsets of T cells distinguished by their mutually exclusive expression of CD4 and CD8 molecules (6,7 and 8). $CD4^+8^-$ (CD4) T cells generally outnumber $CD4^-8^+$ (CD8) cells by 2:1. In most species, ab TCR-bearing CD4 and CD8 T cells comprise more than 90% of the T cells found in the lymphoid tissues and in blood and lymph. The specificity of typical ab T cells is not directed to native antigens but to peptides bound to cell-associated MHC molecules (4,5). As discussed later, CD8 T cells recognize peptides bound to MHC class I (MHC I) molecules, whereas CD4 T cells bind peptide-MHC II complexes.

A few ab T cells show features of natural killer (NK) cells (9). These NK T cells have highly restricted TCR specificity and preferentially recognize lipids and glycolipids bound to MHC I–like CD1 molecules (10,11). Another small subset of T cells expresses gd TCR heterodimers (12,13); gd T cells tend to accumulate in nonlymphoid tissues and are mostly $CD4^-8^-$. Whereas ab T cells recognize “processed” antigens, the specificity of gd TCR molecules resembles that of immunoglobulin G (Ig) and is directed to a spectrum of native antigens. The biologic functions of ab NK T cells and gd T cells are still unclear and are not covered in this chapter.

Naive and Memory T Cells

Most ab T cells are small resting cells (8,14,15). These cells can remain in interphase for prolonged periods and do not require constant replenishment from the thymus. Typical naive resting T cells characteristically express high-molecular-weight isoforms of the CD45 molecule (e.g., CD45RA) (16,17,18 and 19). Resting T cells also express high levels of the lymph node homing receptor, CD62L (see later); certain other surface molecules, such as CD44, are expressed at low levels (14,15). Activated T cells have a different phenotype. These cells show diminished expression of high-molecular-weight CD45 molecules and instead express a low-molecular-weight isoform termed CD45RO. Activated T cells downregulate expression of CD62L but upregulate CD44 expression. Activated T cells also show upregulation of other markers. Many of the markers on activated T cells are also expressed on memory T cells, that is, the cells controlling responses to recall antigens (16,19,20). As implied by their phenotype, memory T cells appear to remain in a state of chronic or partial activation and undergo a much faster turnover than typical naive T cells (15,21). However, some memory cells eventually revert to resting cells and reacquire some of the phenotypic characteristics of naive T cells.

Although naive T cells rarely divide, survival of these cells requires continuous TCR contact with self-MHC–peptide complexes (8,22). TCR signaling is less important for the survival of memory T cells (23,24 and 25). For these cells, their persistence and rapid turnover may be controlled largely by cytokines.

Other Subsets of T Cells

Some T cells are known to suppress the functions of other T cells, and there are many claims that “suppressor” T cells represent a separate lineage (26,27). Unequivocal evidence on this issue is lacking, however, and the current tendency is to explain T-cell suppression in terms of the activity of cytotoxic lymphocytes or the release of inhibitory lymphokines. With regard to lymphokines, accumulating evidence indicates that ab T cells display considerable heterogeneity in terms of their patterns of lymphokine release (28,29). As discussed later, the conditions of T-cell priming can have a decisive influence on the subsequent patterns of lymphokine production by primed T cells.

TISSUE DISTRIBUTION AND MIGRATORY PROPERTIES OF T CELLS

Typical CD4 and CD8 ab T cells are found at their highest concentration in the circulation (blood and lymph) and in the spleen and lymph nodes (14,21,30,31). These T cells also accumulate in certain other lymphoid organs, such as Peyer patches and tonsils. In each tissue, T cells are situated in clearly defined microenvironments, termed T-dependent areas. As discussed later, T-cell migration to spleen and lymph nodes is different (Fig. 5.1 and Fig. 5.2).

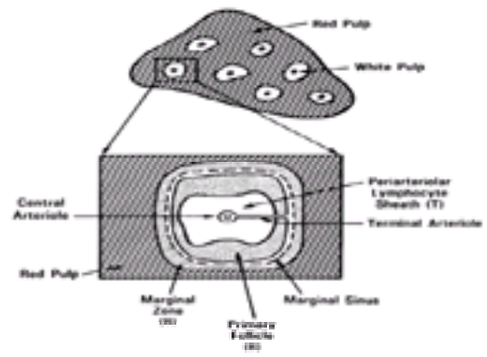

Figure 5.1. Microanatomy of the spleen. T cells are concentrated in periarteriolar sheaths (PALS) in the white pulp. T cells reach this region from the marginal sinus, which receives T and B cells as well as other blood components from the terminal arterioles that branch from the central arterioles. B cells also move from the marginal sinus to the PALS but then localize in the outer portions of the white pulp, that is, in primary follicles and germinal centers (the latter are not shown). B cells also localize in the marginal zone (which forms a layer of cells between the marginal sinus and the red pulp). After passing through the white pulp, T and B cells leave the spleen by percolating through the loose network of sinusoids in the red pulp to reach the tributaries of the splenic vein. Exit through lymphatic channels is limited in most species. (From J Sprent. T lymphocytes and the thymus. In: WE Paul, ed. *Fundamental immunology,* 3rd ed. New York: Raven, 1993:75–109, with permission.)

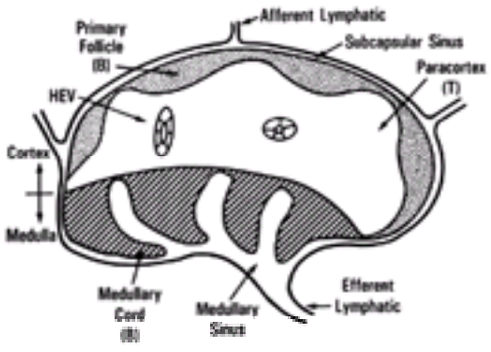

Figure 5.2. Microanatomy of a lymph node (131). T and B cells enter the lymph node (LN) by penetrating the walls of HEV to reach the paracortex. B cells pass through the paracortex to reach primary follicles and germinal centers (not shown) in the outer cortex. In the medulla the medullary cords are packed with plasma cells (i.e., the differentiated progeny of small B lymphocytes). T and B cells leave the LN by moving from the cortex to the medulla to enter the medullary sinus and then the efferent lymphatic vessel. Afferent lymphatic vessels contain macrophages (Mf) and dendritic cells (DC) but few T and B cells (except when the afferent lymphatics receive cells from efferent lymphatics of other LN). (From J Sprent. T lymphocytes and the thymus. In: WE Paul, ed. *Fundamental immunology,* 3rd ed. New York: Raven, 1993:75–109, with permission.)

Spleen

The spleen is partitioned into two distinct types of tissue: red pulp and white pulp. The red pulp contains a full spectrum of lymphohemopoietic cells, including neutrophils and erythrocytes, and the white pulp consists predominantly of lymphocytes. T cells (including both CD4 and CD8 cells) are concentrated in the central regions of the white pulp and form a sheath around arterioles. These periarteriolar lymphocyte sheaths (PALSs) constitute the T-dependent region of the spleen and contain a dense network of interdigitating dendritic cells. As discussed later, dendritic cells play a vital role in presenting antigen to T cells during immune responses.

T-cell entry to the spleen is largely nonspecific. There are no direct anastomoses between the arterial and venous systems in the spleen, and all blood-borne cells enter the spleen at the same site, namely, the marginal sinuses at the junction between the red and white pulp. Whereas most cells are carried directly to the red pulp for eventual exit through the splenic vein, lymphocytes move from the marginal sinuses into the white pulp. B cells migrate to the peripheral regions of the white pulp, and T cells accumulate in the PALSs. After several hours, T cells move back into the red pulp and leave the spleen through the splenic vein.

Lymph Nodes

In marked contrast to the spleen, the arteries and veins of lymph nodes are directly connected through capillaries. Most blood-borne cells are swept rapidly through lymph nodes through capillaries and fail to enter the lymphoid tissue itself. Lymphocytes are the exception. As a reflection of their expression of specific “homing” receptors, lymphocytes can bind to a unique type of venule characterized by a lining of plump endothelial cells; these high endothelial venules (HEVs) are also found in certain other lymphoid organs (e.g., Peyer patches) but are not present in the spleen or in nonlymphoid organs. Through CD62L and other homing receptors, T cells make contact with complementary molecules on the luminal surface of HEVs and then squeeze between the endothelial cells to enter the central region of lymph nodes, the paracortex. B cells move rapidly to follicles under the capsule, whereas T cells remain in the paracortex. This region, the T-dependent area of lymph nodes, resembles the PALS of the splenic white pulp in that it contains a dense network of interdigitating dendritic cells. T cells remain in the paracortex for several hours and then make their way out of lymph nodes by migrating to the large efferent lymphatic vessel in the medulla; in contrast to the spleen, T-cell exit from lymph nodes through the venous system is minimal. Efferent lymphatic vessels drain into the thoracic duct. This vessel terminates in the left subclavian vein in the neck and thus allows lymph-borne cells to reenter the circulation.

Whereas efferent lymphatic vessels contain large numbers of T cells (and B cells), afferent lymphatics contain few lymphocytes (unless these afferent lymphatics are tributaries of efferent lymphatics draining from other lymph nodes). Afferent lymph draining the skin, muscle, and so on is almost devoid of the typical ab CD4 and CD8 resting T cells that predominate in efferent lymph. Most of the few T cells found in afferent lymph are a mixture of gd T cells and memory ab T cells. Afferent lymph also contains immature dendritic cells; these cells lodge in the paracortex and are important for transporting antigen into lymph nodes from nonlymphoid tissues (32,33).

Recirculation of T Cells

The capacity of lymphocytes to migrate from blood to efferent lymph through HEVs is termed recirculation (30,34,35). In animal models, blood to lymph recirculation can be demonstrated by injecting lymphocytes intravenously and by monitoring the appearance of these cells in a cannula placed in the thoracic duct. Typical T cells begin to appear in thoracic duct lymph at about 6 hours after intravenous injection and reach peak levels at 18 hours. This prolonged transit time (which is even longer for B cells) is partly a reflection of slow blood-to-blood migration of T cells through the spleen. Because the spleen has a rich blood supply, a high proportion (about 40%) of T cells injected intravenously lodge initially in the spleen before reaching lymph nodes. These cells leave the spleen after about 6 hours and then gradually accumulate in lymph nodes through arterial blood.

By allowing lymphocytes to penetrate the walls of HEVs, the homing receptors on T cells play a critical role in recirculation. Resting T cells express at least two types of HEV homing receptor (14,36,37). In the mouse, the first receptor is CD62L, which has binding specificity for a complementary molecule on HEVs termed peripheral lymph node addressin (PNAd). The second homing receptor is the integrin a_4b_7, which binds to a different ligand on HEV called mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Homing of T cells into peripheral lymph nodes is mainly mediated by binding of CD62L to PNAd. Migration of T cells into mesenteric lymph nodes and Peyer patches, by contrast, occurs through interactions of either CD62L or a_4b_7 with their respective ligands on HEV; entry into Peyer patches is more efficient with a_4b_7 than CD62L.

Recirculation of T cells is also controlled by a class of small soluble proteins termed chemokines (CCs) (38,39 and 40). CCs are chemoattractive molecules released by endothelial and stromal cells and direct movement of all types of hemopoetic cells by forming a gradient to which the cells respond using their chemokine receptors (CCRs) on the cell surface. For HEV, these vessels release a specific CC, termed secondary lymphoid tissue chemokine (SLC), which attracts lymphocytes and facilitates migration of these cells through HEV. Within lymphoid tissues, CCs also direct movement of lymphocytes to specific microenvironments. Thus, T cells find their way to the T-cell zone with the aid of CCR7 receptors that bind SLC and also another CC, Epstein-Barr virus–induced chemokine (ELC), released by resident stromal cells. CCR7 is also expressed on dendritic cells, and, through SLC and ELC, this receptor allows dendritic cells to enter the T-cell zone. Other CCs enable B

cells to move from the T-cell zone into primary follicles.

After contact with antigen, T cells downregulate CD62L and alter their pattern of CCR expression. As a result, activated T cells lose the capacity to enter peripheral lymph nodes through HEVs. Entry to Peyer patches is retained, presumably through the use of a_4b_7, which allows activated T cells to continue to recirculate. Activated T cells also appear to recirculate by an indirect pathway that involves migration through nonlymphoid tissues followed by entry into afferent lymphatics (36,41); the cells then pass through lymph nodes to reach efferent lymph.

Much less is known about the migratory properties of gd T cells. These cells do recirculate from blood to lymph through lymph nodes but, like activated ab T cells, entry to lymph nodes seems to be predominantly through afferent lymphatic vessels rather than through HEV (36,41). The homing receptors on gd cells have yet to be characterized.

RESPONSE OF MATURE T CELLS TO ANTIGEN

In discussing how typical T cells respond to antigen, it is essential to emphasize that ab T-cell specificity and function are under the strict control of MHC molecules (31,42,43). As mentioned earlier, the specificity of ab T cells is directed to small fragments (peptides) of protein antigens held in a groove on cell-bound MHC molecules (4,5).

Cell-surface MHC molecules normally express a variety of self-peptides derived from breakdown of intracellular proteins. As the result of selective events governing their initial formation in the thymus, mature T cells are unresponsive to these MHC–self-peptide complexes and thus display self-tolerance. When self-peptides are replaced by foreign peptides, however, a few T cells bind to these foreign peptide-MHC complexes with high affinity and are triggered.

At the level of naive resting T cells, triggering of T cells by foreign peptides requires contact with MHC-peptide complexes expressed on the surface of specialized antigen-presenting cells (APCs), particularly dendritic cells and macrophages (33,44,45). As discussed in other chapters, these and other APCs degrade antigens into peptides intracellularly and load these peptides onto MHC I and II molecules. MHC molecules plus bound peptides are then transported to the cell surface. Loading of peptides on to MHC I and II molecules involves different mechanisms (44,46,47,48,49,50 and 51). Peptide binding to class II molecules generally involves an “external” pathway of antigen processing whereby exogenous antigens are ingested and then are degraded into peptides in endosomes; these peptides are then bound by the class II molecules in endosomes and are ferried to the cell surface for recognition by $CD4^+$ T cells. For binding of peptides to MHC I molecules, most of the peptides are generated by an “internal” pathway whereby intracellular antigens (e.g., antigens synthesized by infectious viruses) are conveyed to the endoplasmic reticulum and the Golgi network. Here, peptides are bound to class I molecules, which are then transported to the cell surface and are recognized by CD8 T cells. One subset of APC has the ability to process and present exogenous antigens on class I molecules and to activate $CD8^+$ cells. This process of “cross-priming” is well documented, but the identity of the APC and the exact mechanisms of antigen processing involved are still unclear (52).

Distribution of Antigen-Presenting Cells *In Vivo*

Although antigen processing and peptide loading of MHC molecules has been studied extensively *in vitro,* much less is known about antigen processing *in vivo.* In particular, the relative contributions of macrophages and dendritic cells to APC function *in vivo* are still debated. Dendritic cells are found in the T-dependent areas of the lymphoid tissues and are thus strategically positioned to present antigen to recirculating T cells (32,33,53). Nevertheless, typical dendritic cells are poorly phagocytic and are therefore unsuited to degrading exogenous native antigens into peptides. How then do dendritic cells present antigen? The resolution to this problem seems to be that, at least in lymph nodes, the dendritic cells found in the T-dependent areas arise from a precursor population in the skin and other nonlymphoid tissues. These immature dendritic cells, termed Langerhans cells in the skin, are actively phagocytic but are not capable of stimulating T cells. Uptake of microbial antigens and exposure to inflammatory products, however, induce migration of immature dendritic cells to lymph nodes through afferent lymphatics; en route, the cells differentiate into mature dendritic cells. During this stage, the ingested antigens are degraded into immunogenic MHC-bound peptides, and the cells become potent stimulators of naive T cells by upregulating the expression of “costimulatory” molecules such as B7 and also by acquiring the ability to synthesize certain cytokines.

Whether this situation is applicable to other lymphoid organs, such as the spleen, is still unclear. In addition to mature dendritic cells in the white pulp, the spleen has a dense network of macrophages in the marginal sinuses, and it is likely that these cells, which are strongly phagocytic, play a major role in degrading large particulate antigens for presentation to T cells (44,54). Macrophages are found in other lymphoid organs, such as in the subcapsular sinus of lymph nodes, and are also scattered throughout nonlymphoid tissues. The role of macrophages and blood monocytes in antigen presentation to T cells is controversial. When tested directly *ex vivo,* macrophages generally display poor APC function for naive T cells. However, after ingestion of particulate antigens *in vitro* and subcutaneous injection *in vivo,* monocytes migrate into the T-cell areas of the draining lymph nodes and express dendritic cell markers (55). Hence, some monocytes behave functionally as dendritic cell precursors. Indeed, many of the cells typed as macrophages in the marginal zone of the spleen can migrate to the white pulp after exposure to inflammatory stimuli and differentiate rapidly into mature dendritic cells (56). These and other data suggest that macrophage-monocytes and dendritic cell precursors are closely related and perhaps synonymous.

The wide distribution of dendritic cells throughout the body is often taken to signify that T cells can be stimulated by antigen in nonlymphoid tissues. For naive T cells, this possibility is unlikely because, as discussed earlier, typical unprimed T cells remain strictly within the confines of the recirculating lymphocyte pool. Moreover, only mature dendritic cells, that is, the cells found in the T-cell zones of lymphoid tissues, are strongly immunogenic for naive T cells.

T-Cell Recognition of Antigen: Receptor-Ligand Interactions

The receptor-ligand interactions involved in T-cell recognition of antigen are discussed in detail in other chapters. In brief, MHC-peptide complexes on APCs are recognized by two molecules on T cells: the ab TCR and either CD4 or CD8 molecules (7,8,31). The latter molecules act as coreceptors and have binding specificity for nonpolymorphic, class-specific epitopes on the membrane proximal domains of MHC molecules: CD4 molecules bind to class II MHC molecules, whereas CD8 molecules bind to class I molecules. T cells need to make contact with costimulatory molecules, notably B7 (B7-1, B7-2), and various adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), on APCs (57). These molecules are recognized by complementary molecules on T cells, that is, CD28 for B7 and LFA-1 for ICAM-1. These interactions stabilize TCR-peptide-MHC binding and intensify T-cell triggering. For CD28/B7 interaction, ligation of CD28 delivers a “costimulatory” signal to T cells that is crucial for synthesis of cytokines, notably interleukin-2 (IL-2).

T-Cell Triggering

Although T-cell triggering is initiated by cross-linking of TCR molecules, the intracytoplasmic regions of the TCR are too short to mediate signal transduction. Triggering appears to depend on the formation of complexes between at least four sets of cell-surface molecules: (a) TCR, (b) CD4 or CD8, (c) heterodimers formed from the three chains (gde) of the CD3 family, and (d) heterodimers or homodimers of zh or zz chains (58,59 and 60). Triggering involves the actions of several protein tyrosine kinases (PTKs) that activate proximal signaling molecules through phosphorylation; this process subsequently leads to activation of multiple downstream pathways through key adapter proteins. The proximal signal after TCR cross-linking is initiated by $p56^{lck}$, a Src-family PTK that associates with CD4 and CD8 molecules and phosphorylates CD3z chains. ZAP-70, a Syk-family PTK, then binds to CD3z chains and phosphorylates key adapter proteins, LAT and SLP-76, which, in turn, recruit several other crucial signaling molecules and initiate a cascade of signaling events that ultimately turn on multiple downstream events including phosphoinositide hydrolysis, calcium mobilization, and activation of Ras signaling pathways; T-cell triggering is also influenced by a tyrosine phosphatase associated with the CD45 molecule. Downstream signals are then transmitted to the nucleus to initiate gene transcription, which leads to cell division and synthesis of various cytokines and receptors for these cytokines.

T-cell–derived cytokines, also termed lymphokines or interleukins, play a vital role in promoting and controlling T-cell proliferation and, for CD8 cells, differentiation into cytotoxic cells (28,29,61,62,63 and 64). IL-2 is of particular importance and is synthesized rapidly when naive T cells respond to antigen. IL-2 production is conspicuous for CD4 cells but is less prominent for CD8 cells. With strong TCR stimulation, CD8 T cells can produce their own IL-2 and are termed helper-independent cells; with weaker stimulation, CD8 cells produce little IL-2, and these helper-dependent cells proliferate only in the presence of exogenous IL-2 produced by other cells, notably CD4 cells.

In addition to IL-2, triggering of naive T cells causes synthesis of a variety of other lymphokines, such as interferon-g (IFN-g), IL-4, and IL-10. CD4 T cells use these and other lymphokines to mediate numerous effector functions, such as delayed-type hypersensitivity and interaction with B cells leading to antibody production. Cytokines, notably IFN-g, also augment the capacity of CD8 cells to destroy target cells, such as virus-infected cells. The precise spectrum of lymphokines produced by T cells appears to depend on their stage of differentiation and the conditions encountered during interaction with APC. Naive T cells have the potential to produce many different lymphokines, but primed or memory T cells often show a restricted pattern of lymphokine release. Thus, with APCs releasing IL-12 and IFN-g, activation of CD4 cells leads to generation of type 1 T-helper (Th1) cells producing large amounts of IL-2 and IFN-g but little IL-4 or IL-10. Conversely, exposure to IL-4 by neighboring T cells favors the production of Th2 cells, which are cells showing a reciprocal pattern of lymphokine release. Although the precise factors controlling the differentiation of T cells into Th1 and Th2 cells are still not clearly defined, the changes induced in T cells are often irreversible and reflect expression of unique

transcription factors and loss of certain cytokine receptors.

T-Cell Responses to Antigen *In Vivo*

As discussed earlier, unprimed T cells reside in the recirculating lymphocyte pool and move continuously from one lymphoid organ to another. When foreign antigen enters the body, confrontation with MHC-peptide complexes displayed on APCs in the T-dependent areas of the lymphoid tissues causes antigen-specific T cells to be withdrawn from the recirculating pool (30,65). T cells bind to APCs and are triggered to proliferate. After 2 to 3 days, the progeny of the proliferating cells leaves the lymphoid tissues through efferent lymphatics (for lymph nodes) or venous blood (for spleen) and reenters the circulation as effector cells.

This process of antigen-specific trapping of T cells followed by clonal expansion and reentry into the circulation is most easily visualized when the precursor frequency of T cells is high. For conventional antigens and peptides, the precursor frequency of naive T cells for antigen is low, less than 1×10^4. A different situation applies to MHC alloantigens. Whereas T cells display self-tolerance to autologous MHC molecules (plus the endogenous self-peptides on these molecules), T cells are strongly reactive to foreign MHC molecules. Thus, when T cells are exposed to APCs from an MHC-different individual, as many as 5% to 10% of unprimed T cells display overt reactivity to these alloantigens and enter cell division (30). Under *in vitro* conditions, this response constitutes a mixed-lymphocyte reaction and generates large numbers of blast cells. Similar findings apply *in vivo* (30,35,66) (Table 5.1).

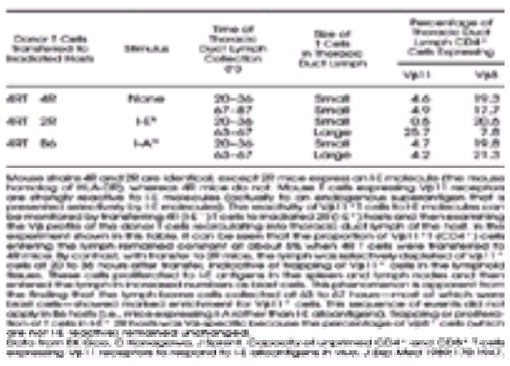

TABLE 5.1. Trapping and Proliferation of Alloreactive T Cells in Vivo Revealed with Vb-Specific Antibody

Hence if T cells from a mouse of MHC^a genotype are injected into an MHC^b mouse, the donor T cells with MHC^b specificity mount a vigorous immune response to the MHC^b-bearing APCs of the host. In this graft-versus-host reaction, the responding T cells, which include both CD4 and CD8 cells, are initially trapped in the T-dependent areas and begin to proliferate extensively. During this period of trapping, the donor-derived T cells recirculating into thoracic duct lymph are selectively depleted of host MHC^b-reactive T cells. After about 3 days, host-specific T cells appear in the lymph as blast cells, and by 4 to 5 days, these blast cells constitute a high proportion of the lymph-borne cells. Indeed, when the host is exposed to irradiation before T-cell injection (which destroys host T cells but spares host APCs), donor-derived host-specific blast cells can account for more than 90% of total lymph-borne cells.

Essentially similar findings apply when T cells respond to conventional antigens, such as to a virus infecting the respiratory system. In this situation, dendritic cell precursors transport viral antigens from the site of infection to the draining lymph nodes. Recirculating T cells encounter viral peptides on mature dendritic cells in the T-cell zones of lymph nodes and then proliferate extensively before reentering the circulation through efferent lymphatics. Because the precursor frequency of naive T cells for viral antigens is low, it is difficult to trace the distribution of the responding T cells in the early stages of the primary response. After clonal expansion of the responding cells, however, tracing the cells is relatively easy, especially with the development of the tetramer approach for detecting individual antigen-specific T cells (67,68 and 69); with this technique, specific T cells can be detected on the basis of their capacity to bind tetramers of recombinant MHC molecules loaded with defined peptides. The tetramer approach has shown that, during viral infections, specific $CD8^+$ T cells divide rapidly (every 4 to 6 hours) and can undergo a 10^4-fold expansion during the first week of infection (69). As the result of this massive proliferation, the precursor frequency of the responding cells increases from undetectable levels to 50% or more of total T cells. These cells enter the circulation as effector blast cells.

The entry of blast cells into the circulation after trapping and proliferation in the lymphoid tissues serves to disseminate effector T cells throughout the body. These cells migrate from the circulation to the original sites of infection, and there the cells destroy infected target cells. Because nonlymphoid tissues are outside the confines of the recirculating lymphocyte pool, entry of effector cells into these sites necessitates a radical change in their migratory properties. Although the mechanism of homing of activated T cells to nonlymphoid tissues is not fully understood, this process appears to involve downregulation of lymph node homing receptors and upregulation of a series of other receptors, which allow T cells to penetrate the walls of typical small capillaries and to percolate through the extracellular matrix to make contact with stromal cells such as epithelial cells (37,40,70).

Elimination of viruses (and other pathogenic organisms) also involves specific antibody. Production of antivirus antibody is initiated by contact of CD4 cells with APCs expressing viral peptides bound to class II MHC molecules. After activation and proliferation in the draining lymph nodes, virus-specific CD4 cells move from the T-dependent areas and make contact with antigen-specific B cells in adjacent germinal centers. Describing the complex events involved in T-cell–B-cell collaboration (71,72,73 and 74) is beyond the scope of this chapter. In brief, B cells bind native protein molecules (e.g., viral proteins) through their specific immunoglobulin receptors. After internalization, the protein is degraded into peptides, some of which associate with class II MHC molecules in endosomes. These peptide-MHC complexes then move to the cell surface. Some of these peptides correspond to the peptides seen by CD4 cells on APCs during initial T-cell activation. Recognition of these peptides on the surface of B cells by CD4 cells results in the delivery of a “helper” signal to the B cells. Under the influence of T-cell–derived lymphokines such as IL-4 and T-cell expression of CD40L, a receptor for B-cell CD40 molecules, B cells then differentiate into antibody-forming cells. The haptenic determinants on the native protein antigen recognized by antibody molecules (and the immunoglobulin receptor on the precursor B cells) are unrelated to the particular breakdown product (peptide) seen by the Th cell. Nevertheless, T-cell–B-cell collaboration depends critically on physical linking of the two epitopes before degradation in the B cell.

Most typical immune responses involve the simultaneous production of CD4 and CD8 effector T cells and the release of specific antibody (31,42). Such combined cellular and humoral immunity is the most effective method for eliminating pathogens. Although the manifestations of cellular immunity by CD4 and CD8 effector cells are often highly complex, virtually all forms of cellular immunity involve the generation of effector cells at sites of antigen concentration, usually the draining lymph nodes, followed by migration of effector cells to the site of infection. Effector cells then destroy pathogen-infected cells by a combination of direct cell-mediated lympholysis and local release of lymphokines. Although the effector functions of CD4 and CD8 cells are broadly overlapping, CD8 cells typically mediate cell-mediated lympholysis, whereas CD4 cells control lymphokine release. In the case of CD4 cells, cellular immunity is principally under the control of Th1 effector cells (29,64,75,76). These cells produce delayed-type hypersensitivity reactions and typically release large quantities of IFN-g.

The ratio of Th1 to Th2 cells can have a major influence on the efficiency of the destruction of pathogen-infected cells. In particular, an excess of Th2 cells can severely inhibit cellular immunity, presumably because IL-10 production by Th2 cells is suppressive for Th1 cells. This situation is illustrated by experimental leishmaniasis, in which, in certain strains of mice, an overabundance of Th2 cells prevents a delayed-type hypersensitivity reaction to *Leishmania*-infected cells.

Although effector T cells can elicit nonspecific (“bystander”) damage through release of lymphokines, the interaction of effector T cells with antigen-bearing target cells is MHC restricted. In fact the MHC-restricted specificity of T cells applies throughout the life history of these cells, even during the stage of initial T-cell formation in the thymus (see later).

T-CELL MEMORY

A characteristic feature of the primary immune response is that, after the infectious agent is cleared, most effector T cells are destroyed by apoptosis (77,78 and 79). Elimination of effector cells involves several mechanisms, including loss of contact with life-sustaining cytokines and delivery of death signals, such as by ligation of cell-surface Fas by a receptor, FasL expressed on other cells (78,80,81). Teleologically, destroying effector cells at the end of the immune response makes sense: the cells are no longer useful, and their long-term survival would clutter the lymphoid tissues.

Despite the widescale death of T cells at the end of the primary response, some of the responding T cells survive as long-term memory cells (16,19,20,82). Immunologic memory is often lifelong, and secondary contact with the pathogen concerned leads to a much more intense T-cell response than is seen during primary infection. The intensity of the secondary response reflects both a higher precursor frequency of T cells and the fact that primed (memory) T cells are more sensitive to

antigen than naive T cells.

The factors controlling the long-term survival of memory cells are controversial (16,19,20,82). Some workers argue that memory cells need to be maintained in a state of chronic activation through contact with residual depots of the primary antigen or by cross-reactive specificity for environmental antigens. However, in animal models, it is now apparent that memory cells survive well in MHC^{-} hosts, that is, where TCR ligation is precluded. As discussed earlier, another possibility is that memory cells receive life-sustaining signals through contact with certain cytokines.

T-CELL SELECTION IN THE THYMUS

Most mature ab (and gd) T cells arise in the thymus (7,83,84,85 and 86), as is apparent from the finding that congenitally athymic (nude) rodents and patients with congenital thymic aplasia (Digeorge syndrome) are almost devoid of mature T cells. Likewise, T cells fail to develop when the thymus is removed early during ontogeny.

The release of T cells from the thymus is maximal during young life and then decreases markedly after the onset of puberty (87,88). T-cell production by the thymus is roughly proportional to the size of the thymus. Although during young life the thymus is a massive organ, after puberty it shrinks progressively through the action of sex hormones. At least in rodents, however, the thymus remains functional until late adult life, as evidenced by the effects of exposing mice to whole-body irradiation, which destroys T cells and other lymphohemopoietic cells. When irradiation is followed by reconstitution with stem cells (bone marrow or fetal liver cells), young adult mice show full restoration in T-cell numbers within 2 months. With old mice, the replenishment of T cells occurs much more slowly but is eventually substantial, provided the mouse has a thymus. When the thymus is removed before irradiation and stem cell reconstitution, the T-cell pool remains severely depleted almost indefinitely.

Removing the thymus in adults without irradiation does not cause a rapid decrease in T-cell numbers. Such a finding is sometimes taken to indicate that T cells in adults arise primarily by an extrathymic pathway. This possibility belies the effects of thymectomy discussed earlier. In rodents, most researchers accept that extrathymic differentiation of T cells, although significant, is limited. The most likely explanation for the persistence of T cells after thymectomy in adults is that, once formed, the mature T-cell pool in largely self-sufficient, and most T cells at a population level are extremely long-lived. The mature T-cell pool is also regulated by poorly understood homeostatic mechanisms that maintain the total size at a near constant level.

In humans, the thymus is functional well before birth. Removing the thymus after birth (e.g., during cardiac surgery in infants) has surprisingly little effect on T-cell numbers and function, presumably because the thymus has already generated large numbers of mature T cells. The same finding applies in mice. In this species, the thymus does not begin to export T cells until around the day of birth. Removing the thymus at this time results in marked and permanent T lymphocytopenia. Thymectomy at 1 week after birth, however, generally causes only minimal T lymphocytopenia, a finding reflecting the substantial output of T cells from the thymus during the first week of life followed by homeostatic expansion of these cells.

Although the rate of production of T cells in the thymus is enormous, relatively small numbers of these cells are released to the extrathymic environment (87,88). In mice, the young thymus produces approximately 4×10^7 T cells per day. Yet the maximal release of T cells from the thymus is fewer than 2×10^6 cells per day. One is thus confronted with the paradox that the thymus produces huge numbers of young T cells and then destroys most of them, apparently *in situ.* At face value, then, T-cell production in the thymus seems to be inefficient. It is now clear, however, that the wide-scale destruction of T cells in the thymus is a reflection of a rigorous process of positive and negative selection whereby only those T cells with the requisite fine specificity to function in the extrathymic environment are allowed to exit to the periphery. Few T cells meet this criterion. The rest are destroyed *in situ.*

To understand T-cell selection in the thymus, it is important to reconsider the specificity of mature T cells. As discussed earlier, T-cell specificity is directed to foreign antigens (peptides) bound to MHC molecules. MHC molecules are highly polymorphic, and in an outbred population each individual generally displays a unique set of these molecules (the chance that any two individuals will express an identical set of MHC molecules is slight). The key point is that the particular MHC molecules expressed in the individual play a decisive role in shaping the T-cell repertoire (42,65,89). Thus, in each individual, the specificity of mature T cells is directed to recognition of foreign antigens presented by the particular self-MHC molecules expressed in the individual. Presentation of antigen by nonself-MHC molecules is much less effective.

Given the enormous polymorphism of MHC molecules, how does the thymus selectively export a T-cell repertoire that is skewed toward recognition of antigen presented by self-MHC molecules rather than by nonself-MHC molecules? Based on studies with bone marrow chimeras and TCR transgenic mice (31,90), the answer seems to be that T-cell confrontation with polymorphic residues on MHC molecules (plus bound endogenous self-peptides) on thymic epithelial cells selects for T cells displaying low but significant reactivity for these ligands (84,90,91,92,93,94 and 95). The level of “physiologic” specificity for self-MHC molecules is delicately balanced. After export from the thymus, T cells are unresponsive to self-MHC molecules (plus bound self-peptides) in the absence of exogenous antigen and thus display self-tolerance. Nevertheless, despite this lack of overt reactivity to self-MHC molecules, postthymic T cells retain *covert* reactivity to these molecules. This specificity is manifested by the strong reactivity of mature T cells to slight perturbations of self-MHC molecules (“altered self” determinants); these epitopes are created when self-peptides are displaced by foreign peptides. As discussed earlier, covert reactivity of T cells for self-MHC–self-peptide complexes is also important for maintaining T-cell survival, presumably by induction of continuous low-level TCR signaling.

As discussed later, T-cell selection in the thymus involves three distinct steps. The first step entails generation of large numbers of immature T cells with potential reactivity for all the various MHC molecules expressed in the species as a whole; this early stage of thymocyte differentiation is independent of MHC. The second step, positive selection, involves screening the immature T-cell repertoire for cells expressing some degree of reactivity for the self-MHC–peptide complexes displayed in the thymus. Positively selected T cells are then subjected to negative selection. This step involves self-tolerance induction and results in the destruction of T cells expressing overt reactivity for self-MHC–peptide ligands. To avoid confusion, it should be stressed that most (more than 95%) T cells differentiating in the thymus are ab T cells; thymic selection of gd T cells is still poorly understood and is not discussed here.

Early Differentiation of Thymocytes

The thymus is divided into two broad areas, the cortex and the medulla (84,85,96,97 and 98). Mature T cells occupy the medulla, whereas immature T cells (which comprise 85% to 90% of thymocytes) reside in the cortex. All thymocytes arise from a minority population of stem cells lacking CD4 and CD8 expression. These $CD4^{-}8^{-}$ cells are the descendants of blood-borne stem cells and form a thin rim under the thymic capsule. Differentiation of $CD4^{-}8^{-}$ cells is controlled by the rearrangement of TCR genes. Rearrangement of the TCR b chain allows $CD4^{-}8^{-}$ cells to undergo extensive proliferation and expression of the pre-TCR, a dimer of the TCR b chain and a surrogate TCR a chain that is expressed without gene rearrangement. Replacing the surrogate a chain with a rearranged conventional TCR a chain causes the cells to express both CD4 and CD8 molecules and to cease to proliferate. These “double-positive” ($CD4^{+}8^{+}$) thymocytes express low levels of mature ab TCR heterodimers on the cell surface but live for only 3 to 4 days unless they are rescued by positive selection.

Positive Selection

Positive selection involves TCR-mediated interaction of $CD4^{+}8^{+}$ thymocytes with self-MHC–peptide complexes expressed on cortical epithelial cells (7,84,85,99,100,101 and 102); these cells, which form a dense network throughout the cortex, express both MHC I and II molecules. When the affinity of TCR-MHC interaction is higher than a certain threshold, $CD4^{+}8^{+}$ T cells receive a protective signal that enables the cell to survive and to differentiate into mature T cells; this process allows the selective survival of T cells displaying physiologic specificity for self-MHC–peptide complexes. Positive selection reflects significant, but probably low-level, TCR signaling and is directed largely to weak (antagonist) peptides. Most thymocytes have negligible specificity for the particular MHC-peptide complexes expressed in the thymus. These T cells fail to receive a protective signal from cortical epithelium and die rapidly by apoptosis; this process is viewed as death by neglect (Fig. 5.3).

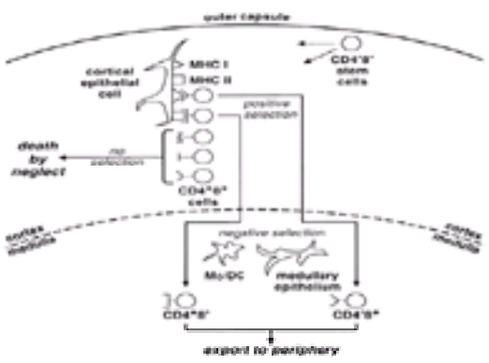

Figure 5.3. Model for positive and negative selection of T cells in the thymus. As discussed in the text, small numbers of $CD4^{-}8^{-}$ stem cells under the outer capsule of the cortex proliferate extensively and generate large numbers of immature $CD4^{+}8^{8+}$ cortical thymocytes. These cells are screened for reactivity to self-MHC loaded with self-peptides through exposure to the class I and class II molecules expressed on cortical epithelium. T-cell receptor (TCR) recognition of polymorphic epitopes of class I and class II molecules loaded with self-peptides is aided by CD8 and CD4 molecules, respectively; these molecules act as coreceptors. T cells able to bind to MHC-peptide complexes on epithelial cells receive a protective signal that enables the cells to survive and move to the medulla. These positively selected T cells downregulate the unwanted coreceptor (CD4 or CD8) and upregulate TCR expression to become typical single-positive $CD4^{+}8^{8-}$ and $CD4^{-}8^{+}$ cells. In the medulla and the corticomedullary junction, $CD4^{+}8^{-}$ and $CD4^{-}8^{+}$ cells are screened for overt reactivity to MHC-peptide complexes expressed on bone-marrow-derived macrophages (Mf), dendritic cells (DCs), and medullary epithelial cells; strong interactions with these cells causes T cells to die by apoptosis. Cells surviving negative selection are exported to the periphery by veins and lymphatics. Positive and negative selection of T cells applies to only a small proportion (< 5%) of thymocytes. Most $CD4^{+}8^{+}$ thymocytes do not have significant reactivity for the particular MHC-peptide complexes expressed in the thymus, and these cells die within a few days by an unknown process.

The CD4 and CD8 molecules on double-positive thymocytes determine the MHC class specificity of positive selection. Thus, when T cells undergo positive selection to peptides bound to MHC class I molecules, TCR binding to MHC class I epitopes plus peptides is strengthened by simultaneous binding of CD8 molecules to MHC class I molecules; as mentioned earlier, CD8 molecules bind to a nonpolymorphic site on the membrane-proximal region of MHC class I molecules, and this interaction strengthens the affinity of TCR-MHC class I interaction. Combined TCR-CD8 binding of T cells to MHC class I molecules is followed by downregulation of CD4 molecules and differentiation into mature CD8 ($CD4^{-}8^{+}$) cells expressing a high TCR density. Conversely, positive selection in response to MHC class II molecules is controlled by CD4 molecules. Here, combined TCR-CD4 binding of T cells to MHC class II molecules results in downregulation of CD8 molecules and differentiation into mature CD4 ($CD4^{+}8^{-}$) cells.

Negative Selection (Tolerance Induction)

Of the T cells undergoing positive selection, many of the cells have the appropriate covert physiologic specificity for self-MHC–peptide complexes required by postthymic T cells; after maturation in the medulla, this subset of thymocytes is released into the periphery by veins and lymphatics and feeds into the recirculating lymphocyte pool. Other thymocytes have stronger reactivity for self-MHC–peptide complexes, and these potentially autoaggressive T cells must be destroyed *in situ.* Negative selection of these T cells involves clonal deletion (apoptosis) and appears to take place largely in the medulla through contact with bone-marrow–derived APCs, especially dendritic cells (103,104,105,106,107,108,109,110,111 and 112).

Because dendritic cells are restricted to the medulla, most T cells undergoing negative selection are presumed to have passed through a prior stage of positive selection. However, although negative selection generally occurs later than positive selection, this is not invariably the case. Thus, in certain experimental models, negative selection can occur in the cortex in parallel with positive selection.

Given the enormous range of self-antigens (and the peptides derived from these antigens), how are T cells rendered self-tolerant to these diverse antigens? When considering this question, one must distinguish between circulating and noncirculating self-antigens. Many self-components, such as serum proteins and the surface molecules of lymphohemopoietic cells, readily enter the circulation. These circulating self-antigens have access to the medullary region of the thymus and are presumably degraded there into peptides by thymic APCs for presentation to newly formed T cells. T cells reactive to these peptides are then deleted *in situ.* Intrathymic deletion of T cells specific for circulating self-antigens is extremely efficient, requiring only minute doses (10^{-8} to 10^{-9} M) of antigen, a finding that explains why these antigens are rarely, if ever, the targets of (T-cell–mediated) autoimmune disease.

A different situation applies to tissue-specific self-antigens (e.g., antigens unique to the brain). These antigens are presumed to enter the circulation in negligible quantities, with the result that T cells generally display little or no tolerance to tissue-specific antigens. This situation is exemplified by the relative ease of inducing experimental organ-specific autoimmune disease to these antigens (e.g., by injecting rodents with myelin basic protein) (113).

Although it is well accepted that tissue-specific antigens are the main targets for autoimmune disease (experimentally induced or spontaneous), one must account for the relative rarity of autoimmune disease. Thus, if T cells are nontolerant to tissue-specific antigens, one could expect T cells to enter tissues such as the brain and to mount autoimmune reactions there. Yet such reactions are uncommon. The simplest explanation for this paradox is that tissues such as the brain are not accessible to normal resting T cells. As discussed earlier, resting T cells remain in the recirculating lymphocyte pool and are excluded from entering nonlymphoid tissues. Accordingly, tolerance to tissue-specific antigens may be largely a reflection of “ignorance.” It is also possible that tolerance to tissue-specific antigens is partly a reflection of central tolerance, that is, as the result of trace levels of these antigens that reach the thymus from the circulation or are synthesized *in situ* (114,115 and 116). Such intrathymic expression could be sufficient to delete high-affinity T cells and could leave only less dangerous low-affinity cells to escape to the periphery. This model could explain why autoimmune disease is relatively uncommon. Despite the foregoing possibilities, many workers believe that tolerance to tissue-specific antigens involves postthymic mechanisms (117). This topic is discussed in the next section.

Postthymic Tolerance

As a supplement to clonal deletion in the thymus, induction of T-cell anergy is currently the most popular backup mechanism for self-tolerance. Anergy is postulated to be the result of mature, unprimed T cells that encounter antigen on “nonprofessional” APCs (i.e., on cells other than macrophages or dendritic cells) (118,119 and 120). In this situation, T cells recognize antigen in the absence of the second signals required for T-cell stimulation. As a result, T cells undergo a form of partial triggering in which the cells are refractory to subsequent stimulation by “professional” APCs.

Although T-cell anergy is well documented *in vitro,* whether anergy plays an important role in inducing or maintaining tolerance *in vivo* is controversial. There are many examples of T-cell stimulation *in vivo* that lead to a refractory state (121), but whether this form of anergy is attributable to T-cell contact with antigen on nonprofessional APCs is unclear. The alternative possibility (for which evidence is clear) is that T cells can transiently enter an anergic state even after optimal stimulation (see later). The key issue is whether induction of anergy can explain the rarity of organ-specific autoimmune disease.

This question has been addressed by preparing transgenic mice in which foreign MHC molecules or viral antigens are displayed selectively in organs such as the pancreas, for example by placing the transgene under the insulin promoter (122). In this situation, the foreign antigens are expressed on nonprofessional APCs (i.e., on pancreatic b cells). Unfortunately, the results of this ingenious experiment are variable (122,123,124,125,126,127 and 128). The general finding is that the transgenic mice fail to develop spontaneous autoimmune disease, a finding indicating operational tolerance. The mechanism of tolerance, however, is still unclear. Some groups find little or no tolerance at the T-cell level but observe strong induction of autoimmune disease when the antigen is injected in immunogenic form, for example, as part of a live virus. Under these conditions, contact with antigen expressed on professional APCs in the lymphoid tissues breaks tolerance; the T cells proliferate and express new homing molecules, and this allows the cells to enter nonlymphoid tissues, such as the pancreas, and thus elicit autoimmune disease. These observations imply that tolerance to tissue-specific antigens is largely a reflection of ignorance. However, other studies with this model have shown significant tolerance at the level of peripheral T cells. In some situations, tolerance at the T-cell level may reflect minor expression of the transgene in the thymus, thus inducing central tolerance. In other situations, tolerance seems to reflect an abortive immune response directed to leakage of antigen from the pancreas that reaches the draining lymph nodes; perhaps because of inadequate expression of costimulatory molecules on APC, the responding T cells are rapidly eliminated or are rendered anergic.

Although inconclusive, the foregoing data indicate that T-cell tolerance to tissue-specific antigens can involve various different mechanisms, including clonal deletion in the thymus, ignorance, and postthymic induction of anergy and clonal deletion. In addition, autoimmune reactions are often subject to immunoregulation, such as by subsets of T cells that produce inhibitory cytokines (129). Collectively, these various mechanisms usually ensure that T-cell responses to self-antigens are minimal or undetectable. Therefore, the implication is that, when it occurs, the development of overt autoimmune disease reflects breakdown of one or more of the mechanisms that normally hold nontolerant T cells in check. As typified by extensive studies on the nonobese diabetic mouse, however, the origin of autoimmune disease is highly complex and is still largely obscure (130).

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6 ANTIGEN PROCESSING AND PRESENTATION

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A fundamental step in immune induction is *antigen presentation,* the process whereby an *antigen-presenting cell* (APC) takes up protein antigens, processes them, and presents fragments to the T cell. The reason that this process is of central importance in immune induction is that T cells do not recognize antigens directly, but only fragments or peptides bound to molecules of the major histocompatibility gene complex (MHC). MHC molecules are peptide binding proteins that rescue protein fragments from intracellular catabolism and select peptides that are then presented to the T cells (1). The T-cell receptor for antigen (TCR) is a molecule that recognizes the peptide fragments and at the same time contacts the MHC molecules (2). Thus, it is a receptor that has dual specificity: recognition of the peptide and recognition of the self-MHC molecule. The two major sets of T cells, CD4 and CD8, recognize peptides, but on the two major classes of MHC molecules: CD8 T cells on class I MHC molecules and CD4 T cells on class II MHC molecules (Chapter 3). Class I and II molecules of the APC interact with peptides derived primarily from cytosolic proteins, whereas class II molecules interact with those peptides derived primarily from the vesicular endocytic compartments. In this way, MHC molecules alert the T-cell system of the composition of its intracellular environment. Because the APCs are phagocytic cells that sample the extracellular milieu, any disruption of their intracellular environment, such as by the uptake of viruses or bacteria or extracellular toxins, is rapidly flagged to the T-cell system by way of MHC molecules.

Finally, to generalize, the molecular basis for the two major types of antigens, the thymus-dependent antigens, which are proteins and glycoproteins, and the thymus-independent antigens, which are the polysaccharides, is based on their capacity to associate with the two major sets of MHC molecules. The thymus-dependent antigens are capable of donating peptides to MHC molecules and therefore can form the epitope that engages the specific T cells. In contrast, thymus-independent antigens do not bind to MHC class I and II molecules (3), and they do not bring T cells to the reactions. (Polysaccharides can directly trigger some B cells.) Table 6.1 compares the response to these two sets of antigens and underscores the importance of antigen presentation in inducing a full immune response.

	Pure Polysaccharide	Globular Protein
Recognition by BCR	Yes	Yes
Recognition by TCR	No	Yes, after processing
Binding to MHC	No	Only after processing by antigen-presenting cells
Involvement of CD4 T cells	No	Yes
Memory	Poor	Strong
Affinity maturation	Poor	Strong

[a] The B-cell receptor (BCR), a membrane-bound antibody molecule, can interact with either pure polysaccharides or proteins. However, pure polysaccharides do not bind to MHC molecules, so the ligand for the T-cell receptor (TCR) cannot be formed. Consequently, responses to polysaccharides are not influenced by the helper function of CD4 T cells that takes place during antigen presentation.

TABLE 6.1. Comparison of the Response to Carbohydrates and Proteins[a]

Besides the classic MHC class I and II molecules, other nonconventional MHC or MHC-like molecules appear to have a role against special sets of molecules represented in microbes. Thus, H-2 M3 is a murine nonpolymorphic class I molecule that binds short peptide sequences having a formylmethionine at the amino-terminal (4). The CD1 family of molecules binds lipids and presents glycolipids to T cells, including mycolic acid from the tubercle bacillus (5,6). The role of these presenting molecules in now being evaluated and compared with the classic class I and II MHC proteins (Table 6.2). Although comparisons are made in this chapter between the features of class I and class II presentation, the major emphasis is to review primarily the basis of antigen presentation by the class II MHC system (Chapter 18 discusses the effector role of CD8 T cells.)

	MHC-I	MHC-II
Expression	Most cells	Primarily in antigen-presenting cells and in some specialized epithelial
Structure	A heavy transmembrane chain (~44 kd) together with β2-microglobulin (12 kd)	Two transmembrane chains α and β of ~30 kd
Peptide binding	Usually 8–10 residues in length	Normally large peptides, >15 residues
Peptide loading	In endoplasmic reticulum	In vesicles
Site of catabolism of the protein	Cytosol: proteasome	Vesicles: lysosomal
Auxiliary molecules	Tapasin, calreticulin	Invariant chain, DM
Biology	Antiviral and tumor immunity	Immunity to intracellular pathogenesis and foreign proteins
	Interaction with CD8 T cells	Interaction with CD4 T cells

TABLE 6.2. Comparison of Class I and II Major Histocompatibility Complex (MHC) Molecules

The APC interaction with T cells is an indication of the strong relationship between the cellular elements of the innate system of defense and the adaptive immune system. The innate system, represented by monocytes and macrophages, the family of dendritic cells (DCs), the natural killer (NK) cells, and the polymorphonuclear leukocytes, constitutes an early system of defense against a variety of microbes and foreign material (7,8). The innate system is the cellular system first identified by Eli Metchnikoff, a discovery that led to the first major controversy in immunology, centering on how immune reactions operated. Recognition of the microbe by the innate system is followed by a cascade of intracellular events leading to activation of the cells and the release of inflammatory molecules, including cytokines. These molecules produce inflammation and partially control the growth of the microbe. Probably the best example of the innate system operating alone can be found in mice with genetic abnormalities that result in their lack of development of lymphocytes. Such mice challenged with bacteria and viruses show partial resistance to the pathogens resulting from a variety of reactions that include phagocytosis, activation of leukocytes by cytokines, particularly interferon-g, activation of the complement system, and mobilization of leukocytes and sequestration of the pathogen (9).

In contrast to the innate system is the adaptive or lymphocyte system, represented by B and T cells with their antigen-specific receptors. We now recognize that both systems depend on each other and operate in a symbiotic manner (10). The APCs of the innate system by way of antigen presentation (and also by the release of cytokines) select, engage, and activate the T-cell adaptive system. The molecular basis of communication between both cell systems is through the MHC molecules during antigen presentation. Once activated after antigen presentation, the T cells release a plethora of cytokines that mobilize and activate the cells of the innate system, thus making the elimination of the pathogen a highly effective process, hence the term *symbiosis* to describe the interactions of the two cellular systems.

ANTIGEN PRESENTATION IN TISSUES AND IN THE THYMUS

Antigen presentation takes place at two stages in the natural history of a T cell. Mature T cells encounter APCs that have taken up antigen to which their TCR has specificity. This encounter can take place in lymphoid or in nonlymphoid environments, that is, in the extracellular spaces of many tissues: APCs, particularly DCs, and monocyte-macrophages, are found in all tissues and are constantly sampling their extracellular milieu. This encounter of APCs with antigen and the presentation of the peptide-MHC complex results in the selection of the T-cell clones, and with it, their expansion and activation. This encounter may take place between monocyte-macrophages or DCs, as APCs, and CD4 T cells or between B cells and CD4 T cells. The result is the reciprocal activation of both cells. In the case of macrophages and DCs, their activation is characterized by the release of cytokines and inflammatory radicals, and particularly with macrophages, in microbicidal activity. In the case of B cells, the result is B-cell expansion and differentiation with antibody formation.

However, T cells also encounter the peptide-MHC complex during the process of differentiation that takes place in the thymus gland (Chapter 5). There, the immature T cells bearing both CD4 and CD8—the double positive thymocytes—can interact with cells bearing MHC molecules. This interaction depends on the specificity of their TCR, which is expressed during differentiation as a result of rearrangements of the various gene segments that encode for it. The cells that bear MHC molecules are the epithelial cells of the cortex and the monocytes and DCs of the medulla. In the case of the thymus, their APCs are presenting peptides derived from the processing of autologous proteins. Foreign antigens enter the thymus in only limited amounts, if at all. Thus, the function of presentation in the thymus is to control the repertoire of immature T cells, whereas that of antigen presentation in the periphery is to select and activate the mature T cells (11,12). A thymocyte that expresses a TCR that does not have any degree of complementarity to the MHC-peptide complex eventually dies in the gland and does not peripheralize. Moreover, a thymocyte that expresses a receptor that matches perfectly with the ligand likewise is eliminated by a process now termed *negative selection,* identical to the central tolerance described in the classic experiments since Medawar's original work. The T cells that are selected are mainly those with TCRs that have affinities to self-MHC, but weakly for one of the self-peptides. However, such receptors have higher affinity for, but strongly against, a foreign peptide. These T cells mature and circulate among tissues and lymphoid organs, and they express a TCR that has affinity for self-MHC and against a peptide of a foreign protein.

These features of differentiation of thymocytes lead to the phenomenon of *MHC restriction* during antigen presentation; that is, the MHC, a gene complex with a high degree of allelic polymorphism, *restricts* the T cells that recognize the foreign peptides. This phenomenon was discovered in the early 1970s when either CD8 T cells or CD4 T cells were found to recognize only antigens presented by their own APCs (by their own MHC molecules) and not by APCs from another genetically disparate individual containing an unrelated allelic MHC molecule. MHC restriction was discovered in the experiments of Zinkernagel and Doherty when they tested cytolytic T cells against viral antigens (13). At about the same time, the same phenomenon was found for interactions of macrophages and T cells (14), as well as for B and T cells during antibody formation (15,16). The experiments of Rosenthal and Shevach, the first to show MHC restriction with macrophages, are presented in Table 6.3.

Macrophages		T lymphocytes (cpm × 10^{-3})		
Strain	PPD	Strain 2	Strain 13	Strain (2 × 13) F1
2	–	0.9	5.7	1.6
2	+	26.4	8.6	7.0
13	–	4.6	1.7	1.8
13	+	3.1	19.9	7.8
(2 × 13) F1	–	1.9	4.3	1.7
(2 × 13) F1	+	12.4	11.8	12.6

[a] The inbred strains of guinea pigs, which differ at their class II MHC gene locus, were immunized with complete Freund adjuvant. Their T cells were isolated and cultured with macrophages from normal guinea pigs in the absence or presence of PPD of *Mycobacterium tuberculosis*. Strain 2 lymphocytes responded only to PPD presented by macrophages of strain 2, and the same applied to lymphocytes from strain 13. Note the mild mixed lymphocytic reaction when lymphocytes from strain 2 or 13 are incubated with macrophages of strain 13 or 2, respectively.
Modified from Rosenthal AS, Shevach EM. Function of macrophages in antigen recognition by guinea pig T lymphocytes. *J Exp Med* 1973; 138:1194, with permission.

TABLE 6.3. Rosenthal-Shevach Experiment Showing Major Histocompatibility Complex (MHC) Restriction for the Presentation of Purified Protein Derivative (PPD)[a]

ANTIGEN-PRESENTING CELLS

For a cell to present antigen, it needs to (a) be able to take up the foreign molecule, (b) process the foreign proteins, (c) express histocompatibility molecules, and (d) express auxiliary molecules that foster the interaction with the T cell. The last requirement falls into three sets: adhesion molecules, costimulatory molecules, and cytokines that regulate T-cell function. We briefly comment on the APCs involved in physiologic interactions with T cells, which are the DCs, the monocyte-macrophages, and the B cells.

DCs have been the center of much attention (17,18). Derived from the blood monocytes, DCs are found in many tissues. Particularly important is their localization in lymphoid organs, where they form an antigen-trapping network in the deep cortex of the lymph nodes and in the periarteriolar lymphoid sheath of the spleen. DCs also circulate in small numbers. In the skin, an immature form of DC is found among epidermal cells, the Langerhans cell. Langerhans cells are the cells responsible for contact sensitivity: they take up antigen that enters through the skin, and they migrate through the local lymphatics into draining nodes, to localize to the deep cortex, where they present antigen to T cells (19). In the thymus, DCs are found in the medulla, where they present self-antigens, as described earlier. When they are isolated from tissues, DCs are heterogeneous and bear different surface markers. DCs have been divided into two major sets, myeloid and lymphoid, depending on the presence primarily of the CD8 a chain in the latter. Their interrelationship is the subject of much attention at this time (20).

DCs are the most effective cells in presenting antigen among the APC family, and they are responsible for the initial recruitment and activation of the T cells. DCs are particularly effective in interacting with T cells by favoring contacts by way of thin cytoplasmic extensions, called veils or dendrites: DCs express high levels of MHC molecules, as well as adhesion and costimulatory molecules, and they release early cytokines that modulate T-cell activity. In culture, DCs have proven to be highly effective in stimulating both CD4 and CD8 T cells (17,18). In fact, DCs are also effective in stimulating allogeneic T cells and are thought to be the central cell in sensitizing against organ allografts in transplantation reactions.

Monocytes can be made to differentiate *ex vivo* as well as *in vivo* into DCs, by specific cytokines, particularly granulocyte-macrophage colony-stimulating factor (GM-CSF) (21). The easiest procedure for isolating DCs is to generate them from cultures of monocytes or bone marrow stem cells by the addition of GM-CSF together with IL-4 (22). Other cytokines that favor their survival include TRANCE, a member of the tumor necrosis factor (TNF) family (23). Evidence indicates that monocytes differentiate to DCs as they cross endothelial beds (24).

DCs pass through different stages of maturation in their antigen-presenting function. The “immature” DCs are actively phagocytic but are less effective in processing (25). The “mature” DCs result from their stimulation by cytokines such as interleukin-1 (IL-1) and TNF-a, endotoxins, double-stranded RNA, apoptotic cells, or gram-positive or gram-negative bacteria (22,23,25,26 and 27), or even by T cells through interaction with their CD40 ligand (28,29). Mature DCs are less phagocytic, but yet they are highly effective in presenting antigen. Thus, in DCs, the two stages of presentation, uptake and presentation, can be dissociated.

DCs are mobilized during antigen presentation and represent the cells responsible for the recruitment and early activation of the naive T cell (30,31 and 32). The mobilization of T cells and DCs involves complex interaction among chemokines produced by various cells in the lymphoid organs. Some features of this complexity are now being identified. For, example, as DCs mature, they upregulate the chemokine receptor CCR7 that directs their migration to T-dependent areas of lymph nodes and where the two ligands SLC and ELC are being generated. As mature DCs accumulate, they themselves produce these two chemokines that, in turn, bring in more activated T cells (30,31 and 32).

Macrophages also serve as presenting cells. In fact, the first cells to be studied as APCs were peritoneal macrophages from mice (10). As with DCs, macrophages also derive from blood monocytes (33). The major cytokine that favors the monocyte differentiation to macrophages is macrophage colony-stimulating factor (M-CSF). Thus, the cytokine milieu is the key element in regulating the differentiation of monocyte to either DCs or to macrophages. In tissues, macrophages differentiate further, to express particular functions, such as happens in bone, with the osteoclast, or in lung, with the alveolar macrophages. Macrophages have other functions besides antigen presentation in that they are actively involved in uptake and degradation of particulate material, in tissue remodeling, and in cytocidal activity. Macrophages are also highly secretory cells, releasing cytokines such as IL-12, IL-1, and TNF that regulate lymphocyte function. Macrophages bear a large array of surface molecules that allow them to interact with many different microbes. Macrophages, like DCs, are also mobilized to sites of infectious foci where they can be activated by T-cell cytokines. Highly responsive to interferon-g, macrophages become cytocidal to intracellular microbes by way of release of reactive oxygen and nitrate intermediates. Indeed, the activated macrophage is the central cell in bringing about resistance to intracellular pathogens. Thus, macrophages form the cells of delayed hypersensitivity reactions and of infectious granulomas. Macrophages express class I and II MHC molecules. Their expression is also highly regulated by interferon-g,

which increases it by severalfold (10).

B cells express constitutively both sets of MHC molecules and can present antigen, particularly B cells that bind to their surface-bound antibody molecules (34,35). Bound to surface immunoglobulin (Ig), the complex is effectively internalized and can be processed so peptides can be selected and bound to class II MHC molecules.

Concerning B cells, the function of presenting antigen is the first step in the interaction between B and T cells that results in antibody formation: in a two-step process, the B cell first binds, internalizes, and processes the antigen and, in a second step, presents the peptide-MHC complexes to the CD4 T cells. The end result is reciprocal activation: the T cell proliferates and differentiates, and the B cell undergoes clonal expansion and differentiation to an antibody-forming cell. This two-stage interaction explains the classic results of immunization with globular proteins. Immunization with globular proteins induced mostly antibodies that recognized conformational determinants of the protein; that is, the protein was recognized before it was handled by the normal catabolic processes, whereas the T cell recognized linear determinants, after intracellular processing, as we now understand during antigen presentation (10). Indeed, the native antigen selected those B cells with high-affinity antibody molecules that interacted with the antigen molecule in its unfolded form. Once it was processed, the T cell then recognized a linear segment of the molecule in the form of a peptide bound to class II molecules.

B cells vary in their presenting activity depending on their stage of differentiation. Immature B cells tend to be poor APCs, but their activation improves this function, most likely because of their increased expression of adhesion and costimulatory molecules. In lymphoid tissue, the situation that is favored is of a first interaction of antigen molecules with the DCs of the deep cortex leading to activation of CD4 T cells, followed by the interaction between B cells and T cells, as described earlier (36). The B cells may capture the antigen draining into the deep cortex of the lymph nodes (or into the periarteriolar lymphoid sheath in case of the spleen). Investigators have also suggested that macrophages or DCs can present unprocessed antigen to the B cell (37). Regardless, the first interaction of B cells with the activated T cell takes place outside the B-cell follicle (38,39). Once the B cell presents to the CD4 T cells, it becomes competent to translocate into the follicles, where subsequent activation takes place, with differentiation and class switching. The interaction in the follicles involve the B cells with follicular DCs that capture antigen by way of complexes with antibody and complement proteins, in a process independent of MHC presentation (39). Some T cells have been found in the follicles, but their precise role at that site is not clear.

Aside from the three sets of APCs described earlier (i.e., the “professional” APCs), other cells may have the potential to present antigen. Under normal conditions, most epithelial and mesenchymal cells do not express class II MHC molecules, and their levels of class I molecules are low. In humans in particular, class II proteins expression is found in some endothelia and some epithelia. For example, some of the cells of the renal proximal tubules or the gastrointestinal mucosa express a small level. This lack or low level of expression of class II and the lack of adhesion or costimulatory molecules ensure that most cells in tissues do not present antigens, therefore avoiding autoimmunization (40). Investigators have speculated that abnormal tissue conditions that result in inflammation and interferon-g production may make these cells effective in presentation and could be an element in autoreactivity. Whether this is the case has yet to be determined.

STEPS IN ANTIGEN PRESENTATION

Antigen presentation is made up of several steps. At the level of the APC, the process starts with the internalization of the protein antigen, its traffic through intracellular vesicles, and its processing. At some point during the intracellular sojourn of the protein, the products of processing meet with the class II MHC molecule, and the assembly of the peptide with class II MHC takes place. Eventually, the MHC-peptide complex is transported to the plasma membrane. Once the peptide-MHC complex—the TCR ligand—is displayed, the issue becomes one of selection for a T-cell clone with a TCR that has complementarity to the ligand. The issue of how in a clonal system such encounters take place has not been settled, and it probably involves complex changes at the level of the tissue environment by cytokines and chemokines that may regulate the flow of T cells through networks of APCs (see the previous section).

For antigen presentation to be effective in activating T cells, several molecular interactions need to take place at the surface of both interacting cells. The essential one that gives specificity to the interaction is, of course, that of CD4 T cells recognizing through their TCR the peptide-MHC complex. In this initial interaction, the CD4 molecule plays an important role because it interacts with class II MHC molecules and fosters the binding of the two cells. (CD4 molecules are also involved in the intracellular scaffolding of molecules around the TCR, and this leads to signaling of the cell to proliferate and express different cytokine genes.) Other interactions that are required for optimal T-cell activation include those among adhesion molecules and among costimulator molecules. Adhesion molecules most prominently involved are those involving the b_2 integrin molecules. Costimulators consist of membrane molecules that regulate the final fate of the T-cell–APC interaction. Finally, during the APC–T-cell interaction, cytokines are released that influence each of the interacting cells.

Uptake and Processing

Peptides that bind to class II MHC molecules are generated from proteins that enter the APC as soluble proteins, as part of large macromolecules, or as constituents of microbes. Processing requires the internalization of the protein antigen. No pathway of entry of the protein antigen in the APC is unique in the sense of donating or not donating peptides for complexing with class II MHC molecules, although the efficiency may vary (10,35,41,42). Antigens can enter the cell by fluid-phase endocytosis or by binding to cell-surface receptors that include the Fc and C3 receptors (which bind to antigen-antibody complexes, containing Ig or C3, respectively), or to a variety of surface ligands, such as scavenging receptors or mannose receptors. In the case of the B cell, binding to the extracellular protein by its surface-bound antibody molecule markedly increases the amount taken into the cell and improves the degree of presentation (35). Generally, the higher the amount of antigen entering the APC, the more peptide-MHC complex is generated.

Processing is a fast event. For most protein antigens, the peptide-MHC complexes are detected on the membrane of the APC, within the first hour after its uptake (10,43,44 and 45). The biochemical steps that take place leading to the peptide fragment bound to the MHC molecule may depend on the structural and chemical features of the protein antigen: they could involve partial fragmentation of the protein before or after a step of denaturation of the protein. The protein antigens travel through vesicular compartments that are progressively acidic and that contain cathepsins or acid hydrolases, many of broad specificity (41). Inhibition of lysosomal catabolism usually translates into a reduction in the amount of peptide-MHC complex that is generated (41,46). A substantial amount of the protein processing takes place in a deep vesicular compartment in the APC. A situation favored by some investigators is that the protein first unfolds, acquiring more conformational flexibility, and the MHC then binds to segments that contain a sequence favored for binding (47). Subsequently, the portions of the protein that extend beyond the combining site of the MHC molecules can be trimmed by amino and carboxy peptidases.

Peptides bound to MHC molecule are highly protected from intracellular catabolism. Indeed, although most peptides or denatured proteins are extensively catabolized to the level of amino acids, an MHC-bound peptide for the most part survives the sojourn through highly acidic, proteolytic vesicles and reaches plasma membrane. Overall, the efficiency of processing tends to be low; most internalized proteins are catabolized, and only a few of those internalized associate with MHC molecules (43).

The possibility that protein processing by APC involves a first step of internalization of the protein followed by its release from the APC to the extracellular milieu does not appear to take place with most antigens. However, cellular antigens from an nonpresenting cell can be presented by APCs, in what is termed *cross-priming.* Cross-priming is a term first used by Michael Bevan when testing responses to minor histocompatibility antigens: the antigens from one cell could be transferred to a presenting cell (48). Cross-priming has been studied extensively in the response of peptides presented by the class I MHC system and also takes place in the class II system (49). (Cross-priming may be particularly important in the presentation of allogeneic cellular antigens in transplantation reactions.) How the transfer takes place may vary and is not entirely clear. The antigen from the donating cell may be released as a macromolecule or as part of a fragment taken up by the APC, or the whole cell may be phagocytosed by the APC and the antigens processed (27,50). Indeed, necrotic or apoptotic cells or their fragments can be internalized by APCs, and their antigens can be processed and presented.

Role of the Major Histocompatibility Complex

The role of the MHC molecules is to bind peptides creating the structure, the peptide-MHC complex, that engages the TCR. Class II MHC molecules are made up of two chains, the a and b chains (of about 30 and 25 kd, respectively), that are assembled in the endoplasmic reticulum (ER) and are transported after a sojourn through the Golgi complex and the trans-Golgi network into endocytic compartments. Involved in the transport of the ab dimer is the invariant chain (Ii), so called because in contrast to the a and b chains, it exhibits no genetic polymorphism. (The b chains are highly polymorphic; the a chain has limited polymorphism in humans, but it does show allelic differences in the mouse. The selective pressure for the high degree of allelism is peptide binding; indeed, the amino acids responsible for the allelic differences are represented in the areas of the molecule that form the combining site for peptides.) Ii expresses various physical forms brought about by differential splicing of the gene, as well as by posttranslational changes (51). However, most of the protein is represented in a 33-kd single chain. In the ER, Ii forms a complex with the ab dimers with three chains associating with three dimers (52). A segment of the Ii actually folds over the combining site of the dimer. This segment is termed CLIP, for class II–associated invariant peptide, and encompasses residues 83 to 109 (51,53). The interaction of Ii with the ab dimer takes place through various sites in the Ii proximal and distal to the CLIP segment. During transport through ER-Golgi complex, the CLIP segment inhibits binding of peptides and therefore protects the combining site until it reaches an endocytic compartment (53). Ii, a type II transmembrane molecule, contains cytoplasmic motifs that target most of the complexes to endocytic compartments, this being one of its important functions (41,52,54). (There is a minor traffic route by which the I-ab is transported to plasma membrane and is rapidly internalized.) Ii is degraded in endocytic compartments through the actions of various cathepsins, the acidic proteolytic enzymes typical of lysosomal vesicles (41,55,56). Although several cathepsins are involved in a stepwise process of degradation, cathepsin S, and, in some APCs, cathepsin F and L are major enzymes in

the final process of degradation of Ii (55,56,57 and 58). In thymus, cathepsin L plays a role in Ii degradation (58). Degradation of Ii allows for the ab dimer to become competent to bind peptides.

Other proteins help in the assembly of the peptides with the ab dimer of class II. Most prominent is the HLA-DM or, in the mouse, H-2DM molecule. From here on, we refer to both as DM (59,60 and 61). The gene encoding the a and b chains of DM are found in the large MHC gene complex (62,63). DM has a general structure similar to a class II MHC molecule. However, structural analysis shows that the combining site does not contain peptides because the two helices come near each other to close the groove (64,65). In the APC, DM is localized to the same vesicular compartment as class II molecules. DM assembles with the CLIP–class II dimer in ways that are unclear at this time: nonetheless, this association results in the faster release of the CLIP peptide (66). The CLIP peptide has relatively weak binding for the ab dimer of class II, but its spontaneous rate of dissociation depends on particular class II alleles. By DM's favoring the release of CLIP at a faster rate, the dimer becomes competent to bind to polypeptides in the vesicular compartment (66,67 and 68). Thus, DM can be viewed as a catalytic protein that favors the dissociation or off rate of peptides bound to class II molecules (69). The stronger the peptide interaction with class II, the lesser is the effect of DM on the complex. Thus, the effect of DM is to edit the peptides selected by class II molecules. In the absence of DM, the class II molecules are restricted in the diversity of peptides they contain, because they are mostly occupied by CLIP peptides. Another protein, DO, has also been identified (70); it associates with DM and perhaps regulates the effective free concentration of DM available for peptide exchange (71).

The vesicular compartment in which Ii proteolysis takes place and the proteins are processed has been the subject of much analysis (72,73 and 74). In general, class II molecules are found in various vesicular compartments, including vesicles involved in the recycling of membrane proteins. At each of the various sites, class II molecules have the potential to bind peptides. Peptides or unfolded proteins can bind to MHC molecules in early endocytic vesicles where class II recycling may be taking place. However, deep vesicular compartments or prelysosomal vesicles containing lysosomal enzymes contain class II molecules with Ii and DM. It is believed that in these compartments, termed MIICs, Ii is degraded and much of the peptide assembly takes place. MIICs are characterized by containing multilamellar profiles (75).

The class II molecules bind peptides by their combining site found in the membrane distal domains of both their a and b chains (Fig. 6.1). The combining site has features in common with those of the class I MHC molecule (76,77,78,79 and 80). It is made up of a platform of b-pleated sheets surrounded by two a helices. The peptide sits stretched between the two helices. Most of the amino acids of MHC molecules responsible for allelic polymorphisms are located either in the b sheets or in the a helices. The fine ultrastructure noted by x-ray crystallography has identified the main features responsible for the binding of the peptide. The class II–bound peptides are found in an extended polyproline-type helix in which some side chains are contacting the residues in the helix or the b-pleated sheets while some residues are solvent exposed to contact the TCR. Peptides bind to class II MHC molecules through several chemical interactions. A large network of hydrogen bonding involves the peptide backbone with conserved amino acid residues along the combining site. In general, the extent of contribution to binding energy of the hydrogen bond network depends on the length of the peptide. In contrast to peptides bound to the class I MHC combining site, peptides bound to class II MHC are usually long, 14 residues or more. A key factor in binding peptides is the interaction between side chains and sites or pockets in the combining site that are formed by the polymorphic residues. These side chain interactions determine the degree of binding and the sequence motif of the peptides that class II molecules select. For many class II molecules, a single side chain from the peptide can be responsible for the binding and selection of peptides. This has been shown to happen with HLA-DR1 (81) and I-A^k (82), in which a single residue at the P1 pocket can be responsible for the binding (a tyrosine or an aspartic acid, respectively) (47). Although other peptide side chains may not contribute to binding energy, they will or will not permit the peptide chain to fold properly in the combining site (83).

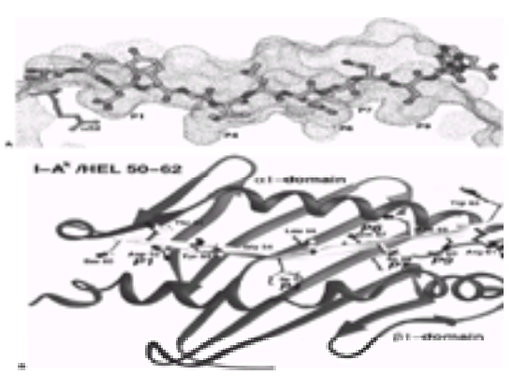

Figure 6.1. Peptide 52-61 of hen egg white lysozyme binds to I-A^k. **A:** Side view of the peptide. **B:** Ribbon diagram of the peptide as it is found on the I-A^k protein. The sequence of 52-61 is DYGILQINSR. The Asp52 residue is responsible for the selection of the peptide from HEL as well as in the binding affinity. Asp52 forms a salt-bridge with Arga52 situated at the base of the P1 pocket. Mutation of Asp52 to Ala52 reduces binding affinity severalfold. (Fremont DH, Monnaie D, Nelson CA, et al. Crystal structure of I-A^k in complex with a dominant epitope of lysozyme. *Immunity* 1998;8:305–317, with permission.) (See Color Figure 6.1A.)

The interactions between the TCR and the peptide-MHC complex have been examined at the structural level by x-ray crystallography using TCR complexed to either class I or class II peptide-MHC complexes (2,84,85,86 and 87). Some structures show the TCR in a diagonal position relative to the peptide, whereas in other cases, the position is orthogonal to it. The TCR contacts residues of the peptide that are solvent exposed mainly through the complementary determining regions that show most variability, the CDR3a and b. The important feature is that regions of the TCR contact the peptide, whereas others contact the MHC; indeed, both MHC and peptide form part of the interacting face with the TCR, an explanation for the phenomenon of MHC restriction mentioned earlier (2).

Class II Major Histocompatibility Complex Binding

The assembly of peptides with MHC induces changes in the macromolecular complex that depends on the binding features of the peptide. With strong binding peptides, the complex becomes highly stable (88,89 and 90) and resistant to proteolytic enzymes (91,92). In contrast, weak peptides form more labile complexes in which the dimer becomes more sensitive to proteolysis. These features are of biologic importance in that they determine the extent to which the complex survives in the APC, that is, its half-life, which, in turn, reflects in the immunogenicity of the complex in the APC surface (47).

The binding of peptides to class II MHC molecules can be studied in free solution using purified proteins and peptides (1,93,94). The combining site of the MHC molecule can accommodate only a single peptide. This finding implies that for these molecules to have biologic significance, they must bind to a wide diversity of them. This is the case because the specificity for peptides of any MHC molecule is broad (47,94,95,96 and 97). The binding affinities depend on the peptide, usually in the range of nM to mM. Some peptides do not bind. Binding can have slow on rates but extremely slow off rates. Indeed, for some MHC-peptide combinations, the reactions are practically irreversible. By using peptides in which amino acids are changed at each position, usually with alanines, the identification of the key residues responsible for the binding can be made (47).

The biologic significance of peptide binding is clear. From complex proteins, several epitopes are usually selected (see later), and this selection establishes the degree of immunogenicity of the protein. Historically, it is interesting to mention how this physiologic role of MHC was identified. Early observations made primarily by McDevitt, Sela, and Benacerraf indicated that some peptides of limited heterogeneity did or did not induce responsiveness, and this property was linked genetically to the MHC class II genes (98,99 and 100). McDevitt and Chintz went on to identify the genes locus responsible for the control as that encoding for class II MHC molecules (101). These results were subsequently explained by the interaction between peptides and MHC molecules, that is, by the formation, or lack thereof, of the peptide-MHC complex (93). These studies led to the discovery of the role of MHC in normal immune responses to proteins, a function distinct from its role in transplantation reactions where this gene locus was first identified.

Class II Major Histocompatibility Complex Selection of Peptides

From a protein that is taken by APC, many peptides can be generated that become associated with a class II MHC molecule. The MHC alleles determine the composition of the peptides that are selected. This “determinant selection” was first indicated in the now classic studies of Barcinski and Rosenthal when they evaluated the response to insulin by guinea pig macrophages: each allelic form of the guinea pig class II system presented different segments of the molecule (102). These observations were later confirmed by other investigators and were extended noticeably as the various peptides reactive with T cells could be identified. Thus, from a single protein, various segments are selected for processing and are found expressed as peptides bound to MHC (47,103,104). A peptide that is not selected by one allele may be selected by others (105). Overall, the most important factor so far identified in peptide selection is the MHC genotype. From a protein, some peptides may be expressed at a high density, and these are chemically dominant, whereas others may be expressed severalfold lower (47). Presumably, those peptides that are strongly represented are those that stimulate more T-cell clones.

MHC molecules have been isolated from APCs, and the peptides bound to them have been released and studied (106,107,108,109 and 110). The method of choice to

examine for MHC-bound peptides is electrospray tandem mass spectrometry, in which peptides, even at the femtomole range, can be identified and sequenced (109,111) (Fig. 6.2). The procedure consists of isolating APCs, releasing by detergents the MHC molecules, and purifying them by immunoaffinity chromatography with anti-MHC antibodies bound to insoluble matrices. When these purified MHC molecules are denatured, the peptides are released and can then be fractionated, usually by reverse-phase high liquid pressure chromatography, and identified by mass spectrometry combined with bioassays. This technical approach has identified hundreds of self-peptides bound to MHC molecules. Many peptides are represented extensively, and these usually have binding motifs that favor the interaction. However, many peptides are at low density of a few per APC. In our own experience studying the I-A^k molecules, about 60% of the peptides are found in few numbers per APC, whereas the remaining range up to a few thousand molecules (47). In essence, the MHC molecules do not discriminate between foreign peptides and self-peptides. Both self-peptides and foreign peptides share the same binding properties (and, in fact, compete among each other). For example, the major dominant epitope of chicken lysozyme antigen for the murine I-A^k binds strongly, and so does the murine equivalent peptide. The final self-nonself discrimination of a peptide therefore is made by the T cell.

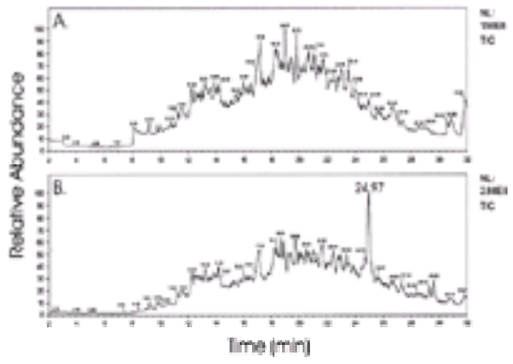

Figure 6.2. Chromatogram of peptides isolated from antigen-presenting cells (APCs) bearing I-A^k molecules. **A:** The line C3.F6 (a B-cell line) was cultured, the cells were lysed, and the I-A^k molecules were isolated. Shown are the profiles of the most abundant peptides. Many of these autologous peptides bear Asp or Asn at the fourth or fifth residue from the amino terminus. **B:** The cell was cultured with lysozyme. At 24.97 minutes, there is a large peak represented by HEL (48,49,50,51,52,53,54,55,56,57,58,59,60,61 and 62) (Fig. 6.1). (From Gugasyan R, Vidavsky I, Nelson CA, et al. Isolation and quantitation of a minor determinant of hen egg white lysozyme bound to I-A^k by using peptide-specific immunoaffinity. *J Immunol* 1998;161:6074–6083, with permission.)

In the class II system, in contrast to the class I, peptides are usually selected as families (47,107,108,109,111). A family can be made up of up to 30 members and consists of peptides having a “core” segment and “flanks.” The core portion is about 9 residues in length and binds to the MHC combining site, from the P1 to the P9 pockets that constitute the allele specific sites. Flanks in the amino-terminal and carboxy-terminal are of variable lengths, and this variation determines the number of peptides in a family. Structurally, the binding sites of the class II MHC molecules are more open at their ends, so peptides can extend beyond the end pockets. Although the core segment is responsible for the selection, the extensions or flanks are of considerable importance: the flanks contribute to binding affinity of peptides (82), specificity of the TCR (112), and to the persistence of the peptide-MHC complex in the APC (113).

The peptides bound to class II MHC derive from three sources of proteins: those from the media where the APCs are cultured, those that are constituents of the vesicular system of the APCs, and, perhaps surprisingly, those normally residing in the cytosol. The cytosolic peptides found associated with class II MHC molecules are long, like those derived from exogenous proteins. Clearly, the pathway from cytosol into the class II MHC system is distinct from that taken by peptides that bind to the class I MHC molecules. These peptides are of limited size—eight to ten residues—and are processed from cytosolic proteins in proteasomes and transported to the ER by way of the transporters of antigen processing (TAP) transporters.

Binding of Autologous Peptides

The presence of self-peptides bound normally to MHC proteins is, of course, of major importance in the context of antigen presentation and the cause of autoimmune diseases. The current thinking is that autoimmune diseases develop as T cells that escape deletion in the thymus recognize the self-peptides expressed by the MHC molecules of APCs located in the tissues. Depending on the particular MHC genotype, some self-peptides may be preferentially displayed over others, and these may be the target of recognition because of their amounts bound to MHC, or because of other factors, such as local tissue conditions induced by inflammation and cytokines. Indeed, autoimmune diseases have an association with a particular MHC allele, that is, individuals with a particular allele will have a higher relative risk in developing the disease (114). A most thoroughly discussed example is autoimmune or type I insulin-dependent diabetes mellitus (IDDM). The MHC gene complex is most strongly linked to susceptibility to IDDM. Persons bearing the DR3 and DR4 alleles show a relatively higher risk of developing IDDM. Genetic studies have shown that this linkage is most strong with DQ alleles that show changes in one or two key residues of the b chain (115). Similar changes are found in the I-A^{g7} class II molecule of the mouse NOD, a strain of mice that spontaneously develops IDDM (116). The diabetes-susceptible class II molecules are distinguished by having a serine instead of an aspartic acid at residue 57 of the b chain; Asp57 found in most other alleles forms a salt bridge with Arg76 in the a chain. These two residues help to form part of the P9 pocket in the combining site for peptides (117,118). Although the identification of the peptides responsible for triggering IDDM remains unidentified, the spectrum of self-peptides displayed by the non-Asp 57 alleles (i.e., those with a propensity for IDDM) is markedly different from the wild type. The non-Asp prefers peptides in which an acidic residue is present to interact at the P9 site and ion pair with the unpaired Arg 76 residue.

Continuing to take autoimmune diabetes as a representative example, the situation for presentation would be as follows: the b cells do not express class II MHC molecules and therefore cannot be the presenting cell: however, the antigen passes from b cells to those APCs found in the draining peripancreatic nodes or in the islets. The nature of this antigen passage is unclear, but the presentation that activates the autoreactive diabetogenic T cell takes place in the APCs at the local sites (119). Of course, many events have to come together not only to activate the T cell but also to bring about the effector side of the reaction. Presentation of the self-b cell antigen is only the initiating event.

ANTIGEN-PRESENTING CELL–T-CELL INTERACTION: CELLULAR EVENTS

Adhesion and Synapse Formation

During presentation, close contact exists between the T cell and the APC (120,121 and 122). T cells remain associated with the APC for several hours, during which time the intracellular events that lead to their activation and expression of different gene programs are taking place. The T cell eventually leaves the APC and enters DNA cell cycle and undergoes differentiation. Investigators believe that during the time of contact there are repeated interactions of the TCR with the peptide-MHC complex (122). Certainly, TCRs are lost from the T-cell surface in amounts unrelated to that of peptide-MHC complex displayed on the APC.

The amounts of peptide-MHC complex required to trigger the T cells depend on the biology of the T cells, indeed, on their state of differentiation and their past experience, that is, whether naive or memory T cells. The immature T cells in the thymus gland, those expressing both coreceptors CD4 and CD8, are unusually sensitive to peptide-MHC complexes and can undergo apoptosis by engagement of as few as ten or fewer. In our own studies using hen egg-white lysozyme, we directly calculated as few as three MHC-peptide complex per APC (123,124). This sensitivity is expected if antigen presentation in the thymus is the means to eliminate T cells that are autoreactive to circulating proteins, if one takes into account the blood–thymus barrier and the relatively inefficiency of processing.

In contrast, the mature T cell that has not encountered antigen requires a few hundred complexes to be stimulated; however, activated T cells require many fewer. So although 3 complexes per APC induced death of double-positive thymocytes, a mature T cell required about 300 complexes, whereas an activated T cell required about 30 to 50 per APC (125,126) (Fig. 6.3).

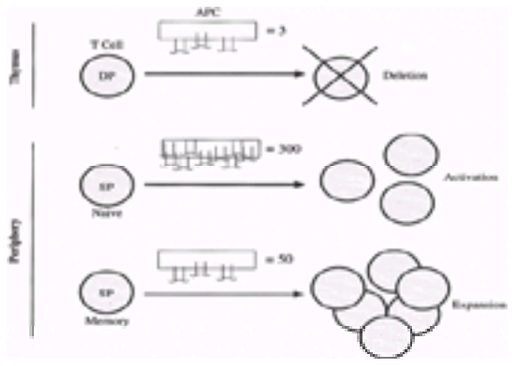

Figure 6.3. The amount of peptide–major histocompatibility complex (MHC) required to activate T cells depends on the state of the T cell. In the thymus, the amounts of complexes that delete double-positive ($CD4^+$ $CD8^+$) T cells is extremely low. This amount is about 100-fold more for stimulating mature T cells. Thus, a biochemical margin of safety protects the individual from self-immunization against peptides expressed in peripheral lymphoid tissues. An activated T cell requires fewer complexes. (Data from Peterson DA, DiPaolo RJ, Kanagawa O, et al. Negative selection of immature thymocytes by a few peptide-MHC complexes: differential sensitivity of immature and mature T cells. *J Immunol* 1999;162:3117–3120; and Peterson DA, DiPaolo RJ, Kanagawa O, et al. Quantitative aspects of the T cell repertoire that escapes negative selection. *Immunity* 1999;11:453–462.)

The interaction of the T cell with the APC results in a rapid reorganization of the molecules in both interacting cell surfaces, in what is termed the *immunologic synapse* (127). During the contact between the two cells, the TCR needs to interact with the few peptide-MHC complexes among plasma membrane proteins of different sizes and structures. Shortly after the cells come together, the center of the contact area of the T cell is occupied by the TCR forming an extensive cluster or aggregate that is surrounded by other molecules on the outer side (128,129,130,131 and 132). On the APC side, the center is occupied by the cluster of peptide-MHC complex (128). This central cluster also contains CD28 and CD4 molecules and is surrounded by the intercellular adhesion molecules (ICAMs). This reorganization of membrane molecules allows for the segregation of molecules that differ greatly in their dimension and otherwise would have difficulty in associating with their complementary protein. Thus, in the synapse, the small TCR can then associate with the MHC spanning a distance of about 15 nm. In contrast, the ICAM interaction with the integrin lymphocyte function antigen-1 (LFA-1) spans about 40 nm. The same situation develops with the long CD45 molecules. This reorganization of plasma membrane molecules in the synapse allows for more effective contact and takes into account the relatively low density of peptide-MHC complexes as well as the low affinity of a TCR for the complex. Indeed, in solution, TCRs bind to peptide-MHC complexes with affinity constants of about 10^{-4} to 10^{-5} M (129). The clustering of TCRs at the synapse creates a matrix that enhances binding by cooperativity of binding sites. Probably the synapse allows for continuous signaling, as well as for the reorganization of the cytoskeleton and the polarization of the T cell. Evidence indicates that the molecule CD28 is important in the reorganization that constitutes the synapse (122,132).

Costimulation and Cytokines

Important in the interactions between CD4 T cells and the APCs are certain auxiliary molecules that favor and regulate the two interacting cells. Two important features to consider in this discussion are the roles of the various membrane or soluble secreted molecules in the regulation of the two interacting cells and the interrelationships among them, in time and after various stimuli, important for the outcome of antigen presentation. The situation favored by many investigators is that the expression of the peptide-MHC complex as a result of antigen processing and selection by the class II molecules is then followed by the selection and engagement of the appropriate T cell, issues discussed earlier. The interactions that follow require the engagement of the costimulatory or auxiliary molecules that are normally expressed at low levels on either the APC or the T cell. The increased activation of the family of costimulatory molecules can be accomplished by two routes. The first route is a result of the antigen itself (e.g., a microbe), which may have properties that activate the APC to release cytokines and to express costimulatory molecules. The second route is by the interaction of the TCR-peptide-MHC complex, which, depending on amount, stimulates the APC to increase costimulatory molecule expression and to release cytokines. Involved here is the CD40 molecule of the T cell. Thus, the nature of the antigen and its amount become critical for allowing antigen presentation to be productive (Fig. 6.4).

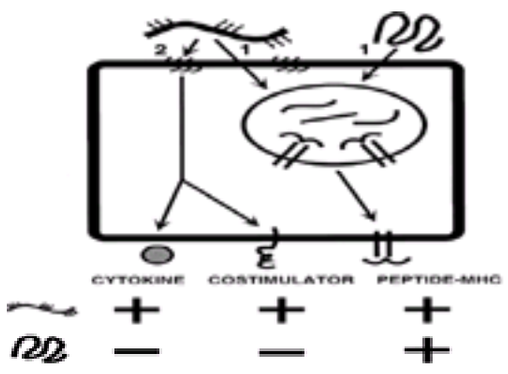

Figure 6.4. Differences in presentation of pure proteins or proteins from microbes. A bacterium **(left)** interacts with Toll-like receptors (TLRs) of the antigen-presenting cell (APC) and stimulates the APC (marked as *2)* to release cytokines and to express costimulators. The protein is processed (indicated as *1)* in vesicles and yields peptides bound to class II major histocompatibility complex (MHC) molecules. In one example, a protein **(right)** enters the APC by fluid-phase endocytosis: it is catabolized to yield peptides, but by itself the protein does not stimulate the APC. In the latter case, interaction with the T cell CD40L is required to activate the APC to express costimulators and cytokines.

Costimulator molecules stimulate T cells once the TCR-MHC interaction has taken place. Although several costimulator systems exist, we mainly consider the CD28 molecules on the T cell, which interact with the B7-1 and B7-2 molecules on the APC. We also review the interactions between another pair of critical molecules, the CD40 ligand (CD40L), which is found on T cells, and CD40 on the APC membrane.

Interactions of CD4 T cells with APCs under situations in which costimulator molecules are absent or their function is blocked result in a weaker T-cell response. These results have led to the *two-signal hypothesis,* which states that the T cell requires two fundamental interactions for activation, one involving the TCR with the peptide-MHC complex and the other involving costimulatory molecules (133). In culture, T cells that are exposed to the first signal (usually in the form of antibodies to the TCR or its associated molecules like CD3) do not proliferate well and become unresponsive because of poor production of IL-2 (134). These T cells are thought to be “anergized.” Anergy does not develop when the CD28 molecule of the T cell is engaged, either by antibodies to it or, physiologically, by engaging the B7-1 and B7-2 molecules of the APCs (134). The relationship of anergy as induced in culture to forms of tolerance *in vivo* is not definitively established (135). Indeed, mice with genetic ablation of B7-1 and B7-2 or of CD28 have defects in T-cell responses, but the T cells are usually not anergized. Nevertheless, engagement of CD28 is important and fundamental for T-cell responses that take place during antigen presentation.

B7-1 and B7-2 (i.e., CD80 and CD86, respectively) are two molecules of the Ig superfamily expressed in low amounts on APCs. This low expression is noticeably augmented by interaction of APCs with cytokines or with microbial products (136,137). Their level also increases during interaction with the CD4 T cells during presentation by the class II–peptide complexes, in a reaction that involves two other important pairs of molecules, the CD40L of the CD4 T cell and the CD40 of the APC (28,29,138,139 and 140). Thus, in a resting situation, the APC is in a relatively dormant state regarding its expression of the molecules involved in presentation. The interaction with the antigen or the T cell regulates the APC and, in turn, activates the T cell.

CD28 is a homodimer with a single Ig-like domain capable of interacting at relatively low affinity with the B7-1 and B7-2 molecules. Engagement of CD28 favors certain cellular and metabolic responses, which include fostering the T cell–APC adhesion, regulating the formation of the synapse, as discussed earlier, and transducing intracellular signals by the phosphorylation of intracellular molecules including the tyrosine kinases lck and itk. CD28 can also activate the jun pathway as well as nuclear factor-kB (NF-kB). Thus, CD28 engagement appears to stimulate multiple metabolic pathways in the T cell (141,142 and 143). CD28 engagement also promotes T-cell survival, most likely as a result of induction of the antiapoptotic molecule bcl-xL (144). Finally, B7-1 and B7-2 also engage the CTLA-4 molecules of T cells in an interaction that stops T-cell activation (136,137). CTLA-4 (CD152) is expressed in small amounts for a brief period, late after T-cell stimulation (145). Genetic ablation of its gene results in uncontrolled T-cell proliferation (146).

Other molecules are involved in the regulation of the T-cell response during the interaction with the APC. Included here are the CD2 molecule of the T cell that also confers costimulation similar to CD28 (147). CD2 is a member of the Ig superfamily and interacts with, on the APC side, LFA-3 (in humans) and CD48 (in the mouse).

The CD40 molecule is a member of the TNF receptor family and binds to its ligand the CD40L (or CD154), with homology to TNF and expressed as a trimer. CD40L is expressed in the resting T cells in small amounts, but it increases during antigen presentation (148,149). It interacts with the CD40 molecule expressed on B cells, DCs, or monocytes. This interaction results in important changes on the side of the presenting cell (28,29,138,139,140,150). In the case of the B cells, it results in their activation, involving both proliferation and antibody class switching (151,152). In fact, the important role of the CD40L was discovered from studies of patients with the immunodeficiency termed hyper-IgM syndrome (149). The disease is caused by mutations in the gene encoding for the CD40L that result in nonfunctional proteins. These patients produce few IgG antibodies and consequently suffer infections with extracellular bacteria. The importance of the CD40L-CD40 interaction is found during antigen presentation involving B cells, as well as DCs and monocytes-macrophages. With these APCs, depending on the antigen, as mentioned earlier, the interaction modulates the expression of the B7-1 and B7-2 expression as well as the release of cytokines.

Finally, during the APC–T-cell interaction, cytokines are released both by the APC and by the T cell. These cytokines profoundly influence the response to antigen presentation. Particularly critical is IL-12, which prepares the responding T cell to express the interferon-g genes. The extent of release of cytokines by the APCs depends on the nature of the antigen. Interactions of bacteria with membrane receptors on the APC can signal the cell to release IL-1, TNF, and IL-12. The TLR family of receptor molecules has been identified as an important component of this response to microbial stimuli. TLR stands for Toll-like receptors, indicating their homologies to proteins of invertebrates that are involved in innate resistance (7,8). TLR molecules are key proteins that signal the APCs to activate and release the cytokines that will influence the outcome of antigen presentation. With regular protein antigens, the stimulus for the release of early cytokines is the interaction between TCR and the peptide-MHC complex. Important again in this respect is the interaction with the CD40L of the T cell (28,29).

Summary

In conclusion, antigen presentation is one of the seminal steps in immune induction, and it represents an eloquent demonstration of the cooperativity and symbiosis between the cells of the innate system—the phagocytes—and those of the adaptive immune reactions—in this case, the T cells. Careful and extensive analysis of this phenomenon allows us to understand many facets of immune induction and to learn how to manipulate it. We are now in a position to understand immunogenicity and to remove the many empiricisms that have dominated this area: the actual chemistry of the immunogens can be determined and their immunogenicity explained in molecular and quantitative terms. Thus, we should be able to create more meaningful vaccines by knowing the structures of MHC-bound peptides. By identifying the molecules involved in various stages of the interaction and the ways in which they influence outcome, we can proceed to foster or to inhibit the interaction. This is now happening by manipulating the adhesion and costimulator molecules such as those of the CD40 or CD28 group (Chapter 87). Finally, we are starting to understand the molecular basis of autoimmunization, as the identity of self-peptides and the role of the MHC are being disclosed.

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7 IMMUNOGENICITY AND ANTIGENICITY

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This chapter summarizes the basic principles as currently understood on the requirements for immunogenicity and antigenicity of protein and peptide antigens, and to a limited extent carbohydrate and lipid antigens and haptens, for antibodies, $CD4^+$ T cells, and $CD8^+$ T cells. These issues depend strongly on concepts developed in more detail in subsequent chapters in this section, in particular those on immunoglobulin, T-cell receptors (TCRs), T-cell biology, antigen presentation, and major histocompatibility complex (MHC) structure and function. Some of these concepts must of necessity be introduced briefly here, but the reader is referred to Chapter 3,Chapter 4,Chapter 5 and Chapter 6,Chapter 9,Chapter 13 for a more detailed treatment.

IMMUNOGENICITY VERSUS ANTIGENICITY

It is first important to indicate some definitions and to clarify the distinction between immunogenicity and antigenicity, which are so often confused. *Immunogenicity* refers to the ability of a molecule to elicit a new immune response against itself, whereas *antigenicity* signifies only the ability to be recognized by the product of a preexisting immune response, be it antibody or T cell. Both concepts also imply that the response is specific for the molecule in question. Specificity, of course, does not preclude some degree of cross-reactivity with other related molecules, but it implies only that the response is limited to chemical structures in some way resembling the molecule in question. Thus, for example, a mitogen such as lipopolysaccharide (LPS or endotoxin) that stimulates a polyclonal B-cell response would not be considered immunogenic unless that response includes antibodies specific for the LPS itself. Similarly, a superantigen such staphylococcal enterotoxin B would not be considered immunogenic because it acts as a mitogen for a subset of T cells with a certain family of receptors, but it does not elicit an immune response directly specifically to itself.

It should be apparent from these definitions, and from clonal selection theory, that antigenicity is a prerequisite for immunogenicity. The theory of clonal selection, as proposed by Burnet (1), states that immune cells preexist in a organism with a broad repertoire of receptors, each specific for a limited number of molecular structures, before a foreign antigen is ever encountered. The foreign antigen then merely selects from this preexisting repertoire to expand those cells with receptors specific for itself. Implicit in this theory, then, is the notion that for a molecule to be immunogenic, that is, able to elicit an immune response, it must also be antigenic, that is, able to be recognized by the specific receptors on preexisting immune cells. When this theory was first proposed, the distinction between T and B lymphocytes had not yet been made, but the theory and this corollary to it apply equally well to B cells with surface immunoglobulins and to T cells with surface antigen-specific receptors.

However, the converse is not necessarily true: that is, it is possible for a molecule to be antigenic without being immunogenic. For example, small organic molecules may be bound by antibodies, but they may not be able to elicit an immune response unless they are attached to a larger molecule, called a carrier. Such molecules that can bind antibodies but not by themselves elicit them are called haptens. Similarly, some short peptides may be antigenic for antibodies or T cells without being immunogenic. Thus, binding to an existing receptor on an immune cell is necessary for immunogenicity but is not sufficient. In the case of polysaccharide antigens, sufficient multivalency to obtain extensive cross-linking of receptors may be the only other requirement, but in the case of protein antigens, the additional requirement is usually the induction of help from a helper T cell to activate either a B cell or a cytotoxic T cell (CTL). The provision of antigenic sites eliciting T-cell help is probably the major contribution of the carrier, although it can also provide some degree of multivalency.

Finally, it is worth clarifying the use of the terms antigenic determinant, antigenic site, and epitope, from the beginning. As originally understood for antibodies, these terms have been generally used as synonymous, applying to the portion of a molecule interacting with the antibody combining site. However, as applied to T cells, the term epitope has been given a narrower meaning in the nomenclature proposed by Heber-Katz et al. (2), to refer only to that portion of the antigenic determinant interacting with the TCR, in contrast to the portion that interacts with an MHC molecule. Antigenic site remains approximately synonymous with antigenic determinant, and both imply the whole structure that must simultaneously interact with the TCR and the MHC molecule to trigger a T cell. Although T-cell epitope is often still used to mean the whole antigenic determinant recognized by a T cell, I use the terms T-cell determinant and antigenic site to avoid ambiguity when discussing T cells.

ANTIGENICITY FOR ANTIBODIES

Because antigenicity is a requirement for immunogenicity, it is appropriate to discuss antigenicity first and then to discuss the additional requirements for immunogenicity. In the case of antibodies, all that is required of a molecule for antigenicity is the ability to bind to the antibody combining site with sufficient affinity to be considered specific in some binding assay, that is, an affinity significantly higher than the affinities of random unrelated control molecules. Thus, operationally, antigenicity depends on the threshold for detection of binding in a particular assay, and on the panel of control molecules chosen. However, in most cases, the affinity is sufficiently high that little ambiguity exists.

Surface Exposure

If binding is the *sine qua non* of antigenicity, then it follows that exposure on the surface of a molecule is a necessary condition for a portion of a molecule to be an antigenic site, at least in the native molecule (3). Some would argue that it is the only requirement; that is, any portion of a protein that is exposed can be bound by some antibody. Certainly, it is the only absolute requirement, but other properties of a structure may make it more likely to bind antibodies, that is, to bind a larger proportion of antibodies. Here, the distinction between antigenicity and immunogenicity becomes less clear, because if a substructure of a molecule binds a larger proportion of the antibodies against the molecule than do other substructures, the implication is that this substructure elicited more antibodies in the first place and therefore was more immunogenic.

Exposure on the surface of a macromolecule can be determined in several ways. Experimentally, one can measure exposure by accessibility to reagents that bind to or modify structures on the macromolecule. Indeed, antibodies can be used in other contexts to define exposure, but in the current context, such a result would be considered a tautology. Ideally, one would like a three-dimensional structure of the macromolecule, such as from x-ray crystallography. However, even with a three-dimensional model, exposure is not always obvious by inspection. Because macromolecules are not smooth spheres, but have bumps and crevices, some

portions of the surface may be accessible to small molecules such as water, but not to a large molecule such as an antibody. Different degrees of exposure can be defined by rolling a sphere of defined radius over the van der Waals surface of the macromolecule (4,5 and 6). A sphere the size of a water molecule contacts more surface area than does a sphere the size of an antibody. However, antibodies are not spheres either, so such comparisons represent only gross approximations. From the crystal structures of known antigen-antibody complexes in which the antigen is a protein (7,8 and 9), the buried surface area on each molecule at the contact site is generally around 800 $Å^2$. The contact surface may be relatively flat but may include sections in which bumps on the antibody fit into crevices on the antigen, as well as the reverse. Therefore, the antigen does not necessarily have to fit inside some large, concave pocket of the antibody molecule. From such studies, it is apparent that many structures not accessible to a sphere the size of an immunoglobulin, or even the size of a pair of immunoglobulin variable (V_H + V_L) domains, could still make contact with amino acid side chains of an antibody combining site. The net affinity of the interaction actually corresponds to a small difference between large free energies of attractive and repulsive interactions over this large, buried surface area (3,10). Thus, small changes in the antigen or antibody structure can greatly affect affinity even though most of the contacts remain unchanged.

Exposure can also be measured by the degree of protrusion of parts of a protein, from a known crystal structure, beyond an ellipsoidal solid enveloping the majority of the protein. Such a protrusion index has been shown to correlate with known antigenic sites (11). However, this result may be caused by the use of peptides to identify antigenic sites corresponding to continuous segments of the protein sequence (see the later discussion on assembled topographic sites).

Hydrophilicity

Hydrophilicity, or a preponderance of hydrophilic amino acids, was proposed as an indicator of regions of protein sequence likely to be antigenic (12,13 and 14). Statistically, the correlation appears to be valid. However, some debate exists on the mechanism. Because water-soluble proteins tend to fold in their minimum energy conformation to bury hydrophobic residues in the interior, away from the aqueous solvent, and to expose hydrophilic residues to the water at the surface, one would expect hydrophilic residues to be exposed more frequently than hydrophobic ones, and indeed this is the case (15). Thus, a segment containing a high proportion of hydrophilic residues is more likely to be on the surface of a protein and to be accessible to an antibody. Therefore, hydrophilicity may be an indicator of antigenic sequences in a protein only secondarily, as a locator of surface-exposed sequences, and not an independent contributor to antigenicity. However, because hydrophilic side chains can participate in types of bonding that hydrophobic residues cannot, there is reason to believe that hydrophilic residues may contribute to the specificity of the interaction and may determine the likelihood of binding by specific antibodies beyond their usefulness as predictors of surface exposure. In either case, because crystal structures are not available for most proteins, and surface exposure cannot be determined from amino acid sequence alone, it remains useful to search for hydrophilic regions of a protein sequence in, for instance, deciding what segment to prepare as a synthetic peptide to produce antibodies that are more likely to interact with the whole protein.

Mobility

Mobility was proposed as a factor in antigenicity of protein segments, based on two complementary types of studies. In the first group of studies, antibodies raised against short peptides corresponding to more mobile segments of the protein were more likely to bind to the native protein than those raised against peptides corresponding to more rigid segments (10,16,17 and 18). In the second group of studies, antibodies raised to the native protein and tested for binding to short peptides from the sequence of the protein bound primarily to peptides corresponding to the more mobile segments of the protein (19,20). However, both sets of results deal more with cross-reactivity between peptides and proteins than they do with antigenicity or immunogenicity *per se* (3,20). In the first case, more mobile segments of the protein can more easily assume conformations like those of the short peptides against which the antibodies were raised than can rigid segments of the protein. In the second case, antibodies to more rigid segments of the native protein may not be detected by screening on short peptides because the antibodies are specific for conformations not assumed by the short peptides. Proof of the latter was obtained when longer peptides were used for screening the same antiprotein antisera (21). The longer peptides (about 20 residues) detected antibodies in the serum that bound to more rigid segments of the protein, that were present all the time but had not been detected using extremely short peptides (of only 6 residues). Thus, these rigid segments were immunogenic as well, but to detect them the probes had to be longer peptides. However, antibodies may also prefer to bind to more mobile segments because these have more flexibility to accommodate themselves to the surface of the preexisting antibody combining site, in an induced fit, independent of the question of detection with short peptides.

In any case, prediction of mobility can contribute to the selection of peptide sequences from a protein for raising of antipeptide antibodies as probes of the native protein. Of course, because mobility can best be determined by x-ray crystallography, if enough protein is available to crystallize, there should also be enough to immunize with the intact protein. However, when all one has is a sequence translated from the DNA sequence of a gene, and no purified protein, antibodies made against synthetic peptides corresponding to parts of this sequence may be especially useful for isolating the protein product of that gene. In such cases, prediction of mobility becomes particularly valuable, but it also is the most difficult to accomplish. One approach is based on the finding that interdomain segments tend to be more mobile than segments within globular domains. Because protein domains tend to correspond to exons in the genomic sequence, it is useful to make peptides close to exon boundaries, as well as at the N and C termini of the protein (17).

Glycosylation

Glycosylation appears to be a mechanism by which some organisms shield their protein antigenic determinants from the immune system. For example, the head of the influenza neuraminidase molecule is a target for antibodies, whereas the stem is not, and this finding corresponds to the fact that the stem is almost completely covered with carbohydrate (22). Although *O*-glycosylation sites are not simply predicted, potential *N*-glycosylation sites are identifiable in a sequence as a tripeptide motif, N-X-S or N-X-T (23). Thus, avoiding such segments in selecting peptides for immunization may be useful.

Assembled Topographic Sites

All the approaches based on raising antibodies to synthetic peptides or testing antiprotein antibodies on short peptides apply only to antibodies that bind primarily to a single continuous segment of polypeptide chain. These are called segmental antigenic sites (24). However, many antibodies, sometimes most, raised to native proteins bind to a region of the protein surface that consists of more than one section of polypeptide chain. These sites are called assembled topographic sites because they are assembled from segments of the protein chain that may be far apart in the primary sequence, but they are brought together on the surface of the native protein by the way it folds (3,24). These may also be called discontinuous sites. For example, 30% to 40% of antimyoglobin antibodies in immune sera from several species of animals were found to bind only to the native protein but not to any of the three cyanogen bromide cleavage products of the protein that span the whole sequence (25). Furthermore, crystal structures of protein antigens bound to monoclonal antibodies show that the surface of the antigen in contact with the antibody consists of more than one segment of the protein sequence (6,7 and 8). A likely explanation comes from studies of residues that neighbor each other on the surface of a protein. Investigators have estimated from known crystal structures that the probability that all neighbors within a given distance of a particular residue are from the same continuous segment in the protein sequence becomes vanishingly small when the distance is greater than 10 Å (26). Therefore, because the area covered by an antibody combining site is much larger than this, one would expect that most antibodies would cover more than one continuous segment of polypeptide. An exception may occur when a single loop of polypeptide protrudes from the surface of the protein, and this finding may account for the correlation of segmental antigenic sites with protruding structures, as discussed earlier (11).

IMMUNOGENICITY FOR ANTIBODIES

Mechanisms of T-Cell–B-Cell Cooperation

To understand the requirements for immunogenicity to elicit antibodies, it is first necessary to understand the basic mechanisms of T-cell help for an antibody response. B cells express surface immunoglobulin corresponding to the antibody they will secrete when activated. Thus, a B cell has a high-affinity surface receptor to bind and take up the antigen for which it is specific. However, in contrast to the original notion of a hapten-carrier bridge between the B-cell surface immunoglobulin and the TCR, the surface immunoglobulin does not display the antigen to the T cell, but captures it and facilitates its internalization through receptor-mediated endocytosis. In the resulting endosomes, the internalized antigen is proteolytically degraded into peptide fragments, which bind to class II MHC molecules that are either recycling from the surface or are newly synthesized. The peptide-MHC complexes are then exported to the cell surface, where they serve as a recognition signal for helper T cells specific for the peptide-MHC combination. Investigators have shown that antigen-specific B cells can take up antigen at 1,000-fold lower concentrations than B cells not specific for that antigen, as measured by stimulation of the relevant helper T cell (27,28 and 29). The peptide that binds to the class II MHC molecule is not necessarily related to the antigenic determinant bound by the surface antibody, and indeed, it may be more effective if these are some distance apart. However, the determinant bound by the surface antibody must be physically attached to the peptide that will be presented to the T cell, to take the latter with it into the cell. This explains the requirement for a hapten, that can bind to an antibody but cannot itself bind to an MHC molecule to elicit help, to be attached to a carrier that can be recognized by helper T cells. Such help produced by an attached carrier is called cognate help, in contrast to bystander help, which can be mediated by the secretion of lymphokines in response to a separate stimulus. To elicit such cognate help, the B-cell and T-cell antigenic determinants do not have to be covalently attached, but only physically associated. For example, noncovalent charge complexes of strongly acidic and strongly basic polypeptides also work (30). Moreover, association in a particulate structure, such as a virion, may be sufficient if the whole virion is small enough to be internalized with the surface immunoglobulin. This phenomenon has been called intermolecular intrastructural help (31), and it is exemplified by the ability of helper T cells specific for the core protein of hepatitis B to help B cells specific for the surface protein of the virus (32). It has also been observed with T-cell and B-cell determinant peptides noncovalently associated in liposome membranes (33).

Requirements for Immunogenicity

Antigenicity

Because a B cell from a preexisting repertoire must bind the antigen with its surface immunoglobulin to be activated and to take up the antigen and process it to induce T-cell cognate help, as just discussed, antigenicity is clearly a prerequisite for immunogenicity.

T-Cell Help

In the case of T-dependent antigens, such as most proteins, T-cell help is also required. This means that the B-cell determinant must be physically attached to a helper T-cell determinant, as discussed earlier, to bring the latter into the cell where it can become bound to class II MHC molecules and presented to T cells. However, because the class II MHC molecules are polymorphic in many of the residues in their peptide-binding groove (34,35), the class II MHC molecules expressed on a particular B cell determine which peptide fragments of the protein antigen or carrier protein can bind to those MHC molecules and can be presented by that B cell. These requirements are discussed further in the later section on helper T cells and in Chapter 3 and Chapter 6. The peptide binding specificity of MHC molecules thus accounts for MHC-linked *Ir* gene control of immune responses, in which the same antibody determinant or hapten may be immunogenic or not, depending on the helper determinants to which it is attached and the MHC molecules of the animal being immunized (36,37).

Difference from Self

A structure may be antigenic in that it can bind to antibodies raised in another animal, but it may not be immunogenic in a particular animal because it resembles some autologous protein to which the animal is tolerant. Self-tolerance and the regulation of the repertoire are discussed in detail in Chapter 9. Here, it is worth pointing out that when one examines the antigenic structure of a mammalian protein used to immunize another mammal, the regions of difference from the homologous self-protein are the primary targets of antibodies (24). This has been clearly shown for cytochrome *c* (24,38) and for myoglobin (39). Even a single amino acid difference is sufficient to permit recognition as nonself (24,38).

Influence of T-Cell Help on Antibody Specificity

Two types of influence have been described. First, helper T cells specific for some antigenic determinants may more efficiently provide help for some B-cell antigenic determinants on the same protein than others. This has been observed by using defined populations of helper T cells to help an antibody response, by noting the differences in antibodies produced (40,41,42 and 43), and by using B cells with defined surface antibody to stimulate T-cell clones specific for different determinants on the same protein (44). Investigators have also observed that macrophages taking up immune complexes consisting of the same antigen bound to different antibodies preferentially stimulate different helper T cells (45). The mechanism thus proposed is that what an antigen-specific B cell takes up to process is not just free antigen, as in the case of a macrophage or dendritic cell; rather, it is an immune complex of the antigen with a monoclonal antibody, namely, the B cell's own surface immunoglobulin, and the bound immunoglobulin influences the way the antigen is proteolytically processed (46). Many antigen-antibody complexes are stable at the pH of the endosome (in the range of pH 5 to 6). Furthermore, *in vitro,* antibody has been shown to protect its antigenic determinant from proteolysis sufficiently to allow mapping of an antigenic determinant by analysis of the proteolytic fragments produced in the presence and absence of antibody (47). Finally, direct proof of this hypothesis has been obtained by the demonstration that the products of antigen processing by B cells depend on the epitope specificity of the surface immunoglobulin of the monoclonal Epstein-Barr virus–transformed B cells used (48). Moreover, the same surface immunoglobulin can enhance presentation of one determinant and can hinder presentation of an adjacent determinant, both within the footprint of the antibody (49). This mechanism may explain how MHC-linked *Ir* genes, which control T-cell specificity, can also control the fine specificity of an antibody response (46,50,51). It also has practical implications for the use of specifically designed immune complexes as immunogens to steer the immune response and to enhance responses to subdominant epitopes. This influence may also contribute to epitope spreading (52), in which the response to one antigenic site facilitates induction of responses to other sites.

The second type of influence has to do with the way short peptides are attached to carrier proteins to make them immunogenic. Investigators have observed that the antibodies raised against these complexes are predominantly specific for the end of the peptide that is not tethered to the carrier protein (53). Thus, attaching a peptide through its carboxy-terminal favors antibodies to the amino-terminal, and *vice versa.* Attaching a slightly longer peptide by both ends favors antibodies to the middle. This observation can be useful in designing appropriate coupling methods for particular peptide-carrier complexes.

Immunogenicity of Polysaccharides

Unlike proteins, polysaccharides have not been found to bind to and be presented by MHC molecules to T cells, and in general, T cells do not recognize carbohydrate (54). Therefore, for a polysaccharide to be immunogenic, it has to be either sufficiently polyvalent to elicit a T-independent response or attached to a protein as a carrier. Polysaccharides are the prototypical antigen for a T-independent response, probably because they tend to have multiple copies of the same structure to allow sufficient cross-linking of the B-cell surface immunoglobulin to trigger such a response. However, more than cross-linking is involved. Some studies have shown that T-independent responses are primarily mediated by a specialized subset of B cells bearing specific surface markers such as Lyb 5 (55,56). Because such B cells appear late in ontogeny, they are absent from human umbilical cord blood and from newborns during the first few months of life, and thus newborns are defective in their response to polysaccharide antigens (57,58). This situation has serious practical implications for the development of polysaccharide vaccines against bacterial pathogens in newborns, and it has forced vaccine designers to use bacterial polysaccharides attached to a protein carrier to create a T-dependent vaccine that is effective in newborn children (57).

IMMUNOGENICITY AND ANTIGENICITY FOR $CD4^+$ HELPER T CELLS

Because immunogenicity of protein antigens for B cells generally requires induction of helper T cells as well, immunogenicity for such $CD4^+$ helper T cells is necessary for the protein antigen to be immunogenic for both B-cell and T-cell responses. Immunogenicity for $CD4^+$ T cells, which generally recognize complexes of peptide fragments of proteins bound to class II MHC molecules, requires a segment of the protein that can survive proteolytic processing of the protein into fragments, generally in endosomes, and can bind to both a class II MHC molecule and a TCR of a helper T cell that has not been deleted or anergized by self-tolerance. Each of these requirements is addressed in turn.

Role of Antigen Processing

The pathways of antigen processing are discussed in detail in Chapter 6. Here, the focus is the impact of processing on T-cell recognition. The apparent purpose of proteolytic processing of antigens is to produce a structure that can interact with the peptide-binding groove of an MHC molecule and to have relevant portions accessible to interaction with a TCR (59,60 and 61). Most globular proteins or domains of proteins are too bulky, and if a polypeptide segment on one exposed surface of the protein could interact with the MHC molecule, the other side of this segment would likely be buried in the interior of the protein and would be inaccessible to a TCR. Thus, if exposure of all sides of a polypeptide segment is the critical requirement, it is conceivable that simply unfolding a small protein or protein domain may be sufficient to accomplish this goal. Indeed, several early studies indicated that unfolding a protein was sufficient without proteolysis (60,62,63 and 64). Moreover, even a short peptide that was constrained by disulfide bonds required reduction and unfolding to allow T-cell recognition (65), so short length is not sufficient in the absence of unfolding. The ability of longer, unfolded polypeptides to bind to class II MHC molecules with the ends hanging out of the peptide-binding groove has been confirmed by x-ray crystallography (66,67,68,69 and 70).

However, in most cases, unfolding is accomplished by proteolytic digestion of the protein into smaller fragments. The exact size of these fragments has not been determined in most cases, but because peptides eluted from class II MHC molecules generally range from 13 to 18 residues, with some much longer (71,72), it is unlikely that these fragments correspond exactly to the synthetic peptides widely used to study T-cell recognition. Indeed, the naturally processed fragments may be considerably larger than the actual T-cell antigenic determinant. A crystal structure study of a TCR-peptide-MHC class II molecular complex shows that the length of the peptide contained within the MHC II groove and interacting with the TCR is about 9 residues, as in the case of class I molecules, but additional peptide may extend from both ends of the groove. These additional residues may not be merely extraneous, but they may actually interfere sterically with binding of the peptide to the MHC molecule or to the TCR (73,74 and 75). In one such case, the hindrance was MHC specific, so the naturally processed fragment of a protein was presented by one class II MHC molecule but not by another, even though a synthetic peptide corresponding to the antigenic determinant contained within the natural fragment could be presented by both MHC molecules (73). In a second example, a 10-residue peptide was presented, but a 15-residue peptide containing this was not (74). Mutations in the additional 5-residue extension, or within the original 10-residue sequence, could restore function. This result suggests that interaction between residues in the extension and the original peptide led to obstruction of interaction between the antigenic determinant and either the MHC molecule or the TCR. Another situation in which the structure of the naturally processed fragment can influence recognition is the competition among antigenic determinants on the same fragment for binding to the same or different MHC molecules (61,75,76). High-affinity binding of one determinant to one MHC molecule may prevent binding of a different determinant on the antigen fragment to a different MHC molecule. Thus, the location at which a peptide is cleaved during processing can profoundly affect both antigenicity and

immunogenicity of a determinant for T-cell recognition, and that effect may be different in individuals of different MHC types, even if the processing is the same.

Finally, the determinant must not be destroyed by processing. Thus, otherwise potentially recognizable determinants, if they contain a cleavage site for a protease involved in processing, may be immunologically silent. For this reason, it may be important that many proteins are processed in endosomes in which proteolysis is not complete and the antigens are not exposed to the full proteolytic capacity of the cell in lysosomes, although cases may also occur in which the more vigorous proteolysis of lysosomes is required (77,78 and 79). In addition, for both antigenicity *in vitro* and immunogenicity *in vivo,* proteases in serum may mediate extracellular processing that can lead to degradation of the antigenic determinant (80,81), or in some cases even activation of the determinant by appropriate processing (81,82).

For all these reasons, a more complete characterization of the enzymes involved in processing, and of their sequence specificity, would greatly aid in predicting the availability of sequences for presentation, and their susceptibility to degradation. Cathepsins B, D, L, and E have been implicated in processing different proteins (83,84,85,86 and 87). Cathepsin S has been implicated primarily in cleavage and degradation of the MHC class II invariant chain in both murine and human antigen-presenting cells, and so it affects presentation of peptides by class II molecules by contributing to the removal of invariant chain that protects the peptide-binding pocket (88,89). Thus, protease inhibitors that inhibit antigen presentation may be affecting either the degradation of the antigen itself or the processing of invariant chain to allow peptide binding. Unfortunately, the specificity of these cathepsins is not as sharply defined as that of restriction endonucleases, or even that of trypsin.

Binding to Class II MHC Molecules

As discussed in Chapter 3, both class I and class II MHC molecules are polymorphic within most mammalian species, and in particular are so in humans. Moreover, the structural differences among such MHC molecules have been found to occur primarily within the groove identified by x-ray crystallography as the likely site for peptide binding (66,67,68,69,70,90,91,92,93,94,95,96,97 and 98). This variability in the peptide binding site in large measure accounts for the phenomenon of immune response *(Ir)* genes (37,99), which are defined as genes that determine in an antigen-specific fashion whether an individual or inbred strain of animals will respond to a particular antigen. Many of these genes, defined functionally by the magnitude of antibody or T-cell responses to specific antigens, were mapped to the MHC and, for antibody and $CD4^+$ T-cell responses, were mapped to the region encoding the class II MHC molecules (36,37). Indeed, the original name of the class II molecules, Ia antigens, stands for *Ir*-associated antigens. However, it was only by use of structural mutations in the class II MHC molecules, and by production of class II transgenic mice, that investigators finally proved that the *Ir* genes were the structural genes for class II MHC molecules, and not merely genes mapping nearby (37). Indeed, studies of single residue mutations in the peptide binding groove of class II MHC molecules show that structural changes in the groove affect not only whether a peptide binds, but also how it binds, that is, in what conformation or orientation (100,101). Similar findings have been made for class I MHC molecules (102). These differences in conformation or orientation of the peptide induced by the structural differences in the MHC groove also affect T-cell recognition of the peptide.

The important functional result of the class II MHC polymorphism is that each MHC molecule binds a different (although possibly overlapping) set of peptide fragments from a given protein. If few or no peptide fragments from a protein bind to any of the class II molecules of an individual, then that individual is a low or nonresponder to that protein. Therefore, immunogenicity of a protein varies among individuals of a species, depending on their MHC type. In this context, immunogenicity is not the inherent property of a structure, but it depends on the recipient of the immunization.

For this reason, attempts have been made to identify sequence motifs that are predictive of binding to specific MHC molecules (103,104,105,106,107,108 and 109). Several such motifs have been defined for a few murine and human class II molecules (103,104,106,108,109,110,111,112,113 and 114), but they are much less clear-cut and consistent than motifs defined for peptides binding to class I MHC molecules (see later). This may in part relate to the variability in length of peptide binding class II MHC molecules. As noted earlier, in contrast to peptides naturally bound to class I MHC molecules (8 to 10 residues; see later), naturally bound peptides eluted from class II molecules appear to be much longer, ranging from 13 to 18 residues (71,72). The reasons for this greater permissivity in length are that both ends of the class II molecule's peptide-binding groove are more open than those of class I molecules, and the peptide thus can extend from both ends (66,67,68,69 and 70,96,98). The nonpolymorphic peptide-binding residues of MHC class II molecules interact with the peptide main chain backbone, rather than with the amino-terminal and carboxyl-terminal, as in the case of the class I MHC molecule, so the ends of the peptide do not have to be buried. The result, however, is that it is more difficult to define precise binding motifs, because the key peptide anchor residues that fit in pockets of the MHC class II molecule may occur at any random distance from the amino-terminal of the peptide, in contrast to peptides binding to class I molecules in which the anchor residues occur at fixed distances from the ends. Thus, sequencing of unseparated eluted peptides from class II molecules is less likely to reveal clear patterns (see later). Furthermore, in the $I\text{-}A^d$ molecule, specific high-affinity binding occurs without any large anchor residues fitting into pockets in the class II MHC molecule (98). The groove of the class II molecule may also be more flexible, because it is assembled from two separate polypeptide chains, rather than from two domains on the same polypeptide chain as in the case of class I molecules. Nevertheless, motifs for certain MHC class II molecules have been reported (108,110,111,112,113 and 114), as reviewed in (109,115,116 and 117), and have proved useful for prediction of antigenic determinants of medically relevant proteins (112,118,119,120 and 121).

Even before motifs were found for peptides binding to specific MHC molecules, sequence patterns were observed that occurred more commonly in peptides recognized by T cells than those that were not (103,122,123,124,125,126,127 and 128). As pointed out by DeLisi and Berzofsky (122) and Cornette et al. (125,126), all these patterns tend to represent periodicities in hydrophobicity of amino acid residues consistent with sequences that fold as amphipathic helices. An amphipathic structure is one that can be separated into hydrophilic and hydrophobic regions. Thus, an amphipathic helix is one in which the hydrophilic residues are aligned preferentially on one side of the helix, and the hydrophobic residues are aligned on the other. Such helices are found in native proteins, especially among helices in which one face of the helix is buried in the protein and the other is exposed to aqueous solvent (15). Amino acid sequences that would be amphipathic if folded as a helix were found to be statistically significantly more frequent among known T-cell antigenic sites than among random segments of the same proteins in which the T-cell sites had been identified, by several different statistical comparisons (123,124,129). A computer program was developed to search protein sequences for such sites (124). As the number of known antigenic sites for helper T cells has increased exponentially, the statistical significance of the correlation has remained high ($p < .001$) (125,126,129). Thus, the statistical correlation has remained robust, a finding suggesting that this property reflects something important about the chemistry of T-cell recognition.

However, the crystal structures of class I and class II MHC molecules have revealed extended peptides in the MHC grooves, not helices (66,67,68,69,70,91,92,93,94,95,96,97,98,130,131,132,133,134,135,136 and 137). How, then, can one explain the statistical correlation with periodicity of hydrophobicity? Two explanations have been suggested. First, in class II molecule crystal structures, the peptides have been found to lie in the groove with a 130-degree twist, like that of a type II polyproline helix (67,69), and the hydrophobicity of these sequences shows a 130-degree periodicity (129). Although not identical to an a helix (100-degree periodicity) or a 3^{10} helix (120-degree periodicity), this 130-degree twist is close enough that computer algorithms designed to detect such periodicity of hydrophobicity would detect them (129). Second, the spacing of the anchor residues that bind to pockets in the MHC molecule (92,132,135,136,138,139) tends to be consistent with the spacing between residues on the same side of an a helix. The anchor residues of class I MHC–binding peptides (that are best defined) are most commonly at positions 2, 5, and 9 (for different MHC molecules 2 and 9 or 5 and 9), and the spacing between these approximates the distance between one or two turns of an a helix (3.6 or 7.2 residues). Because most anchor residues are hydrophobic, if other residues are more random, such spacing often results in a periodicity of hydrophobicity like that of an amphipathic a helix even though the peptide is bound in an extended conformation (129). Effectively, the spacing of pockets in the MHC groove enforces a spacing of hydrophobic anchor residues that, in turn, creates the observed periodicity in hydrophobicity. Thus, these hydrophobicity sequence patterns represent the first sequence motifs detected to T-cell antigenic determinants, and they define an overall pattern that encompasses most of the motifs known for peptides binding murine and human MHC molecules and that may continue to be useful.

Recognition by a T-Cell Receptor

Once bound to a class II MHC molecule, the peptide must still be recognized by a TCR. Although the potential repertoire of TCRs is enormous, probably comparable to that of antibodies despite the lack of somatic mutation, it is not infinite. Holes in the repertoire have been reported (140). However, such holes are rare (141). There is considerable plasticity in the repertoire, so if one family of TCR genes is deleted that normally predominates in a response to a given antigen, a response to the same antigen can be made using other families of TCR genes (142,143). For this reason, although some cases have been reported of limited use of particular a or b chain TCR variable regions in the response to particular antigens (144,145,146,147,148,149,150 and 151), such constraints do not appear to have a major impact on immunogenicity. Indeed, the repertoires of murine and human TCRs are sufficiently broad that peptide binding to MHC molecules tends to be the limiting factor, so in the case of CTL responses to peptides presented by class I MHC molecules, CTL responses in human leukocyte antigen A2.1 (HLA-A2.1) transgenic mice have been found to predict antigenic determinants recognized by human CTLs in association with HLA-A2.1 (152).

A much greater limitation than holes in the potential TCR structural repertoire imposed by the availability of TCR genes and by chemical constraints on the structure is the narrowing of that potential repertoire by self-tolerance, as well as possibly by positive selection for recognition of peptides bound to particular MHC molecules (so-called self-MHC restriction). These functionally imposed holes in the repertoire must be sizable to exclude reactivity to a host of normal self-proteins. Thus, a protein identical to a normal protein of the animal or person being immunized would not be expected to be immunogenic. Moreover, a related protein that differs only in one or two amino acid residues from a self-protein would be expected to be immunogenic only if those amino acid substitutions occur in peptide fragments that can bind to and be presented by class II MHC molecules of the responding person or animal. Thus, some differences from self may be detected in individuals of some MHC types but not in individuals of other MHC types. This MHC-linked difference in the ability to break tolerance by particular mutations may play a role in the MHC-linked propensities to particular autoimmune diseases.

This last point is related to the notion that self-tolerance is MHC restricted (153,154 and 155). By this, I mean that one can be tolerant to only those peptide fragments of self-proteins that bind to self-MHC molecules and thus lead to tolerance. Peptides of self-proteins that do not bind to any self-MHC molecule would be

immunologically silent. However, they may be recognized as part of an alloantigen, because the complex of a self-peptide with an allo-MHC molecule to which it can bind is a different antigen from the complex of the same peptide with a self-MHC molecule, given that the MHC molecule contributes to the total structure bound by the TCR. It is conceivable that a mutation could arise in a self-protein that affected only the ability of a peptide fragment to bind to a self-MHC molecule, so a formerly silent peptide, to which one was not tolerant, could now become immunogenic. Such events could also therefore contribute to autoimmune disease.

Immunogenicity of Peptides, Haptens, and Carbohydrates

The immunogenicity of short peptides for $CD4^+$ T cells depends on the same considerations as that of whole proteins, except the short peptide may not need processing. However, it still must survive the processing enzymes, and even extracellularly, it may be susceptible to degradation by proteases in serum (80,81 and 82). Most peptides that bind to class II MHC molecules can elicit a $CD4^+$ T cell-response. However, some peptides bind to a class II MHC molecule, but the complex produced fails to activate a T cell, and it serves as an inhibitor of T cells specific for a structurally related peptide-MHC complex. Such peptides are called antagonist peptides or altered peptide ligands and are reviewed in detail elsewhere (156). In between fully agonist peptide-MHC complexes and antagonists are some peptide-MHC complexes that are partial agonists, that can induce only a partial signal, and thus produce a subset of the responses of which the T cell is capable (156,157,158,159,160,161,162,163,164 and 165). One potential mechanism for such partial responses is a reduced dwell time of the peptide-MHC complex on the TCR, insufficient to permit transduction of the complete signaling process (166,167,168,169,170 and 171). Thus, this phenomenon is at least partly related to the affinity of the peptide-MHC complex for the TCR. If such antagonist or partial agonist peptides are produced by pathogens, they could contribute to induction of anergy in T cells that could otherwise contribute to protection. They may also skew the cytokine phenotype and thus alter the outcome of the immune response produced.

Haptens, which are small molecules that themselves are not immunogenic, but require attachment to a carrier, almost by definition are not molecules that can bind directly to MHC molecules and be presented to T-cells. How then can one account for apparent cases of hapten-specific T cells (172)? At least one explanation has come to light, by the demonstration that MHC-restricted trinitrophenyl (TNP)-specific T cells are actually recognizing a TNP group attached to an amino acid residue of a peptide that does bind to the MHC molecule in question (173,174). Furthermore, apparent differences in T-cell specificity in this system appear to relate to the position along the length of the peptide at which the TNP group is attached. Thus, in this case, the hapten-specific TCR is binding to a conventional peptide-MHC complex, and the TNP group is serving merely as a modified side chain of one of the peptide amino acids. Similarly, T cells can be specific for carbohydrate moieties (175,176,177 and 178) or even metal ions (179,180,181 and 182) bound to peptides (or proteins that can be processed into peptides) in analogous fashion.

IMMUNOGENICITY AND ANTIGENICITY FOR $CD8^+$ CYTOTOXIC T LYMPHOCYTES

Many of the same considerations apply to $CD8^+$ CTL recognition that applied to $CD4^+$ helper T cell-recognition (183,184). The main differences arise because the class of MHC molecule presenting to $CD8^+$ cells is usually class I rather than class II, with their different binding constraints, and because the pathway for processing of antigens to be associated with class I MHC molecules is different from that for class II. In addition, a fourth step influencing specificity, between antigen processing and MHC binding, applies in the case of $CD8^+$ T cells, because a specific active transport mechanism translocates processed peptides from the cytosol to the endoplasmic reticulum, where they are loaded onto nascent class I MHC molecules. The transport is mediated by the transporter associated with antigen processing (TAP) 1 and 2 proteins, which shows preferences for peptides of certain size and sequence.

Antigen Processing

In general, class I MHC molecules bind peptide fragments of proteins synthesized within the cytoplasm of the cell or introduced there in some other way, rather than proteins taken up from outside the cell, as in the case of class II MHC molecules (185,186,187,188 and 189). This difference arises from the finding that peptide fragments produced in the cytoplasm are transported by a specific transporter mechanism into the endoplasmic reticulum (190,191,192,193,194,195 and 196), where they encounter nascent class I MHC molecules that actually require bound peptide to be stabilized in their native conformation and to be transported out to the surface of the cell efficiently (189,197,198,199 and 200). In contrast, class II molecules in the endoplasmic reticulum cannot bind peptide because they are complexed to an invariant chain, which is not dissociated until the class II molecule reaches an endosome, where fragments of exogenous antigens are produced (201,202,203,204 and 205). This mechanism, which is described in more detail in Chapter 6, helps to segregate the two pathways to allow class I molecules to sample primarily the proteins in the cytoplasm of the cell and class II molecules to sample the proteins taken up from outside into endosomes. The functional importance of this segregation is that class I molecules present their peptides to $CD8^+$ CTLs, which provide immune surveillance to destroy cells producing inappropriate proteins, such as viral proteins in a virally infected cell, or mutant proteins in a cancer cell, whereas class II molecules present their peptides to helper T cells that activate the presenting cell, be it a B cell to make immunoglobulin or a macrophage to trigger its cytolytic or oxidative machinery. Clearly, one would not want a B cell that had finally encountered the antigen for which it was specific to present it to a $CD8^+$ T cell and be destroyed instead of positively selected and expanded. Conversely, one would not want a virally infected cell to present viral antigen to a helper T cell and be activated to produce more virus, rather than being destroyed. Thus, a clear rationale exists for the segregation of the two pathways.

From our perspective in terms of immunogenicity, this segregation of processing pathways implies that immunization with an intact protein that suffices for induction of $CD4^+$ helper T cells and for antibody production may not be sufficient for induction of $CD8^+$ CTLs. The most effective general method for inducing a class I MHC–restricted response to an antigen is to have the antigen expressed by a virus with which one can infect the recipient, so the antigen in question is expressed in the cytoplasm of the infected cells. For this purpose, many recombinant viral vectors have been prepared, such as the many recombinant vaccinia viruses now in use (206,207). More recently developed DNA vaccines accomplish the same goal by expressing the relevant antigen in the cytosol of professional antigen-presenting cells (208,209,210,211,212,213,214,215,216,217,218,219,220,221,222,223,224,225,226,227,228 and 229), such as dendritic cells or macrophages (217,221,230). Townsend and coworkers opened up new approaches in 1986 by showing that short synthetic peptides could bind directly to class I MHC molecules on the surface of cells without entering the class I processing pathway (231). This approach was initially applicable to antigenicity, rather than immunogenicity, because most early attempts to use such short peptides to immunize failed. However, the approach was extremely valuable for mapping epitopes with short synthetic peptides. Subsequently, methods of immunizing with peptides to induce $CD8^+$ CTLs were reported, using either free peptides in adjuvant (232,233,234 and 235), peptides attached to a lipid tail (236,237), or peptides coated onto cells that were then injected (238,239,240,241,242,243,244 and 245). In some of these cases, the peptide immunization actually led to protective immunity (234,238,240,241,242 and 243).

The limitation on using whole native purified proteins can be circumvented as well. For example, incorporation of the whole protein into ISCOMs, which are immunostimulatory complexes consisting of 35-nm particles containing the protein with cholesterol and Quil A, a mixture of natural plant saponins (246), could induce $CD8^+$ CTLs to human immunodeficiency virus type 1 (HIV-1) envelope protein or influenza hemagglutinin, whereas whole protein in saline or complete or incomplete Freund's adjuvant did not (247). Although it was originally supposed that the detergent-like properties of the ISCOM allowed the antigen to enter the cytoplasm of the cell and thus the class I processing pathway, the difficulty in using ISCOMs to sensitize targets for recognition by CTLs *in vitro* has hampered the elucidation of the mechanism (unpublished data). However, investigators were able to show that ISCOMs containing whole measles fusion protein could deliver the protein to the endogenous processing pathway for recognition by class I–restricted CTLs (248). In any case, protein in ISCOMs consistently induces CTLs that are specific for the same antigenic determinants that are dominant in the response induced by live viral vectors (247). QS21, a less toxic component of Quil A that retains adjuvant activity, can also facilitate induction of $CD8^+$ CTLs with soluble whole proteins (249,250) and peptides (251,252). Similarly, proteins in liposomes can be used to immunize to elicit $CD8^+$ CTLs. Particulate antigens can also escape from endosomes into the cytosolic class I processing pathway (253,254 and 255). Viruslike particles are especially useful in this regard (256,257 and 258). Finally, whole proteins coated onto the surface murine spleen cells and injected intravenously can induce $CD8^+$ CTLs (259,260 and 261). It appears that the cells, or the antigen they carry, are processed and are represented by some cell in the recipient, because parental spleen cells bearing antigen injected into an F_1 hybrid recipient induce CTLs restricted to both MHC types of the F_1, not just the one of the injected cells (260). The mechanism likely involves uptake of apoptotic bodies of the injected cells by dendritic cells of the recipient (262). This method induces CTLs that have the same epitope specificity and same MHC restriction as those induced by live viral immunization (261).

As in the case of $CD4^+$ helper T cells, recognition of a determinant with a class I molecule requires that it not be destroyed by the processing mechanism and that it be made accessible to both the class I MHC molecule and to the TCR. In this regard, investigators observed in one case that flanking residues adjacent to the minimal determinant inserted into the sequence of a fusion protein expressed by a recombinant viral vector influenced whether the minimal determinant was recognized (263). However, a similar study with another protein found no effect of flanking residues (264), so it is not clear how often this effect is a problem.

Studies with specific inhibitors of proteasomes indicated that these large proteolytic complexes are the primary machinery for cytosolic proteolytic processing of proteins to produce peptides for the class I pathway (265,266,267 and 268). The relative efficiency of proteasomes to cleave cytosolic proteins at different sites can significantly influence the relative dominance of different antigenic determinants of a protein for CTL recognition (183,184,269). In particular, the MHC-encoded LMP-2 and LMP-7 subunits of the proteasome can influence the site of cleavage (266,270,271 and 272), thus affecting the relative amount of cleavage after hydrophobic or basic amino acids versus acidic ones. Because class I MHC–binding motifs of peptides typically involve hydrophobic or basic, not acidic, residues at the C terminus (see later), these subunits can enhance the production of peptides that can potentially bind to class I MHC molecules. The production of these subunits is induced by interferon-g (270), so interferon-g can enhance processing of relevant peptides for CTL recognition. Another interferon-g–inducible protein called PA28, which is associated with the proteasome, may influence the length of the peptide produced and thus may also affect the repertoire of peptides available for binding to class I MHC molecules (273,274). Another study using purified proteasomes for digestion of well-characterized antigens showed a predominant length of peptide produced in the 7- to 10-residue range, and a distribution of terminal residues corresponding to that for class I MHC binding (275,276). Furthermore, the production of a

immunodominant epitope far exceeded that of a subdominant epitope, a finding suggesting a role for proteasome specificity in determining immunodominance (276). This proteasome specificity preceded the evolution of the MHC and the vertebrate immune system, consistent with the possibility that the MHC evolved taking advantage of the preexisting proteasomal system (277). A more recent analysis of naturally processed peptides indicated that the cleavage depends most heavily on the residues on either side of the cleavage site, but this is especially notable at the C terminus, presumably because the N terminus is subject to further trimming in the endoplasmic reticulum (278). Thus, the specificity of the proteasome, which can be altered by components such as LMP-2, LMP-7, and PA28, which are induced by interferon-g, is a key factor in the first step of the multistep pathway for selection of antigenic determinants recognized by $CD8^+$ T cells. It is relevant, therefore, that the size of the peptides produced and the nature of the C-terminal residues are compatible with those of peptides binding to class I MHC molecules.

Finally, when peptides are used exogenously to bind to MHC molecules on the surface of target cells, peptides longer than appropriate for binding to the class I MHC groove (see later) may be effective in serum, but not without serum, because proteases in serum process the peptides down to a functional size (81,82,279). Examining presentation by purified MHC molecules in the absence of presenting cells allowed the identification of one serum protease that processes antigenic peptides as the angiotensin-converting enzyme, inhibitable by angiotensin-converting enzyme inhibitors such a captopril (81). However, if the minimal length peptide is used in serum, it may actually be less potent than in the absence of serum, because serum proteases can further divide it into smaller peptides (80,81). Thus, for example, the minimal 10-residue peptide of the HIV-1 envelope, RGPGRAFVTI, presented by $H\text{-}2D^d$, is about 10-fold more potent on a molar basis than the 15-residue peptide RIQRGPGRAFVTIGK in the presence of serum, but it is more than a millionfold more potent in the absence of serum (81).

Transport into the Endoplasmic Reticulum

The second selective step influencing which potentially antigenic determinants are available for recognition by $CD8^+$ T cells is transport of peptides from the cytosol to the endoplasmic reticulum (ER) by an active transport mechanism mediated by the transporter associated with antigen processing (TAP) 1 and 2 complex of proteins. TAP 1 and 2 are 70-kd proteins of the adenosine triphosphate–binding cassette family of transporters, such as multidrug resistance protein and the cystic fibrosis transporter (280,281 and 282). The observed association of class I MHC heavy chain–b_2-microglobulin complexes (but not free heavy chains) with TAP in both murine and human cells suggested that TAP may facilitate peptide loading of class I MHC molecules in the endoplasmic reticulum by transferring peptides it transports directly to bound empty class I MHC molecules (283,284 and 285). This process may be facilitated by a third molecule, tapasin, which appears to mediate the association between class I MHC molecules and TAP and may facilitate peptide loading, although it may not be essential (286,287 and 288).

Relevant to the issue of antigenicity and immunogenicity, the TAP molecules appear to bind and transport some peptides more effectively than others, and they contribute a second step during which potential antigenic peptides are selectively winnowed (183). The functional significance of this was demonstrated by the observation that a polymorphism in TAP in the rat that affected binding of peptides with different C-terminal residues led to MHC-linked histoincompatibility in strains of rats that had identical MHC molecule sequences (289). TAP selectively transports peptides of 9 residues or longer in the mouse (290), and in the human TAP preferentially transports peptides of 8 to 11 residues and rarely transports peptides shorter than 7 residues or longer than 24 residues (291). Using glycosylation of an indicator peptide as a marker of transport into the endoplasmic reticulum, and using competition with other peptides, investigators showed that the sequence specificity of the TAP complex is much broader than the binding specificity of MHC class I molecules (291), as could be expected because the same TAP molecules must function with multiple class I MHC molecules. Nevertheless, selection against peptides with Pro at positions 2, 6, or 9 was found in the rat (292), and an assay measuring binding of peptides to human TAP showed some specificity for peptides, especially involving residues 2, 3, and 9 (293,294). Another study using combinatorial peptide libraries for measuring binding to TAP indicated that the three N-terminal residues and the C-terminal residue were the most critical in determining TAP binding (295). It may not be coincidental that these residues are often the key anchor residues in peptide binding to class I MHC molecules and that the lengths of peptides preferentially transported by TAP correspond to the lengths that bind to class I MHC molecules (see later). At the C-terminal position, TAP appears to select against Glu, which is also not generally a C-terminal anchor residue for class I MHC molecules. Human TAP appears to transport peptides with Arg at position 2 and Phe, Tyr, Leu, Arg, or Lys at position 9, consistent with the residues involved in peptide binding to HLA-B27, whereas it disfavors peptides with Pro at position 2, such as those that bind to HLA-B7 (293,294,296). Therefore, the specificity of TAP transport could favor the loading of some HLA molecules more than others. Thus, the peptide specificity of TAP, although not as limiting as that of the MHC molecules themselves, contributes to the selective process determining which antigenic determinants dominate the $CD8^+$ T-lymphocyte response.

MHC Binding

Major advances have been made in defining the structural requirements for peptide binding to class I MHC molecules. The most striking finding is a severe length limitation, apparently imposed by the closure of both ends of the peptide-binding groove in the case of class I MHC molecules, apparent from the crystal structure (91,93), as is not the case for the class II MHC molecule based on the class II crystal structure (66,67,68,70,95,96,97 and 98). Investigators first found that small, truncated peptides contaminating the major population of synthesized longer peptide could be the active species, so for example, a 9-residue fragment may be responsible for all the activity, even though it is but a minor contaminant in a 12-residue peptide that is actually inactive (297). A similar length constraint was found by the independent approach of eluting natural peptides from class I MHC molecules on cells and sequencing them (105,298,299). The natural peptides bound to class I molecules were mostly nonamers, with a few octamers. Subsequent studies also identified decamers (300). In contrast, elution of peptides from class II MHC molecules gave exclusively longer peptides with a broader range of lengths, from 13 to 17 residues (71,72). The latter are consistent with the observation that the ends of the class II molecule peptide binding groove are open, thus allowing varying lengths of peptide to extend from both ends (66,67,68,70). In support of the length restrictions on peptide binding to class I MHC molecules, a study of the effect of length of a nested series of peptides from influenza nucleoprotein on binding to $H\text{-}2D^b$ revealed a strong dependence of affinity on length (301). The highest affinity peptide was 9 residues long, and increases in length resulted in a lower affinity and shorter half-time for stability of the complex. Furthermore, the study cited earlier in which a 15-residue peptide appeared to be active in serum, but was not active in the absence of serum, whereas a 10-residue peptide contained within it was active even without serum (81), illustrates the point that even when using purified MHC molecules without cells, the apparent activity of a longer peptide can depend on protease contaminating the system.

However, one should not conclude that only peptides of 8 to 10 residues can be used for immunization. Most of the cases in which peptide immunization was successful (referenced earlier) used longer peptides. Therefore, longer peptides can be just as effective, and in some cases they are more immunogenic because they stimulate helper cells as well (see later), provided they can be processed appropriately by cellular or serum proteases to a size that can bind to class I MHC molecules.

Besides the length limitations, the next most striking finding is the presence of well-defined sequence motifs for specific binding to a particular MHC class I molecule. Many of these were found by sequencing the mixtures of peptides eluted from a class I molecule and by finding that, at certain positions in the sequence, a single amino acid occurred predominantly, whereas at other positions, many amino acids were found (105). This method would work only if the peptides had a strict length limitation on the N-terminal side of the motif. Otherwise, among peptides all sharing the same motif, the motif would not always start at the same position in the peptide. Other motifs were found by eluting peptides from class I MHC molecules, by purifying the major peptides in the mixture, and sequencing these individually (298,299), or by analyzing the residues required for presentation of a particular antigenic peptide (302). When a motif was found using two different methods, such as in the case of $H\text{-}2K^d$, the same motif was found by both methods, a finding providing independent support for the motif (105,302). Further support for this motif concept was provided by the prospective prediction of an antigenic site, in which 1 of 16 peptides synthesized on the basis of this motif for K^d was found to be antigenic (303).

Most of these motifs involve only 2 or, at most, 3 residues within the peptide, usually at positions 2 and 8 or 9, or sometimes at positions 5 and 9. These key residues have been called anchor residues because it is thought that they anchor the peptide by binding in specific pockets in the floor of the peptide-binding groove, thus leaving more flexibility in the other residues in between, some of which must interact with the TCR. This concept of anchor residues binding in pockets has been confirmed by x-ray crystallography (92,93,131,132,133,134,135,136,137,304,305). The presence of more sharply defined motifs for peptides binding class I molecules than for those binding class II molecules may result from the greater length restrictions, which force all peptides binding to a given MHC molecule to line up in a similar position and therefore to have similar side chains to fit into pockets in the MHC groove at defined positions. These motifs may prove extremely useful in locating within protein sequences peptides that should be presented by a given class I MHC molecule. A correlation has been found between the affinity of peptide-binding to class I MHC molecules and the ability to raise CTLs to that peptide, that is, the immunogenicity of the peptide (306).

However, the predictive success rate of only about 30% for such motifs, based on primary anchor residues (303,307,308), can probably be improved by taking into account the role of other peptide residues that serve as secondary anchor residues, as defined for HLA-2.1, for example (309). This approach has been expanded to take into account a weighted value for each peptide residue in a matrix approach for predicting binding (120,310,311 and 312), and it has been applied to class II MHC molecules as well (114,313,314). It is also useful that MHC molecules have been found to fall into families that bind similar peptides containing what has been called a supermotif (117,315,316 and 317). A detailed structural analysis of each of the class I MHC binding pockets that shows how the class I MHC molecules fall into families that can be defined independently for each pocket, as if similar pockets have been resorted among different alleles, allows prediction of anchor motifs for other class I MHC molecules for which empiric data are lacking or are too limited (139). Motifs for peptides binding to class I and class II MHC molecules have been summarized by Rammensee et al. (109).

Although in all the published cases in which a peptide could be clearly discerned within the groove of a class I MHC molecule by x-ray diffraction the peptide appeared to be in an extended conformation (93,94,131,132,133,134,135,136,137,304,305), the spacing of the anchor residues 7 residues apart or 4 residues apart in most motifs would place them exactly 2 turns or 1 turn of an a helix apart if the peptides folded as helices, because each turn of an a helix has 3.6 residues. Moreover, in the case of class I MHC molecules, although fewer peptide binding sequences are known than for class II, 33 of 51 or 65% could fold as amphipathic helices, and the correlation was significant compared with other sequences from the same proteins (129). Similarly, 7 of the 11 natural peptides eluted from HLA-B27 by Jardetzky et al.

(298) would be amphipathic if they were folded as a helix (126). For comparison, 2 control sets of 11 control peptides were selected at random from the protein sequence database, with the only constraint besides similar length being that they shared the Arg at position 2 shared by all the B27-binding peptides, and only 2 of 11 and 3 of 11 were helically amphipathic ($p < .02$). Thus, even though the conformation adopted by the peptide when bound by the class I MHC molecule is not helical, the periodicity in hydrophobicity that resembles that of an a helix is imposed by the spacing of the pockets in the MHC groove that, in turn, enforce a spacing of peptide anchor residues similar to the spacing of residues of like hydrophobicity on one side of a helix (129), considering that most class I anchor residues are hydrophobic (109). Thus, this periodicity of hydrophobicity is predictive of MHC-binding peptides in a relatively allele-independent fashion, thus creating perhaps the broadest of supermotifs (129).

Finally, the polymorphism of class I MHC molecules within the peptide binding groove, like that of class II MHC molecules, leads to the phenomenon of *Ir* gene control of responses. For example, response of female mice to the H-Y male antigen occurs in mice expressing D^b because this is the class I molecule that presents the H-Y peptide (318,319 and 320). Strains of mice lacking D^b generally do not respond to H-Y antigen.

Recognition by a T-Cell Receptor

The same issues apply here as for $CD4^+$ T cells discussed earlier. The potential TCR repertoire is enormous, and absolute holes in the repertoire are probably rare (321). For example, the subset of peptides from hepatitis C virus (HCV) containing a motif for HLA-A2.1 binding that actually elicited CTLs in HLA-A2.1–transgenic mice were predictive of the subset recognized by CTLs from HCV-infected human patients, despite the differences in murine and human T cell-repertoire (152). However, holes created by self-tolerance may be significant, and they certainly would be expected to affect recognition of determinants that resemble self.

Requirement for Help

Like a B-cell, a CTL requires some form of T-cell help to be activated. Not all antigenic determinants presented by class I MHC molecules necessarily induce help as well. Although the existence of $CD8^+$ cells that can secrete interleukin-2 (IL-2) and provide help for other $CD8^+$ cells has been demonstrated (322), most $CD8^+$ CTL responses seem to be at least partially dependent on help from $CD4^+$ cells. Thus, an antigenic determinant that can be presented by class II MHC molecules may contribute to the immunogenicity of a peptide for induction of $CD8^+$ CTLs. Studies indicate that adding a helper site to a peptide presented by a class I MHC molecule can contribute to its immunogenicity, as, for example, in the case of determinants from the HIV-1 envelope protein or from HCV (235,251,323,324 and 325). Without attachment of a helper determinant, a peptide containing only a CTL determinant was not immunogenic (251). Similarly, a peptide containing an intrinsic helper determinant as well as a CTL determinant did not require attachment of an extrinsic helper determinant, whereas one that lacked an intrinsic helper determinant required an extrinsic one to be attached to induce CTLs (325). Moreover, improving the level of $CD4^+$ T-cell help can greatly enhance the immunogenicity of a peptide for eliciting $CD8^+$ CTLs (326). The concepts in this section should be applicable to the systematic development of peptide immunogens that are capable of eliciting strong CTL responses.

ENHANCING IMMUNOGENICITY THROUGH IMMUNOPOTENTIATION AND EPITOPE ENHANCEMENT

Given what we know about the mechanisms of antigen recognition by T and B lymphocytes and about antigen processing, what maneuvers can be used to attempt to enhance or potentiate the immune response to a particular antigen, that is, to enhance its immunogenicity? Attaching a small peptide or hapten to a large carrier protein could be considered immunopotentiation and is discussed earlier in this chapter. Here the focus is on three types of approaches in which covalent modifications of the antigen or admixtures to the adjuvant could significantly enhance the response.

Targeting the Antigen to Antigen-Presenting Cells

Investigators have shown, both with antiimmunoglobulin as antigen (327,328) and with antigen-specific B cells that bind antigen through the combining site of their surface immunoglobulin (27,28,29,329), that antigens that bind to surface immunoglobulin of a B cell are presented more efficiently to antigen-specific T cells, often at several orders of magnitude lower concentration. However, most antigens bind only to relatively rare B cells specific for them *in vivo,* and these few antigen-specific B cells are not sufficient to enhance immunogenicity, unless, perhaps, they have already been greatly expanded by prior immunization. To make any antigen more immunogenic by targeting it to such antigen-presenting cells, Kawamura and Berzofsky coupled antigen to antiimmunoglobulin antibodies that would bind specifically to B cells as antigen-presenting cells (328). Both antilight-chain and anti-IgM heavy-chain antibodies enhanced potency of the antigen for presentation by spleen cells to specific T-cell clones *in vitro,* whereas anti-IgA heavy-chain antibodies of the same isotype did not, a finding indicating that this was not a conventional carrier effect or simply binding to Fc receptors. Rather, only antibodies that could bind to B-cell surface immunoglobulin worked. The radiosensitivity of the presenting cell was consistent with B-cell presentation of the antigen. More strikingly, despite the presence of serum immunoglobulin that could potentially compete, these same antigen-antiimmunoglobulin constructs were also much more immunogenic *in vivo,* and they could elicit a detectable antibody response at 50- to 100-fold lower immunization doses (328). Again, the control construct in which the antigen was coupled to anti-IgA heavy-chain–specific antibodies did not enhance immunogenicity, so the immunopotentiation was not simply a conventional carrier effect. As a carrier to elicit immunoglobulin-specific T-cell help, the anti-IgA construct should have functioned equivalently to the antilight chain or the anti-IgM. Therefore, this *in vivo* immunopotentiation must be caused by targeting of antigen to B cells as antigen-presenting cells, which then enhance induction of antigen-specific T-cell help and so increase the antibody response (328). This same immunopotentiation was subsequently shown to occur with antigen coupled to anti-IgD antibodies, even without adjuvant (330). The anti-IgD antibodies have the potential advantage that there is little circulating IgD to compete with the B-cell surface IgD for binding the antigen–anti-IgD complex. Furthermore, this method of immunopotentiation is not unique to antibodies to B-cell surface immunoglobulin. For example, Carayanniotis and Barber found that coupling of antigen to antibodies specific for other surface molecules on antigen-presenting cells, such as class II MHC molecules, can also greatly increase their immunogenicity, even without the use of an adjuvant (331). This approach may therefore be useful in vaccine development, because the same antiimmunoglobulin or anti-MHC molecule can serve not only as a conventional carrier, but also as a specific targeting agent to produce high-affinity binding of antigens to antigen-presenting cells and therefore can reduce the concentration necessary to elicit a response. Moreover, the response would not be just an antibody response using T-cell help to the carrier, but it would also be a helper T-cell response to the targeted antigen presented more efficiently by the target presenting cell.

Incorporation of Cytokines in the Adjuvant

One of the limiting factors in immunogenicity for induction of an antibody response is the ability of the antigen to elicit T-cell help. Failure to elicit help because of failure to bind to a class II MHC molecule is the basis for many cases of *Ir* gene low responsiveness, as discussed earlier (37). Although cognate help, elicited, for example, by coupling the antigen to a carrier containing structures that can be presented by the class II MHC molecules of the animal in question, may be more efficient than noncognate help, it may nevertheless be possible to compensate for inadequate help by the addition of IL-2. IL-2 can help to expand the suboptimal number of T-helper cells present, and it can mimic noncognate help by a direct effect on the B cell (332,333 and 334). Therefore, the question was raised whether incorporation of IL-2 in the adjuvant emulsion would enhance the immunogenicity of a protein in an *Ir* gene low-responder strain of mice (335). Indeed, low responders to myoglobin made antimyoglobin antibodies at nearly the same level as high responders after a single immunization when recombinant IL-2 was incorporated in the adjuvant (335). No increase was noted in the response of high responders above this level, a finding consistent with the interpretation that IL-2 increased or substituted for help in the low responders and that help was not limiting in the high responders. Similar enhancement of immunogenicity was seen for short peptides from the malaria circumsporozoite protein when IL-2 was included with the antigen in the adjuvant (336). In this case, the effect was shown not to simply result from noncognate help specific for the human IL-2 molecule as a carrier, because a mutant of IL-2 that was equally immunogenic but lacked lymphokine activity had no effect (336). An attempt to take advantage of this method by incorporating the gene for IL-2 along with antigen in a recombinant vaccinia virus did not result in increased immunogenicity for the antigen expressed, but it did greatly attenuate the viral infectivity, perhaps by increasing the T-cell response to the virus (337). The use of an emulsion or perhaps other slow-release formulation seems to be important to keep the IL-2 in the same local area as the antigen, because simply injecting IL-2 intravenously multiple times during 3 days after immunization was not sufficient to produce any significant enhancement of antibody response (336; H. Kawamura, S.A. Rosenberg, and J.A. Berzofsky, unpublished data).

The success of this type of approach led to the question whether other cytokines, such as IL-4, IL-12, or granulocyte-macrophage colony-stimulating factor (GM-CSF), could also be incorporated into the adjuvant emulsion not only to enhance immunogenicity but also to steer the immune response in a desired direction, such as toward T-helper cell subtype (Th1 or Th2) cytokine responses or toward CTL induction, or particular antibody isotypes (338). In a comprehensive comparison of eight different cytokines emulsified in incomplete Freund's adjuvant with either of two different HIV-1 peptide vaccine constructs, and tested in mice of different strains, seven different immune responses were analyzed (338). GM-CSF was found to increase most responses without tipping the balance between Th1 and Th2 phenotypes (338), similar to the enhancement of responses observed with this cytokine by other investigators (339,340). In contrast, IL-4 in the adjuvant enhanced Th2 responses, whereas IL-12 in the adjuvant enhanced Th1 and CTL responses (338). The cytokines could also influence the antibody isotypes produced. Strikingly, a synergy was observed between GM-CSF and IL-12 in increasing CTL responses (338), which we now know to result from their action through different mechanisms in which the GM-CSF increases functional antigen presentation, whereas IL-12 increases interferon-g production (340a). A similar synergy for CTL induction was seen between IL-12 and tumor necrosis factor-a (TNF-a), which also synergize to markedly increase the production of interferon-g (338). Triple synergy of IL-12, TNF-a, and GM-CSF was shown to be more effective than any combination of two of these cytokines in inducing CTLs and also in protecting against challenge of mice with a recombinant vaccinia virus expressing the HIV-1 envelope protein (340a, in preparation). A similar enhancement of immunogenicity and steering of the response could be achieved with DNA vaccines by incorporating cytokine genes in the plasmid or an associated plasmid (216,341,342 and 343). Indeed, IL-12 and GM-CSF seem to synergize in DNA vaccines as well (216). IL-12 can also been incorporated in mucosal vaccines to alter cytokine production (344) or to enhance mucosal and systemic CTL

induction (345,346,346a).

Besides cytokines, other molecules can be incorporated into a vaccine to enhance immunogenicity. These include costimulatory molecules (216,347,348), immunostimulatory DNA sequences or oligonucleotides containing CpG motifs (349,350,351,352 and 353), and chemokine molecules (354). Thus, a host of immunomodulatory molecules can be used as needed not only to increase immunogenicity, but also to steer or manipulate the immune system to achieve a particular type of response.

Epitope Enhancement: Modification of the Structure of the Antigen to Increase Immunogenicity

If the immune system exerts natural selection on the evolution of pathogens, one would expect that pathogens evolve to evade the immune system. Thus, the sequences of viral or bacterial proteins would not be expected to be optimally immunogenic. In principle, one should be able to make vaccines that are more immunogenic than the pathogens themselves. One approach to accomplish this is based on the idea that some amino acid side chains that are not involved in interaction with the TCR can adversely affect binding to the MHC molecule, so altering these may lead to improved antigen presentation without loss of T-cell specificity (111,346,355,356,357 and 358). Boehncke et al. observed that removal of a negative charge by replacement of a Glu with an Ala or Gln residue increased the affinity of an HIV envelope helper peptide, T1, for binding to the murine class II molecule, I-E^k, and increased potency for stimulating T1-specific T cells (355). Ahlers et al. therefore tested whether this same modification would increase immunogenicity, and indeed it did, not only for T-cell proliferation, but also for providing help for induction of a CTL response to another epitope attached to this helper epitope (326). A genetic experiment showed that the enhancement of induction of MHC class I–restricted $CD8^+$ CTLs mapped to the class II MHC molecule, a finding indicating that it was indeed caused by increased help from the modified helper epitope. This result provides strong evidence for the importance of $CD4^+$ MHC class II–restricted helper T cells in optimal induction of a $CD8^+$ MHC class I–restricted CTL response. It also serves as proof of principle for the process of epitope enhancement, in which one can modify an amino acid sequence to make a peptide epitope more efficacious. This approach was extended to a human HLA class I molecule, HLA-A2.1, in the case of an HCV core peptide (359). Modification of the sequence of this epitope increased its potency for sensitizing targets for lysis by core-specific human CTLs and increased its immunogenicity for inducing an HCV core-specific CTL response in HLA-A2.1–transgenic mice. Similar findings were made for a peptide epitope from the HIV-1 reverse transcriptase (360), as well as a peptide from the human melanoma tumor antigen, gp100, which, when modified, was more effective at eliciting a $CD8^+$ T cell-response in patients with melanoma (361,362). Thus, the approach of epitope enhancement by sequence modification may turn out to be a useful tool to make second-generation vaccines that are more effective than the pathogens themselves.

A less specific approach to improved immunogenicity of proteins is cationization of the proteins, that is, making them positively charged, by converting carboxyl groups to aminoethyl amide groups. This alteration has been shown to reduce the concentration required to stimulate T cells *in vitro* by several orders of magnitude, as well as to increase immunogenicity *in vivo* even without adjuvant (363,364 and 365). The antibodies and T cells elicited appear to crosss-react with the native protein. Although the exact mechanism of the immunopotentiation is not clear, the finding that the concentration required for activation of T cells *in vitro* is markedly reduced suggests that the cationized proteins are more efficiently taken up or processed by antigen-presenting cells. It seems less likely that the affinity for specific MHC molecules is increased, because the effect is not limited to certain MHC molecules. Moreover, the positive charges would be introduced in different places in the protein, not in the same location with respect to each antigenic determinant. Therefore, some more global effect on uptake or processing seems to be a more likely mechanism. Regardless of the mechanism, cationization appears to be a potentially widely applicable method for enhancing immunogenicity.

CONCLUSION

The selective use of cytokines and the modification of antigens to enhance uptake, processing, and presentation provide new methods of potentiating and steering an immune response that complement the more traditional adjuvants. As we learn to identify the minimal antigenic determinants required for antigenicity, to improve on them by epitope enhancement, and to combine these in a way to make them more immunogenic, for instance, by providing cognate help from T cells specific for the same antigen, we can hope to make artificial vaccines that are more selective for particular responses than would be whole proteins. Other modifications may take advantage of new knowledge on the specificity of proteases performing antigen processing, on the specificity of TAP transport into the endoplasmic reticulum, or on the conformational specificity of antibodies. These engineered constructs, combined with new methods of immunopotentiation such as those discussed earlier, may allow a new generation of vaccines designed to elicit desired immune responses selectively.

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8 LYMPHOCYTE SIGNALING

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T and B lymphocytes are the effectors of adaptive immune responses. Recognition of specific antigens occurs through engagement of either T-cell antigen receptors (TCRs) or B-cell antigen receptors (BCRs), and it leads to clonal proliferation, differentiation, and activation of effector functions in lymphocytes. These events are directed by a complex network of intracellular signal transduction events that is initiated on ligation of either the TCR or BCR. Lymphocyte signaling encompasses a wide array of biochemical reactions that translate alterations in the status of cell surface receptors into the production of activation-induced gene products and the rearrangement of the cytoskeleton. This chapter focuses on the biochemical events generated as a result of either TCR or BCR ligation.

The TCR and BCR are expressed on the surface of lymphocytes as part of multimeric protein complexes that contain both antigen recognition and signal transduction components (1,2,3 and 4) (Fig. 8.1). In both T and B cells, antigen recognition occurs through a polymorphic protein structure that is composed of disulfide-linked subunits. The TCR is heterodimeric, and it can be composed of either a and b chains or, in a minority of cells, g and d polypeptides. The BCR consists of membrane-bound immunoglobulin made of two heavy- and light-chain heterodimers. Random recombination events between multiple gene segments encoding these polypeptides give rise to their polymorphic nature and enable these receptors to recognize a tremendous array of antigens. However, the TCR and BCR do not possess any intrinsic enzymatic activity, and they must therefore rely on associations with other molecules to transduce signals within the cytosol. The signal transducing component of the antigen-receptor complex differs structurally between T and B lymphocytes. In T cells, signal transduction is mediated by six invariant CD3 polypeptide chains that associate with the TCR noncovalently. The CD3 chains are organized into de and ge heterodimers, and zz homodimers (5). Each d, e, and g chain possesses one copy of a motif that is essential for the propagation of signal transduction events arising from the TCR, the immunoreceptor tyrosine-based activation motif (ITAM). In contrast, the z chains each contain three ITAMs (2). Tyrosine residues located within this characteristic YXXL/I-X_{6-8}-YXXL/I motif (where Y designates the amino acid tyrosine, L designates leucine, I designates isoleucine, and X is any amino acid) are rapidly phosphorylated after TCR engagement. Although structurally different, the signal transduction component of the BCR functions in an identical manner. Composed of two separate heterodimers, the invariant Ig-a and Ig-b chains each contain one ITAM that is phosphorylated on BCR ligation (6).

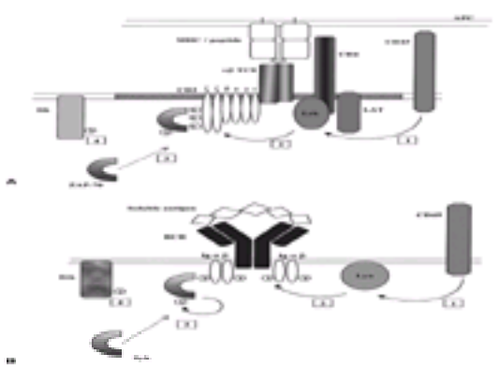

Figure 8.1. A comparison of receptor-proximal protein tyrosine kinase (PTK) activation events in T and B lymphocytes. **A**: Interaction of the T-cell receptor with specific peptide/major histocompatibility complex complexes leads to dephosphorylation of the src PTK, Lck, by CD45 *(1).* Activated Lck then phosphorylates tyrosine residues within immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3 chains *(2).* This induces ZAP-70 translocation to the ITAMs, and allows for its activation by src kinases *(3).* Itk is recruited to the plasma membrane through its PH domain and associations with adapter molecules, and it undergoes phosphorylation-dependent activation by src kinases (4). **B**: Interaction of the BCR with soluble antigen leads to the dephosphorylation of the src PTK, Lyn, by CD45 *(1).* Activated Lyn then phosphorylates tyrosine residues within ITAMs on Ig a and b *(2).* This induces Syk to translocate to the ITAMs and to undergo autophosphorylation *(3).* Btk is recruited to the plasma membrane through its PH domain and associations with adapter molecules, and it undergoes phosphorylation-dependent activation by src kinases such as Lyn *(4).*

Phosphorylation of the ITAM residues is accomplished by members of the src family of protein tyrosine kinases (PTKs). Activation of these kinases is one of the earliest detectable events that follows engagement of either the TCR or BCR (1). Activity of the src kinases is regulated by a balance of phosphorylation and dephosphorylation on key regulatory tyrosine residues. This indicates that phosphatases are also critical mediators of T- and B-cell signal transduction events (7).

After tyrosine phosphorylation of the signal transducing components of TCR or BCR complexes, another family of PTKs is recruited to the complex and is activated. The syk PTKs inducibly associate with CD3 or Ig-a and Ig-b ITAMs by a protein-protein interaction domain referred to as an SH2 (src homology 2) domain (8,9), which mediates binding to phosphorylated tyrosine residues. Once syk PTKs are bound to the ITAMs, they are phosphorylated by src PTKs and become fully active (9). Activated syk PTKs then phosphorylate several important downstream effector molecules and adapter molecules. Adapter molecules are proteins that possess no enzymatic or transcriptional functions, but they coordinate signal transduction by acting as scaffolds around which signaling complexes are formed (10). These complexes effectively bring downstream substrates into contact with their effectors. Therefore, lymphocyte signal transduction is regulated initially by a series of tyrosine phosphorylation events that result in the generation of multimeric clusters of adapter proteins and effector molecules at the site of the engaged antigen receptors.

Although the scheme of signal transduction events initiated by antigen receptors is similar, ligation of the TCR and BCR results in diverse cellular outcomes. These differences are regulated by the specific pattern of kinases, adapter molecules, and downstream effectors, such as transcription factors, that are present and are activated in either T or B cells. To facilitate understanding of lymphocyte signal transduction, this chapter approaches biochemical events in terms of three main categories: activation of PTKs, integration of signal transduction by adapter molecules, and roles of downstream effector molecules. Within each category, both positive and negative regulators of signal transduction are discussed. The major focus is on signaling mediators in T lymphocytes, but throughout the chapter, similarities and differences between T and B cells are discussed.

ACTIVATION OF PROTEIN TYROSINE KINASES

Positive Regulators of Signal Transduction

Src Family Protein Tyrosine Kinases

All downstream biochemical and cellular changes induced by ligation of the TCR or BCR rely on activation of src kinases. Family members Lck and Fyn are thought to be of primary importance in T cells, whereas Lyn and Blk assume prominent roles in B cells (3,4). The src family is characterized by the presence of three functional domains: a kinase domain, an SH2 domain that mediates interactions with phosphorylated tyrosine residues in the context of specific amino acids lying carboxy-terminal to the tyrosine, and an SH3 domain that mediates binding to proline-rich regions on other proteins. In addition, the src kinases possess a myristylated

glycine residue in their amino-terminus that leads to constitutive localization at the plasma membrane (1).

Evidence indicates that src kinases are not randomly distributed across the surface of lymphocytes, but rather are found associated with transmembrane proteins in unique microdomains within the plasma membrane. In resting T cells, Lck is found associated with the cytoplasmic tail of either the CD4 or CD8 co-receptor (Fig. 8.1A). Both CD4 and CD8 are contained within membrane fractions termed glycosphingolipid-enriched microdomains (GEMs) (11). GEMs also possess high concentrations of cholesterol, glycosylphosphatidylinositol (GPI)-anchored proteins, and molecules important for the induction of lymphocyte signal transduction, such as src kinases. It is thought that during the process of TCR ligation, the TCR/CD3 complex, which is normally excluded from the GEMs, rapidly becomes associated with these structures (11). This movement would permit the activated src kinases to phosphorylate ITAM residues. Therefore, the localization of src kinases to GEM regions of the plasma membrane, and their segregation from the TCR/CD3 complex in resting cells, may create another mechanism by which lymphocyte signaling is regulated.

The functional status of src kinases is controlled by a balance of tyrosine phosphorylation and dephosphorylation events. In an inactive state, src kinases are found with a phosphorylated tyrosine residue in their carboxy-terminals. Phosphorylation at this site is predicted to induce an intramolecular association with the SH2 domain, thereby causing a conformational blockade of the kinase domain (12). Therefore, src kinase activation requires dephosphorylation of this negative regulatory residue. In both T and B cells, this is accomplished by the transmembrane phosphatase CD45 (13,14). The importance of CD45 activity has been demonstrated in T-cell clones and variants of the Jurkat T-cell line deficient in CD45 expression. These cells are unable to respond normally to TCR ligation, but they can respond if proximal events, such as src kinase activation, are bypassed by stimulation with pharmacologic agents (13,15). The importance of CD45 function is illustrated in CD45-deficient mice, which display marked abnormalities in thymocyte development and mature T-cell activation (16).

Activation of src kinases may also be influenced by their position in GEMs and their relative proximity to the TCR. Although it is known that CD45 is required to trigger activity of src kinases, the exact mechanisms responsible for this remain unclear. Observations that the phosphatase activity of CD45 does not depend on TCR engagement conflict with evidence that src kinase activity increases significantly only during the process of T-cell activation (17,18).

A possible solution to this dilemma is suggested by work focused on visualizing molecular interactions at the site of contact between T cells and antigen-presenting cells. Several laboratories have shown that cognate engagement of the TCR initiates the dynamic rearrangement of transmembrane surface proteins and intracellular signaling molecules into ordered structures referred to as SMACs (supramolecular activation clusters) (19) or immunologic synapses (20). Results from these studies indicate that Lck and CD45 are repositioned differentially in the plasma membrane, relative to the TCR, after antigen receptor engagement. Further evidence indicates that CD45 may be excluded from GEMs at all times because of limitations in the size of its transmembrane domain (21). Although the exact mechanism of src kinase activation remains unresolved, it seems likely that clues derived from examination of protein localization within the plasma membrane will be essential for understanding these events.

Syk Family Protein Tyosine Kinases

The syk family of PTKs consists of two members: Syk and ZAP-70 (for z-associated protein of 70 kd). Unlike the src kinases, these proteins are cytosolic and possess tandem amino-terminal SH2 domains, multiple phosphorylatable tyrosine residues, and a carboxy-terminal kinase domain. Both ZAP-70 and Syk are expressed in T lymphocytes, whereas only Syk is found in B cells (1).

Phosphorylation of Syk and ZAP-70 modulates not only their enzymatic activity, but also interactions with other proteins. After recruitment to phosphorylated ITAMs, Syk undergoes autophosphorylation and becomes fully active (Fig. 8.1B). In contrast, ZAP-70 requires transphosphorylation by src kinases to become activated (Fig. 8.1A) (2). Many tyrosine residues on Syk and ZAP-70 are phosphorylated after TCR or BCR ligation. Some phosphotyrosines may negatively regulate Syk and ZAP-70 function by allowing an adapter protein known as Cbl to bind through its SH2 domain (22). Models for the role of Cbl as a negative regulator of signal transduction are discussed later in this chapter.

Numerous downstream signal transduction events depend on the activation of Syk and ZAP-70. In T cells, ZAP-70 phosphorylates the adapter molecules LAT (for linker of activated T cells) (23) and SLP-76 (for SH2-domain—containing leukocyte phosphoprotein of 76 kd) (24). These molecules are essential mediators of T-cell signaling (see later). Similarly, in B lymphocytes, Syk phosphorylates a homolog of SLP-76 called B-cell linker protein, or BLNK (25). In addition to these adapter molecules, ZAP-70 and Syk contribute to the phosphorylation and activation of phospholipase C-g (PLC-g) isoforms (2). These enzymes cleave membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (26,27). IP_3 regulates intracellular calcium (Ca^{++}) levels by releasing Ca^{++} sequestered in the endoplasmic reticulum, thereby increasing the cytosolic Ca^{++} concentration. Production of DAG leads to activation of protein kinase C isoforms. The critical nature of ZAP-70 is evidenced in patients with defective expression of this PTK, which is manifested by a loss of T-cell function resulting in a severe combined immunodeficiency phenotype (28). Therefore, syk family PTK function is required for the propagation of signal transduction events initiated by TCR or BCR ligation and src kinase activation.

Tec Family

The third major family of PTKs involved in positively regulating lymphocyte signaling is the Tec family. This family consists of five members: Tec, Btk (or Bruton's tyrosine kinase), Itk (or inducible T-cell tyrosine kinase) also referred to as Emt (expressed in mast and T cells), Rlk or Txk, and Bmx (29). These kinases are cytosolic, but they are rapidly recruited to the plasma membrane after TCR or BCR ligation. For all members except Rlk/Txk (30), this localization is mediated by an amino-terminal pleckstrin homology (PH) domain, which associates with specific lipids produced by the action of another enzyme, phosphatidylinositol-3-kinase (PI3K) (4). Association with the plasma membrane brings Tec PTKs into proximity of the src PTKs, and this activates the Tec kinases by phosphorylating a key tyrosine residue located within the Tec catalytic domain (31) (Fig. 8.1). However, evidence obtained with a ZAP-70–deficient Jurkat T-cell line suggests that ZAP-70 is also indirectly required for Tec family kinase activation (32). In addition to the PH domain, Tec kinases possess one SH2 domain, one SH3 domain, and a carboxy-terminal kinase domain (2). Tec, Itk, and Rlk/Txk are activated in T cells in response to TCR engagement (33,34), and Btk is activated after BCR ligation (29). The requirement for Btk in B-lymphocyte signal transduction is illustrated by loss of function mutations in the protein, which result in X-chromosome–linked agammaglobulinemia (XLA), a disease characterized by profound B-cell immunodeficiency in mice and humans (35). Although early work suggested that mutations in the PH domain of Btk were associated with this disease, a more recent study indicates that mutations in any of the Btk functional domains may cause it (36).

Understanding of the specific contributions of Tec family kinases in both B- and T-cell signal transduction has come from studies using cell lines and mice made deficient for one or more family members. T cells from Itk-deficient mice demonstrate decreased levels of PLC-g1 phosphorylation and IP_3 production after TCR engagement (37). These defects do not substantially alter the amounts of Ca^{++} released from internal stores, but they do impair the opening of calcium channels in the plasma membrane, resulting in a loss of sustained Ca^{++} mobilization in these cells. Examination of activated Itk null T cells shows that z chains are phosphorylated normally, as is ZAP-70 (37). These data suggest that Itk may cooperate with ZAP-70 to bring about PLC-g1 phosphorylation and activation. Similar results have been obtained in a Btk-deficient B-cell line; these cells also demonstrate a decrease in PLC-g phosphorylation and function (38). An inadequate calcium response likely causes the inability of $Itk^{-/-}$ T-helper 0 (Th0) cells to develop into Th2 cells, because a loss of function in the calcium-dependent transcription factor NFAT (for nuclear factor of activated T cells) appears to prohibit interleukin-4 (IL-4) production necessary for the differentiation of Th0 cells into a Th2 phenotype (39). The phenotype of T cells deficient in both Itk and Rlk is more pronounced, and it shows significant losses in the ability of T cells to proliferate and produce cytokines (40), again as a likely result of diminished IP_3 production and calcium responses. Therefore, one role of Tec family kinases is to contribute to the generation of a prolonged rise in intracellular Ca^{++} concentration during the course of T- or B-lymphocyte activation.

The exact mechanism for Tec kinase activation of PLC-g remains unclear. However, studies using mutations in various functional domains of Itk and Btk have suggested potential mechanisms. Loss of the PH domain, for example, prohibits Itk from associating with the plasma membrane after TCR ligation. As expected, this form of Itk is not phosphorylated and remains inactive (41). The SH2 domains of Itk and Btk appear to mediate their interactions with SLP-76 and BLNK, respectively. These associations are not constitutive, but they are observed only after SLP-76 or BLNK become tyrosine phosphorylated in response to antigen receptor ligation (42). Because data indicate that PLC-g isoforms associate with BLNK in B cells (43), this association may provide a mechanism whereby Tec kinases are brought into contact with PLC-g, although such trimolecular complexes have not yet been detected.

Negative Regulators of Signal Transduction

Csk Protein Tyrosine Kinase

Not all PTKs enhance or propagate lymphocyte signaling events. One such example is Csk (or C-src kinase), a cytosolic PTK that opposes the action of CD45 by phosphorylating the negative regulatory tyrosine located in the carboxy-terminals of src kinases (44). By doing so, it negatively regulates T-cell signal transduction.

Csk comprises an amino-terminal SH3 domain, a central SH2 domain, and a carboxy-terminal catalytic domain, much like the src family of kinases. It is ubiquitously expressed, but it is present at higher levels in cells of hematopoietic origin. The critical role of Csk was demonstrated in mice carrying a homozygous deletion of the csk gene, a mutation that results in embryonic lethality (45). To study the effects of Csk on T-cell development, mice carrying a conditional deletion of the csk gene were

generated (46). Thymocytes from these mice mature from the $CD4^-/CD8^-$ double-negative precursor stage through the $CD4^+/CD8^+$ double-positive stage and to the $CD4^+$ single-positive stage without receiving signals from the pre-TCR or TCR. Closer examination of src kinase activity in $Csk^{-/-}$ cells reveals high levels of both Lck and FynT activation, even without TCR ligation. Therefore, without Csk continually to counteract CD45 dephosphorylation of src kinases, even in a resting state, the balance is shifted such that src kinases become constitutively active and initiate inappropriate signal transduction events.

Both the SH2 and SH3 domains of Csk must be present and functional for Csk to phosphorylate src kinases. If either domain is deleted, Csk activity will decrease significantly (47). This finding suggests that associations with other molecules are needed for Csk to function as a negative regulator of src kinase activity. One binding partner that has been identified is a proline-enriched protein tyrosine phosphatase designated PEP (48). The SH3 domain of Csk associates constitutively with PEP, and the formation of a Csk-PEP complex appears to be required for either molecule to inhibit T-cell–signaling events. As part of a Csk-PEP complex, PEP has been shown to dephosphorylate the positive regulatory tyrosine in the src kinase Fyn, thus terminating its activity (49). PEP can also dephosphorylate ZAP-70, at least in a heterologous system using transfections in the COS cell line (49). These results suggest that to understand the regulation of PTK activity in T and B lymphocytes completely, additional work will be required to identify binding partners of PTKs and to determine the functional significance of these interactions.

INTEGRATION OF SIGNAL TRANSDUCTION BY ADAPTER MOLECULES

Positive Regulators of Signal Transduction

LAT

Although PTKs are potent transducers of signal transduction events, their activity is spatially confined to the inner surface of the plasma membrane and the region adjacent to phosphorylated ITAMs within the antigen-receptor signaling complexes. Clearly, additional mechanisms must exist to link these receptor-proximal events to distal effectors that reside in the cytosol and nucleus. It has become evident that the category of proteins known as adapter molecules functions as scaffolds to bring cytosolic proteins into contact with activated PTKs localized at the site of clustered TCRs. In this way, adapter proteins couple TCR-proximal events and downstream signal transduction pathways.

The transmembrane adapter protein LAT illustrates the critical necessity for these molecules in the propagation of signal transduction in T cells. Early studies in the area of T-lymphocyte signaling demonstrated a predominant phosphoprotein of 36 kd that was a substrate of activated PTKs. This phosphoprotein was cloned and was identified as the adapter protein LAT (23). LAT is a small protein that contains multiple cytoplasmic tyrosine residues that are phosphorylated by ZAP-70 after TCR ligation, a feature that makes it an ideal binding partner for proteins with SH2 domains. Although LAT is expressed in natural killer (NK) cells, mast cells, and platelets, it is not present in B lymphocytes.

LAT is targeted constitutively to GEM regions of the plasma membrane by posttranslational palmitoylation on two cysteine residues (50). LAT must be localized within GEMs for it to function as a mediator of T-cell activation (51). Like LAT, other key signaling molecules such as CD4 and CD8-associated Lck are present within GEMs, and LAT associates with these coreceptors (52). This interaction is thought to be important because it presumably enhances the ability of LAT to be phosphorylated by ZAP-70 once the TCR/CD3 complex moves into GEMs after receptor engagement.

Once phosphorylated, LAT associates with several other signaling molecules, both directly and indirectly. Such molecules as PLC-g1 and the p85 subunit of PI3K bind directly to LAT, as does the adapter molecule Grb2, which is discussed later (23). Other molecules, such as the adapter proteins SLP-76 and Cbl, are thought to associate with LAT indirectly. Therefore, LAT appears to assume an essential role as the center of a macromolecular signaling complex that is assembled after the TCR is bound (Fig. 8.2).

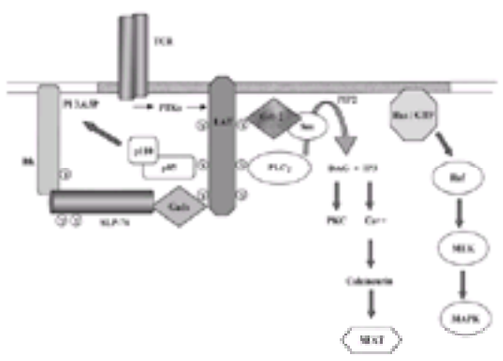

Figure 8.2. LAT (linker of activated T cells)–directed macromolecular complex formation is required for the generation of distal signaling events such as calcium mobilization and induction of the Ras/MAPK cascade in activated T cells. After T-cell receptor ligation, LAT is rapidly phosphorylated by protein tyrosine kinases, a process that allows key signaling molecules such as Grb2, PI3K, and PLC-g to interact with LAT directly. Other proteins, including SLP-76 and Sos, associate indirectly with LAT.

LAT is required for both T-cell activation and development. Mice made deficient for LAT expression illustrate a complete block in thymocyte maturation; thymocytes accumulate at an early $CD4^-/CD8^-$ double-negative stage, and are unable to progress beyond this point to enter circulation in the periphery (53). In response to TCR ligation, two different $LAT^{-/-}$ cell lines show diminished PLC-g1 phosphorylation (54,55), likely from the loss of upstream Itk activation (32). Predictably, subsequent deficiencies in IP_3 production and intracellular calcium mobilization are observed. As a result, transcriptional activity of the calcium-dependent NFAT is lost, and IL-2 promoter activity is absent (54,55). Production of many other cytokines may be deficient as well, because NFAT regulates transcription of numerous genes, although this has not yet been examined. Separate pathways using Ras and its downstream membrane-associated protein (MAP) kinases (MAPKs) are also impaired, leading to a loss of inducible surface expression of CD69. Additionally, SLP-76 phosphorylation is diminished, as is phosphorylation of the SLP-76-associated protein Vav (54,55). Despite these defects, global tyrosine phosphorylation does not appear to be inhibited, a finding indicating that many signaling events proceed in the absence of LAT expression.

LAT is not the only transmembrane adapter protein to be identified in T lymphocytes. Novel proteins such as TRIM (for T-cell receptor interacting molecule), SIT (for SHP-2 interacting transmembrane adapter protein), and KAP 10 also have been described. All three of these molecules are expressed in T cells, and work to elucidate their functions is ongoing. At this time, it is known that TRIM undergoes tyrosine phosphorylation after TCR ligation, and it then associates with the p85 subunit of PI3K (56). KAP 10 also binds to this PI3K subunit, as well as to the adapter protein Grb2 (57). Overexpression studies with SIT indicate that it may negatively affect NFAT activation and perhaps may terminate some T-cell responses (58). As additional transmembrane adapter proteins are identified, it will become increasingly important to understand their functional relevance to each other in terms of competition for binding partners and the effects on downstream cellular responses.

Grb2 Family

Many adapter molecules are cytosolic rather than transmembrane proteins, but they act similarly as scaffolds that recruit signaling molecules to the site of engaged TCRs. Grb2 (for growth factor receptor binding protein 2) is a small, ubiquitously expressed adapter molecule that has been conserved evolutionarily. It consists of an amino-terminal SH3 domain, a central SH2 domain, and a second carboxy-terminal SH3 domain. Grb2 was initially characterized as recruited to phosphorylated PTK receptors (59), and its role in linking growth factor receptors to activation of the Ras pathway is well documented (60). In fibroblasts, Grb2 constitutively associates through its SH3 domains with Sos, a Ras guanine nucleotide exchange factor (61). As Grb2 relocates to the plasma membrane, Sos is brought into contact with Ras. Sos induces Ras to release bound guanosine diphosphate (GDP), thereby promoting interaction with the more abundant guanosine triphosphate (GTP). This process alters the functional state of Ras, which becomes active and initiates a kinase cascade that leads to activation of several transcription factors.

To date, such a definitive relationship between Grb2 and either TCR or BCR complexes has proven more difficult to demonstrate. If Grb2 is recruited to engaged antigen receptors, it may do so indirectly by binding to the adapter molecule Shc (62). Shc is known to associate directly with tyrosine phosphorylated CD3 z chains and ZAP-70 in T cells (63,64). However, the presence of a z/Shc/Grb2/Sos complex has not been demonstrated.

Mounting evidence suggests that in T cells, Sos is not recruited directly or indirectly to the TCR complex. Instead, it may be localized to the plasma membrane as part of a macromolecular complex that forms around LAT (Fig. 8.2). Support for this idea has been provided by several independent studies. For example, it is known that Grb2 interacts inducibly with LAT after TCR ligation (23). Before the identification of LAT, a complex of a 36-kd protein, Grb2, and Sos was implicated in coupling TCR

engagement to activation of the Ras pathway (65). Based on current understanding of T-cell signaling, this early study may have demonstrated a role for LAT in activation of the Ras pathway, an idea that has been substantiated by findings using LAT-deficient T-cell lines (54,55). An equally plausible model is that Sos may be brought into the LAT signaling complex by way of association with a protein related to, but separate from, Grb2.

The proteins Grap (for Grb2-like accessory protein) and Gads (Grb-2 related adapter downstream of Shc) are members of the newly identified Grb2 family of adapter molecules. Like Grb2, both Grap and Gads consist of a central SH2 domain flanked by SH3 domains (66,67 and 68). However, Gads also possesses a proline rich region, and this could facilitate interactions with proteins that would not normally associate with Grb2. Gads was cloned independently by several different laboratories and therefore is also referred to as Grap2, Mona, GrpL, and Grf40 (69,70,71 and 72). Although Gads does not appear to associate with Sos, it does bind to Shc, LAT, and SLP-76 (69,70,71 and 72). Grap does interact with Sos, however, and it also binds to PLC-g1, LAT, and Shc (66,67). Thus, a Grap/Sos complex could be recruited to the plasma membrane through interactions with either Shc or LAT, although this has not been formally demonstrated.

The foregoing discussion has focused on ways in which Sos can translocate to the plasma membrane in T cells. A constitutive association between Grb2 and Sos is observed in B lymphocytes, yet these cells do not express the adapter molecule LAT. Although no homologous transmembrane adapter protein has been identified in B lymphocytes, the novel adapter molecule BLNK may perform an equivalent function.

SLP-76/BLNK

First characterized as an *in vitro* binding partner for Grb2, the adapter molecule SLP-76 is now recognized as an essential mediator of *in vivo* TCR-mediated signal transduction events. SLP-76 is a cytosolic protein consisting of an amino-terminal acidic region that contains multiple tyrosine residues, a central proline-rich region, and a carboxy-terminal SH2 domain (73,74). After ligation of the TCR, SLP-76 is rapidly phosphorylated by ZAP-70 (24), and inducibly associates with other proteins (Fig. 8.3). Although SLP-76 is expressed in a variety of cell types, including NK cells, mast cells, macrophages, and megakaryocytes, it is not present in B cells.

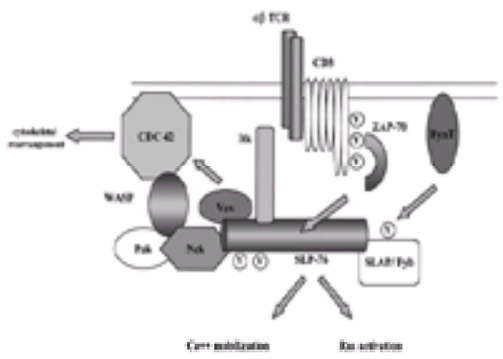

Figure 8.3. The adapter molecule SLP-76 is a central mediator of T-cell receptor (TCR)–induced signal transduction events. After TCR ligation, SLP-76 is tyrosine phosphorylated by ZAP-70. This permits SLP-76 to inducibly associate with such proteins as SLAP-130, and Itk. Binding of Vav and Nck to SLP-76 induces formation of a macromolecular complex that includes Pak and WASP and regulates cytoskeletal rearrangement. The presence of SLP-76 is required as well for downstream events including calcium mobilization and activation of the Ras/MAPK cascade.

The role of SLP-76 as a positive regulator of T-cell signal transduction was suggested from early studies using transient overexpression of SLP-76 in the Jurkat cell line. This work revealed that SLP-76 enhances activity of the MAPK member Erk, as well as transcriptional activity of NFAT and AP-1, resulting in heightened IL-2 promoter function (74,75). Further research showed that phosphorylation of two tyrosines, at positions 113 and 128, mediates the interaction of SLP-76 with the guanine nucleotide exchange factor Vav (76,77 and 78). Unlike Sos, Vav does not modulate Ras function, but acts on members of the related Rho family of GTPases. Overexpression of both SLP-76 and Vav produces a synergistic augmentation of IL-2 promoter activity. However, the interaction between SLP-76 and Vav does not appear to be required for this augmentation to occur, a finding suggesting that the two molecules function in distinct, but overlapping, pathways to enhance NFAT and AP-1 activity (79).

The binding of Vav to SLP-76 does appear to be essential for TCR-induced reorganization of the cytoskeleton. On engagement of the TCR, the cytoskeleton is reoriented and controls antigen receptor capping at the point of contact between the T-cell and stimulatory antigen-presenting cell. SLP-76 and Vav modulate actin polymerization through the formation of a trimolecular complex with another adapter molecule, Nck (80). Nck contains one SH2 domain and three SH3 domains (81). Through its SH2 domain, Nck binds to phosphotyrosine residues 113 and 128 on SLP-76 (82). The Nck/SLP-76 association may be critical, because Nck associates through its SH3 domains with the serine-threonine kinase Pak, and the Wiscott-Aldridge syndrome protein (WASP) (83,84). WASP binds to the activated form of cdc42, a member of the Rho GTPase family, and colocalizes with actin in activated T cells. The loss of WASP prohibits actin polymerization and antigen receptor capping (85). Pak is a serine-threonine kinase that becomes active after binding to GTP-bound cdc42. Studies using specific binding mutants have shown that the trimolecular SLP-76/Vav/Nck complex must be present for actin polymerization and antigen receptor capping to occur (80). Therefore, SLP-76 likely facilitates a cascade in which Vav activation of cdc42 leads to activation of Pak, a process that is possibly caused by the close proximity of substrates and effectors in a multimolecular complex. The results are actin polymerization within the T cell and capping of antigen receptors that may enhance prolonged signal transduction events (Fig. 8.3).

Information about additional functions of SLP-76 was gained by analysis of a Jurkat variant that is deficient in SLP-76 expression. In these cells, phosphorylation of ZAP-70, LAT, and Vav is unaffected by the loss of SLP-76, a finding indicating that these events can occur independently, or upstream, of SLP-76 (86). However, PLC-g1 phosphorylation and activity are entirely blocked. This finding suggests that PLC-g1 binding to LAT is not sufficient to ensure its phosphorylation by PTKs. Events downstream of PLC-g1 activation, including intracellular Ca^{++} mobilization and NFAT activity, are predictably absent in the SLP-$76^{-/-}$ cells. Loss of SLP-76 also negatively affects the Ras pathway, resulting in impaired Erk phosphorylation, decreased AP-1 transcriptional activity, and an inability of TCR ligation to lead to upregulation of the surface marker CD69 (86).

Although much can be learned about SLP-76 function by examining how its absence affects different T-cell responses, identification of molecules that interact with SLP-76 is also revealing. As an example, SLP-76 was initially shown to bind the SH3 domains of Grb2 *in vitro* (73), but this interaction was difficult to observe *in vivo.* An explanation for this discrepancy was provided by the finding that SLP-76 associates with Gads, not Grb2, *in vivo* (71,72,87). Overexpression of both SLP-76 and Gads results in an augmentation of NFAT and IL-2 promoter activity, a finding suggesting that the interaction of these proteins *in vivo* modulates Ca^{++} mobilization and downstream events (71,72,87).

After TCR ligation, SLP-76 inducibly binds through its SH2 domain to another adapter molecule referred to as SLAP-130 (for SLP-76 associated protein of 130 kd) (88) or Fyb (for Fyn-T binding protein) (89). SLAP-130/Fyb is expressed in T and myeloid cells, but it is not found in B lymphocytes. Sequence analysis of SLAP-130/Fyb reveals that it possesses a proline-rich region, multiple tyrosine phosphorylation sites, two nuclear localization motifs, and a carboxy-terminal SH3 domain (88,89). Phosphorylation of key tyrosine residues on SLAP-130/Fyb is accomplished by a T-cell–specific isoform of the PTK Fyn (90). Conflicting evidence has been generated regarding the cellular effects of a SLP-76/SLAP-130 association. Early work performed in the Jurkat T-cell line indicated that overexpression of SLAP-130 inhibited IL-2 promoter activity, a finding that supported a role for SLAP-130/Fyb as a negative regulator of TCR-mediated signal transduction events (88). More recently, however, several studies illustrated positive effects of SLAP-130/Fyb overexpression in Jurkat cells, resulting in augmented IL-2 promoter activity (89). The true nature of SLAP-130 function likely will require use of additional experimental techniques, such as analysis of cell lines or mice made deficient for this adapter molecule.

Although many facets of T-cell activation require the presence of SLP-76, T-cell development in the thymus also depends on SLP-76 expression. This finding was demonstrated through the generation of mice made deficient for SLP-76 by targeted disruption of the gene locus. SLP-$76^{-/-}$ mice possess a complete absence of mature T cells in the periphery (91,92). Thymocyte maturation in these mice is blocked at a $CD4^{-}/CD8^{-}$ double-negative stage during which SLP-76 levels are usually high (93). These data suggest that, as with signaling through the mature TCR, the pre-TCR present on pro-T cells also relies on SLP-76 for propagation of signals. In wild-type mice, the level of SLP-76 expression is downregulated after thymocytes pass through this critical stage of development, and it does not reach equivalently high levels again until T cells become activated in the periphery (93). Therefore, it appears that SLP-76 expression within the T-lymphocyte compartment is tightly regulated as an added means of controlling SLP-76 function.

B lymphocytes express a functional equivalent of SLP-76, BLNK, that also acts as a critical mediator of signal transduction in these cells (25). BLNK, also known as SLP-65 (94) and BASH (95), is a 70-kd protein that is rapidly tyrosine phosphorylated after BCR engagement. Like SLP-76, it consists of a similar functional domain structure: an amino-terminal acidic region containing multiple tyrosine residues, a central proline-rich region, and a carboxy-terminal SH2 domain (25). Homology at the amino acid sequence level, however, is low between the two molecules. BLNK also appears to have a more restricted expression pattern, in that it has been identified

only in B lymphocytes and macrophages.

BLNK shares many functional characteristics with SLP-76, but significant differences between the roles of the two molecules do exist. After BCR ligation, BLNK is phosphorylated on tyrosine residues by the PTK Syk (25). Once this occurs, the SH2 domain of Grb2 mediates binding to BLNK, and data support the presence of a BLNK/Grb2/Sos complex (25). Although Grb2 also binds to Shc in B cells, no evidence has indicated that Shc and BLNK associate indirectly through binding to Grb2. Using a DT40 B-cell line deficient in BLNK expression, it was shown that BLNK is required for activation of PLC-g isoforms (96). Phosphorylated BLNK associates inducibly with the SH2 domain of PLC-g isoforms, and mutations that prevent this interaction lead to a loss of PLC-g phosphorylation and activation (43). Predictably, downstream events, including intracellular Ca^{++} mobilization and NFAT function, are diminished as a result. Because of its structural similarities to SLP-76, and its functional similarities to LAT, it is possible that BLNK acts as a hybrid equivalent of both molecules in B cells.

More recently, it was demonstrated that BLNK is a critical mediator of B-lymphocyte development. Examination of mice made deficient for BLNK expression revealed a block in B-cell maturation at an early pro-B cell stage (97). In contrast to the loss of SLP-76, however, a few B cells are present in the periphery of $BLNK^{-/-}$ mice. These cells possess an altered phenotype from most mature B cells, in that they express increased levels of surface IgM, but do not express IgD. $BLNK^{-/-}$ IgM^{hi} B cells are able to mobilize intracellular calcium and to upregulate B7 expression on the surface after stimulation through the BCR. These findings suggest that other molecules within murine B cells can compensate for the loss of BLNK, albeit only partially. This does not appear to be true for the loss of BLNK in humans. A person with normal numbers of pro-B cells, but a complete loss of mature B cells, was identified as having a splice defect that abrogated processing of BLNK transcripts (98). Therefore, BLNK mRNA and protein were not detectable, and these defects likely were causally related to the absence of B cells in this patient.

Negative Regulators of Signal Transduction

Cbl

Although many adapter molecules function as positive coordinators of signal transduction events in T and B lymphocytes, some act instead to downregulate lymphocyte responses. The Cbl family of adapter molecules comprise perhaps the best-characterized negative regulators of lymphocyte signaling (22). The prototype of the family is a 120-kd cytosolic protein known as Cbl. Cbl is the cellular homolog of the viral, or v-cbl, gene product that has been shown to transform B lymphocytes. It is also a homolog of the *Caenorhabditis elegans* protein Sli-1, a finding indicating that the Cbl protein has been conserved evolutionarily. In addition to Cbl, the family also contains the proteins Cbl-b and Cbl-c, which display varying degrees of amino acid sequence homology to Cbl.

Like all adapter molecules, Cbl possesses multiple domains that allow protein-protein interactions. The amino-terminus of Cbl contains a divergent SH2 domain (99), the central portion includes a ring finger domain and a proline-rich region, and the carboxy-terminal consists of multiple tyrosine residues and a leucine zipper motif (22). Cbl-b is structurally similar to but slightly larger than Cbl. Cbl-c, in contrast, is a smaller protein that lacks carboxy-terminal tyrosine residues and a leucine zipper region.

Cbl is expressed in both T and B cells, and is rapidly tyrosine phosphorylated after TCR or BCR ligation. This is mediated by PTKs of both the Src and Syk families (100,101 and 102). Phosphorylation of Cbl allows it to interact with other SH2-containing proteins such as Vav, the p85 subunit of PI3K, and the adapter molecule CrkL (101,103,104 and 105). Binding to CrkL provides one method by which Cbl interferes with T-cell activation (Fig. 8.4). A ternary complex consisting of CrkL, Cbl, and C3G, a guanine nucleotide exchange factor for the GTPase Rap-1, has been implicated in regulating T-cell anergy (106). It is thought that formation of this complex allows C3G to activate Rap-1. Active Rap-1 can interfere with signaling through the Ras cascade by sequestering Raf, the kinase immediately downstream of Ras. Thus, signals downstream of Ras are prohibited, and blockade of T-cell activation results.

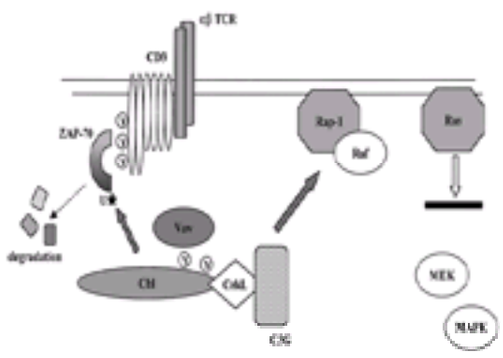

Figure 8.4. Models for the role of Cbl as a negative regulator of lymphocyte signal transduction. A ternary complex consisting of Cbl, CrkL, and C3G may lead to Raf sequestration by Rap-1, thereby blocking progression of the Ras/MAPK pathway. Cbl may also participate in the ubiquitination of Syk and ZAP-70, thereby resulting in degradation of these protein tyrosine kinases.

Cbl also negatively regulates the activity of Syk and ZAP-70, and it may do so through several different mechanisms. Evidence suggests that one method may be the Cbl-dependent ubiquitination and subsequent degradation of these PTKs (Fig. 8.4). In studies examining signals downstream of the platelet-derived growth factor receptor, Cbl was shown to function as an E3-ubiquitin-protein ligase (107,108). E3 molecules bring proteins into contact with E2-ubiquitin-conjugating enzymes, thus promoting degradation of these proteins. Support for the idea that Cbl functions in a similar manner in lymphocytes comes from examination of COS cells made to express Cbl; these cells show a correlation between Cbl expression and decreased levels of detectable Syk protein (109). Cbl has also been shown to bind to negative tyrosine residues on both Syk and Zap-70. This effect appears to be mediated through the SH2 and ring finger domains of Cbl, because mutations of cysteine residues in the ring finger domain abrogate the negative effects of Cbl on Syk function (110).

Cbl also associates with several other key proteins in lymphocyte signal transduction pathways. For example, Cbl associates with the SH3 domains of Grb2 (103), and such an interaction may interfere with the binding of Grb2 to Sos. The proline-rich domain of Cbl also mediates binding to the SH3 domains of Nck, Fyn, and Lck. The interactions with Fyn and Lck are constitutive, but they increase after TCR ligation, a finding implying that SH2 domain-mediated binding may also occur (22). Similarly, constitutive association with the p85 subunit of PI3K also increases after antigen receptor engagement. At this time, however, the physiologic effects of these Cbl interactions are not understood.

Although the exact mechanisms by which Cbl exerts its negative effects on lymphocyte signaling have not been elucidated fully, the role of this adapter molecule as an inhibitor of lymphocyte activation is clear. The most compelling evidence comes through examination of T cells isolated from Cbl-deficient mice. In these cells, TCR and CD3 surface expression is enhanced, and thymocytes show increased levels of tyrosine phosphorylation on ZAP-70, LAT, and SLP-76 (111,112). Downstream events such as activation of the MAPK pathway are also elevated. Mice lacking the Cbl-b molecule illustrate an even more important role for this isoform in both T- and B-cell signaling, because both cell types exhibit spontaneous activation in the periphery, leading to autoimmunity. Closer analysis of these $Cbl\text{-}b^{-/-}$ lymphocytes reveals enhanced signaling through both the TCR and BCR and an ability of the cells to proliferate in response to antigen receptor ligation alone, without a requirement for costimulation (113). These findings support the role of Cbl as a negative regulator of lymphocyte signaling and suggest that it plays a unique role in these cells that cannot be filled in its absence.

ROLES OF DOWNSTREAM EFFECTOR MOLECULES

The signal transduction events initiated by TCR or BCR ligation are propagated by activation of PTKs and coordinated by a variety of adapter molecules. These receptor-proximal occurrences eventually lead to the involvement of downstream effector molecules. Although numerous effectors become activated in T and B lymphocytes after antigen receptor engagement, the remainder of this chapter focuses only on the best-characterized among them. The selected examples demonstrate how ligation of a surface receptor is ultimately translated into transcription of activation-induced genes.

Phospholipase Cg and Ca^{++} Mobilization

One of the primary targets of PTK activation is the enzyme PLC-g. PLC-g functions by cleaving membrane-bound PIP_2 into IP_3 and DAG, a potent inducer of multiple protein kinase C (PKC) isoforms (26,27). The activation of PLC-g correlates with its tyrosine phosphorylation, and this appears to be accomplished coordinately by the activity of ZAP-70 and Tec kinase family members (2,37). Correct cellular localization of PLC-g is required for its phosphorylation to occur. Evidence of this has been provided by examination of T cells that lack either LAT or SLP-76, or B cells that are deficient in BLNK, because these cells display greatly decreased levels of PLC-g

phosphorylation and function (86).

Production of IP_3 controls the release of Ca^{++} from stores in the endoplasmic reticulum. Ca^{++} release occurs when IP_3 binds to ligand-gated ion channels in the endoplasmic reticulum membrane (114). This leads to a rapid, but transient, increase in the concentration of cytosolic, or intracellular Ca^{++}. Many proteins are sensitive to alterations in the level of intracellular calcium and become activated as a result. Two examples are the Ca^{++}–release-activated Ca^{++} current (CRAC) channels located in the plasma membrane, and the serine phosphatase calcineurin. The opening of CRAC channels mediates an influx of extracellular Ca^{++}, and produces a sustained elevation in intracellular Ca^{++} concentration. Activation of calcineurin depends on the function of the Ca-binding protein, calmodulin.

Important substrates of calcineurin include members of the NFAT family of transcription factors. In resting lymphocytes, NFAT proteins are confined to the cytosol. Dephosphorylation of key regulatory residues by calcineurin allows NFAT members to translocate to the nucleus, where they coordinately regulate transcription of activation-induced genes such as IL-2, IL-4, and tumor necrosis factor-a (115). Inhibition of NFAT translocation is mediated by two potent immunosuppressive agents, cyclosporine (Cyclosporin A or CsA) and FK506. These drugs form complexes with the cellular proteins cyclophilin and FKBP, respectively, and subsequently block calcineurin function (116). Thus, treatment of T cells with CsA specifically blocks a downstream effector of the PLC-g/ Ca^{++} pathway, NFAT, and results in complete loss of T-cell function. This underscores the critical importance of PLC-g-mediated events during the course of T-lymphocyte signal transduction.

Phosphoinositide-3-Kinase

The PI3K family of enzymes also assumes a key role in both T- and B-cell signaling. The PI3K family consists of several different isoforms, but the best studied in lymphocytes is composed of two subunits, an 85-kd protein that provides regulatory functions and a 110-kd protein possessing enzymatic activity (117). The regulatory subunit of PI3K is encoded on three separate genes that give rise to the p85a, p85b, and p55g proteins. This discussion focuses primarily on p85a, which comprises two SH2 domains, one SH3 domain, two proline-rich regions, and a p110-binding domain. PI3K is a unique enzyme in that it displays both serine kinase activity and lipid kinase activity. The lipid kinase functions to phosphorylate the D3 position of lipid molecules. Although several different products can be generated by this enzyme, phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5P_3) has been studied most as a modulator of lymphocyte signal transduction.

The lipid kinase activity of PI3K is essential for sustained Ca^{++} mobilization in lymphocytes. This is thought to result from its indirect role in Tec kinase activation. PI3K products, such as PI 3,4,5P_3, are bound by the PH domains of Tec kinases (118,119). This association targets Tec kinases to the plasma membrane, where they can be activated by src PTKs and can subsequently contribute to phosphorylation of PLC-g.

In B lymphocytes, the phosphorylation of PI3K was initially thought to result from activity of the PTK Lyn. However, more recent data suggest that the kinase Syk participates indirectly in PI3K activation through tyrosine phosphorylation of Cbl (120). In this model, Cbl interaction with the SH2 domain of the p85 subunit of PI3K, results in PI3K localization to the plasma membrane. Studies using B-cell lines deficient in Syk demonstrate that formation of D3 phosphoinositides is diminished, as is phosphorylation of a substrate of PI3K, Akt (121).

Akt, also known as protein kinase B, is a serine-threonine kinase that has been implicated in modulating survival signals in a variety of cell types (122). After antigen receptor ligation, Akt is recruited to the plasma membrane through its PH domain, where it binds lipid products generated by PI3K. This allows Akt to become activated through phosphorylation of key serine and threonine residues. It is currently thought that two discrete PI3K-dependent enzymes are responsible for Akt phosphorylation, although only one, PDK1, has been rigorously proven to play such a role.

Akt modulates, both directly and indirectly, the activity of several transcription factors. Examples of indirect effects include regulation of nuclear factor-kB (NF-kB) activity by phosphorylation of the upstream inhibitory kB kinase (or IKK) (123). Phosphorylation of IKK results in degradation of the inhibitory kB protein, which then releases the transcription factor NF-kB so it may translocate to the nucleus. Akt indirectly blocks activation of Erk-dependent transcription factors by phosphorylating a regulatory serine residue on the upstream Raf kinase, rendering it inactive in some cell types (124). An example of Akt directly regulating the activity of transcription factors is seen by the dual serine and threonine phosphorylation of the Forkhead proteins, which prohibits their translocation into the nucleus (122). Thus, PI3K-dependent activation of Akt can either enhance or reduce gene transcription, depending on which transcription factors are involved.

The requirement for PI3K in lymphocyte signaling has been demonstrated by studies examining the phenotype of mice engineered to lack expression of specific PI3K isoforms. In mice with disruption of only p85a, T-cell maturation and activation remain largely unaffected, although PI3K activity is shown to be reduced by as much as 60% in these cells (125). In contrast, B-lymphocyte development is blocked at an early pro-B cell stage. A small population of mature B lymphocytes is present in $p85a^{-/-}$ mice, but these cells display diminished proliferation and antibody secretion in response to stimulation with T-independent antigens such as lipopolysaccharide (LPS). Mice lacking p55a and p50a expression in addition to p85a possess a similar phenotype, with no observable defects in T-cell function, but profound impairment of B-cell development and activity (126). Loss of a PI3Kg isoform produces the reverse phenotype in which T-cell development and function are impaired, whereas B-cell responses appear normal (127). These findings suggest that although PI3K is an important effector of signal transduction in both T and B lymphocytes, specific PI3K isoforms are used differentially by the two cell populations.

Ras and the MAPK Cascade

One of the most intensively studied downstream effectors of antigen receptor engagement is the low-molecular-weight GTPase, Ras. This ubiquitously expressed protein is required for the induction of many different cellular responses in activated lymphocytes. Ras represents just one of many low-molecular-weight GTPase families. Although the various families participate in differing signal transduction pathways, all share a characteristic pattern of activation and inactivation events. Much like the larger heterotrimeric GTPases, Ras cycles between existence in a GDP-bound versus a GTP-bound state (1). In its inactive state, Ras is bound to GDP. Activation of Ras occurs when it releases GDP and binds the more abundant cytosolic GTP. Release of GDP occurs with the assistance of proteins known as GEFs, or guanine nucleotide exchange factors. Once bound to GTP, Ras becomes and remains active, until it hydrolyzes its bound GTP. Hydrolysis of GTP is aided by a second category of proteins referred to as GAPs (for GTPase activating proteins). The generation of GDP again returns Ras to its inactive state.

Once activated, Ras initiates a multistep kinase cascade that traverses from the inner surface of the plasma membrane to the nucleus. Ras proteins undergo posttranslational lipid modifications that result in their constitutive localization at the plasma membrane (128). The protein activated immediately downstream of Ras is Raf, a serine-threonine kinase. The exact mechanism by which Ras activates Raf remains unclear. However, it is known that the normally cytosolic Raf is recruited to the plasma membrane, where it associates with Ras. Activation of Raf is linked to its phosphorylation status, but Ras does not appear to phosphorylate Raf. Instead, experimental evidence supports a model in which binding to Ras permits other kinases to phosphorylate Raf (129). Raf then directly activates a family of dual-specificity threonine-tyrosine kinases known as MAPK kinases (MAPKKs), or MEKs. MEKs, in turn, phosphorylate specific MAPKs, including the Erk 1 and Erk 2 proteins (for extracellular-signal–regulated kinases) (130,131). Activated Erk molecules migrate from the cytosol into the nucleus, where they influence gene transcription by activating a variety of transcription factors.

Although this linear cascade of Ras and MAPK has been well defined, it is becoming increasingly clear that Ras activation participates in a complex network of signal transduction events (129). For example, multiple families of MAPKs, MAPKKs, and the upstream equivalents of Raf proteins, also designated as MAPKK kinases, exist. Ras activation likely contributes to the induction of these kinases cascades, thereby tying together several parallel pathways. The cellular result is that Ras indirectly regulates the activity of numerous transcription factors that lie downstream of not only Erk 1 and 2, but additional MAPK family members, including JNK (for Jun NH_2-terminal kinase) and p38. Positive feedback mechanisms within the Ras/MAPK pathways have also been described in which activated MEKs phosphorylate Raf, leading to an augmentation of its activity. Further complexity is provided by evidence for the involvement of PKC molecules and PI3K in Ras activation, although the molecular basis for these events is unclear. Ultimately, the activation of many different upstream effector molecules converges on the Ras/ MAPK pathways, and the induction of these cascades is essential for a vast array of cellular responses in lymphocytes after TCR or BCR ligation.

SUMMARY

Ligation of antigen receptors on T and B lymphocytes triggers a multitude of intracellular biochemical events. These reactions transduce signals from receptors on the cell surface to the nucleus, where transcription of activation-induced genes occurs. Receptor-proximal activation of PTKs leads to phosphorylation of adapter molecules, which coordinate the formation of multimeric protein complexes. These complexes bring downstream effector molecules, such as PI3K and PLC-g, into contact with the PTKs that regulate their function.

Although this chapter focuses on the signal transduction events that arise from antigen receptor ligation, TCR or BCR engagement does not occur as an isolated event. Instead, costimulatory molecules such as CD28 on T cells and CD40 on B cells are bound simultaneously by their ligands. These costimulatory receptors initiate biochemical changes within lymphocytes that complement signals originating from the TCR or BCR. For a naive T or B lymphocyte, costimulatory signals are essential for activation, and in their absence, cells progress into a state of anergy, or into the apoptotic pathway referred to as programmed cell death. Thus, biochemical pathways initiated by costimulatory receptors provide unique signals to naive lymphocytes that are not present after antigen receptor engagement alone.

The fundamental importance of lymphocyte signal transduction pathways is evidenced by the various immunodeficiencies that develop when the progression of these events are disrupted. Loss of function in a specific signaling mediator may adversely affect T cells without noticeably impacting B cells, as is seen with deletion of SLP-76. The reverse situation is also observed; for example with the loss of Btk resulting in agammaglobulinemia. These findings highlight the differences between T- and B-lymphocyte signal transduction; although both cell types use the same families of effector and adapter molecules, individual members often are used differentially.

Significant advances have been made in elucidating the relationships among various components of lymphocyte signal transduction path-ways; however, much remains to be learned. New adapter molecules continue to be identified, and novel functions are being described for well-known effector molecules. Our appreciation for crosstalk between pathways that were once thought to be discrete is growing. Future efforts focused on understanding the relationships of signaling molecules with respect to each other and to their intracellular localization before and after antigen receptor ligation will enhance our appreciation of the intricate mechanisms that control lymphocyte activation.

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9 T-Lymphocyte Activation, Costimulation, and Tolerance: Signals, Mechanisms, and Clinical Applications

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The mammalian immune system has evolved a complex system of humoral and cell-mediated responses for protection against infectious pathogens. All adaptive immune responses are initiated by the recognition of antigen by specific clones of lymphocytes. However, antigen alone is insufficient to trigger the proliferation of naive lymphocytes and their differentiation into effector cells. The development of effective immune responses usually requires additional signals, which are typically induced by microbes or by innate immune reactions to microbes. In the absence of such signals, the antigen-specific lymphocytes either fail to respond or are shut off. The requirement for these signals ensures that lymphocytes respond to foreign agents but not to harmless substances, such as self-antigens. Elucidating the nature of the signals that influence the choice between lymphocyte activation and tolerance is likely to provide important clues not only about physiologic immunity, but also about pathologic states, such as autoimmunity, that result from a failure of self-tolerance. Furthermore, defining these signals may suggest potential targets for stimulating desired immune responses and for limiting pathologic responses. In this chapter, we describe the nature of the signals that determine lymphocyte activation versus tolerance and the physiologic and potential clinical importance of these signals.

The concept that lymphocytes need stimuli in addition to antigen to mount effective responses dates from 1970, when Bretscher and Cohn postulated that B cells require two signals to become activated (1). More than a decade later, Jenkins and Schwartz showed that T cells also require two sets of signals to be activated (2). The first signal is triggered by antigen, that is, peptide-major histocompatibility complex (MHC) on antigen-presenting cells (APCs), which are recognized by the clonally distributed antigen-specific T-cell receptor (TCR). The second signal is provided by molecules on APCs that were called *costimulators.* These early studies, using cloned lines of $CD4^+$ T cells, also showed that antigen recognition without costimulation led to long-lived unresponsiveness, or anergy, of the T cells. We now know that several molecules on APCs may function as costimulators for T cells. In this chapter, we describe the biology of the two major costimulatory pathways, the B7:CD28 and the CD40:CD40 ligand pathways, and discuss the role of costimulation in regulating T-cell activation versus tolerance.

B7:CD28 FAMILIES OF COSTIMULATORY LIGAND-RECEPTOR PAIRS

The first T-cell molecule found to deliver costimulatory signals was CD28 (3,4). This discovery came from the observation that cross-linking CD28 on human T cells using anti-CD28 mAbs greatly enhanced the response of the T cells to engagement of the TCR/CD3 complex (5,6 and 7). The search for the CD28 ligand led to the next important discovery, that CD28 binds to a molecule called B7 that was thought to be a marker of the B-cell lineage and was later shown to be expressed on most APCs (8,9 and 10). Thus, B7 is a costimulator, and CD28 is its receptor on T cells. As discussed later, the story of costimulation became much more complex and fascinating in the 1990s.

B7 MOLECULES

Two ligands for CD28, called B7-1 (CD80) and B7-2 (CD86), are recognized (10,11 and 12) (Fig. 9.1). These proteins are approximately 25% identical, with greater homology in the extracellular domain than in the cytoplasmic domain, and they have largely overlapping functions (13). The expression of B7-1 and B7-2 has several important characteristics. First, both proteins are expressed on professional APCs, consistent with their roles as costimulators for T cells. Second, the expression of B7-1 and B7-2 is enhanced by microbes interacting with the APCs, by cytokines such as interferon-g (IFN-g) and interleukin-4 (IL-4), which are produced during innate immune responses to microbes, and by activation of the APCs by CD40-mediated signals (discussed later in the chapter) (14,15). Thus, the regulated synthesis of costimulators ensures that they are expressed during the immune response to infection. Third, the kinetics and levels of expression of B7-1 and B7-2 have subtle differences. On most APC populations, B7-2 is present even in the resting state or appears more rapidly after activation and at quantitatively higher levels than does B7-1 (8,16). This finding suggests that B7-2 may be the more important costimulator for initiating immune responses. Finally, B7-1 and B7-2 may be induced on cell types other than APCs. For instance, both molecules are expressed on activated T cells, but the role of T-cell B7 remains uncertain.

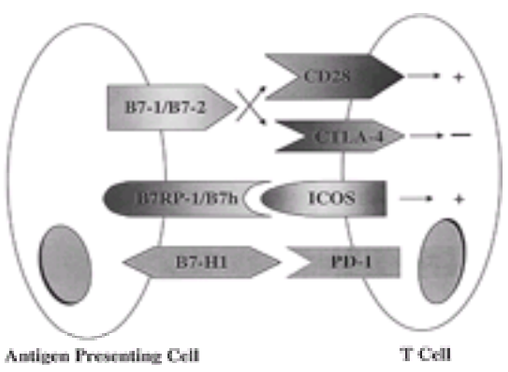

Figure 9.1. B7 costimulatory ligand-receptor pairs. B7-1 and B7-2 on antigen-presenting cells (APCs) interact with CD28 to deliver positive costimulatory signals to T cells, whereas binding to CTLA-4 transmits negative signals. Engagement of B7RP-1:B7h with ICOS provides a stimulatory signal to T cells. B7-H1 is expressed on APCs, and the counterreceptor is PD-1.

Other proteins homologous to B7-1 and B7-2 have been described. One, variously called B7RP-1 (B7-related protein) or B7h (B7 homolog), is expressed on APCs and other cell types in response to endotoxin and cytokines that activate the nuclear factor-kB (NF-kB) transcription factor (17). This response is a characteristic component of innate immunity to microbes. Similar to B7-1 and B7-2, B7RP-1 is a cell surface glycoprotein with costimulatory function for T cells. Two more B7 homologs, called B7-H1 (PD-L1) and B7-DC (PD-L2) have been identified (18). The possible functions of these proteins are described later.

CD28/CTLA-4 FAMILY OF RECEPTORS

The CD28 receptor for B7-1 and B7-2 is expressed on all naive $CD4^+$ helper T (Th) cells and on most naive $CD8^+$ T cells (3). When T cells recognize antigens presented by APCs, CD28 interacts with B7-1 or B7-2. The engagement of CD28 delivers signals to the T cells that initiate immune responses. Thus, CD28 is an activating receptor of T cells.

Several years after the discovery of CD28 and B7, investigators found that T cells express a second receptor for B7-1 and B7-2. This protein was called CTLA-4 (CD152), because it was as the fourth antigen identified in a search for molecules expressed in cytotoxic T lymphocytes (CTLs) (19). CTLA-4 is expressed on activated

CD4$^+$ and CD8$^+$ T cells at lower levels than is CD28, and CTLA-4 is not detectable on resting T cells (20). Importantly, binding of B7 on APCs to CTLA-4 on T cells delivers inhibitory signals that shut off T-cell responses. Thus, engagement of CTLA-4 has the opposite effect on T cells as engagement of CD28, although both receptors bind the same B7 molecules on APCs (Fig. 9.2). How the balance between CD28-mediated activation and CTLA-4-mediated inhibition is achieved during the initiation and termination of physiologic immune responses remains an intriguing question without a clear answer. There are several other differences between CD28 and CTLA-4. CD28 is an integral membrane protein; in contrast, greater than 90% of cell-associated CTLA-4 is present in intracellular vesicles and is rapidly shuttled to the cell surface on antigen recognition by T cells (21). *In vitro,* CTLA-4 binds to B7 with 20- to 50-fold higher affinity than does CD28 (22). The cytoplasmic domains of CD28 and CTLA-4 are also distinct, which may account for the differences in the signals transduced by these receptors (discussed later).

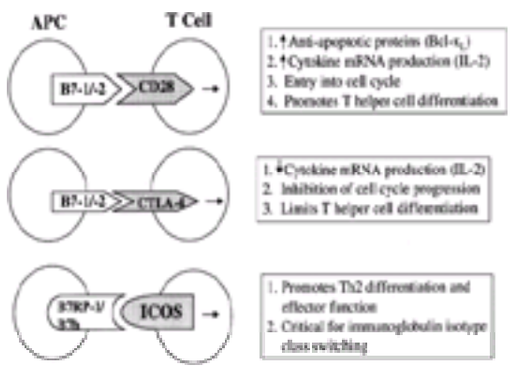

Figure 9.2. Functional effects of B7 costimulatory interactions. Antigen-presenting cells express ligands that engage costimulatory receptors on T cells. The functional effects of B7-1:B7-2 interactions with CD28 and CTLA-4 and B7RP-1:B7h with ICOS are described.

A CD28 homolog called ICOS (inducible costimulatory molecule) has been identified on activated T cells (23). ICOS binds to B7RP-1/B7h and stimulates T-cell responses that are distinct from the responses triggered by CD28. The biologic implications of these multiple receptor-ligand pairs are discussed later.

FUNCTIONAL EFFECTS OF THE B7:CD28 LIGAND-RECEPTOR FAMILY

Binding of CD28 to B7-1 and B7-2 induces a series of biologic responses in T cells that are critical for the initiation of T-cell clonal expansion and differentiation into effector cells. All these biologic responses are the consequence of costimulation functioning together with antigen recognition. In other words, CD28 signals synergize with signals transduced by the TCR/CD3 complex.

Costimulation induces the production of antiapoptotic proteins, notably Bcl-xL, in T cells (24). In the absence of costimulation, T cells die by apoptosis. Thus, costimulation prolongs the survival of antigen-recognizing T cells. At the same time, costimulation stimulates the transcription of cytokine genes and increases the stability of cytokine mRNA, thus leading to increased production of cytokines (13,25,26,27,28,29,30 and 31). The first of these cytokines to be detected in T cells is the growth factor, IL-2, which is largely responsible for the initiation of T-cell proliferation. Costimulation also enhances the expression of high-affinity receptors for cytokines, such as IL-2 receptor. In addition, stimulation through the B7:CD28 pathway promotes cell-cycle progression from the G_1 phase into the S phase by downregulating the cell-cycle inhibitor, $p27^{kip}$ (32,33). Thus, CD28 regulates T-cell expansion through synergistic effects with the TCR, and this leads to production of T-cell survival factors, T-cell growth factors, and cell-cycle progression.

Costimulation enhances the differentiation of antigen-stimulated T cells into effector cells. In CD4$^+$ Th lymphocytes, differentiation is reflected in acquisition of the capacity to produce effector cytokines, such as IL-4, IL-5, and IFN-g (34). CD28-mediated signals appear to be more important for the differentiation of naive T cells into IL-4–producing and IL-5–producing Th2 effectors than in the development of the Th1 subset (35,36). In part, this may be because Th1 differentiation may be induced by other signals such as the macrophage and dendritic cell-derived cytokine, IL-12 (37). In CD8$^+$ T cells, costimulation is required for the development of fully active effector CTLs.

These functional effects of B7:CD28 interactions have been demonstrated by many experimental approaches, such as the use of agonistic and blocking antibodies, and APCs or T cells from knock-out mice. The soluble CTLA-4 fusion protein, CTLA-4Ig, has been particularly effective at blocking B7 interactions with CD28 and CTLA-4 and useful in determining the functional role of this pathway (22). Although such studies clearly illustrate the significance of the B7:CD28 pathway, it is also apparent that many T-cell responses may be partially or largely independent of CD28 signaling. The effect of B7 blockade may depend on the strength of the TCR signal. For example, intensifying the strength of signal by increasing the quantity of specific antigen can overcome the effects of CD28 deficiency in a transgenic autoimmune disease model (38).

Antagonists and gene knock-out mice have proved extremely valuable for analyzing the critical functions of the B7:CD28 pathway during immune responses *in vivo*. Mice lacking both B7-1 and B7-2 are profoundly immunodeficient. These mice fail to exhibit T-cell responses to immunization with protein antigens in adjuvants, are susceptible to microbial infections, and fail to reject vascularized organ allografts (39,40). Likewise, Th-cell–dependent humoral immune responses are impaired, as shown by defects in immunoglobulin class switching and germinal center formation. Mice deficient in B7-2 only respond poorly to immunization with antigens and weak adjuvants, but they respond normally when strong adjuvants are used. Mice lacking B7-1 only do not show significant immunologic defects. These results support the view that B7-2 is the more important costimulator for initiating T-cell responses, but B7-1 and B7-2 serve similar functions. The question whether B7-1 and B7-2 are functionally identical or distinct is controversial. Some earlier studies suggested that these two proteins differ in their ability to induce Th1 or Th2 development. It is likely that such observed differences are related more to the kinetics of B7-1 and B7-2 expression than to intrinsic differences in the way these molecules bind to and signal through CD28.

Surprisingly, mice lacking CD28 show only modest immune defects and no abnormality in many T cell-mediated responses, such as allograft rejection. Basal immunoglobulin levels are reduced, and germinal center formation is blocked in CD28-deficient mice as compared with wild-type mice (41). T cells isolated from CD28-deficient animals exhibit impaired responses to alloantigens and stimulation through the TCR with anti-CD3 mAbs (42). Other *in vivo* studies involving infectious agents have shown that the host immune response is not strictly dependent on CD28 engagement. After LCMV infection, CTL responses and the host-protective delayed-type hypersensitivity reaction are intact in CD28-deficient mice (43). Likewise, CD28-deficient mice infected with *Leishmania major* develop Th2 responses and disease on the susceptible genetic background (44). Differences in results using CD28-deficient and B7-1/B7-2–deficient mice are difficult to explain. Although CD28 is the only known activating receptor for B7-1 and B7-2, CD28 deficiency does not result in the same abnormalities as the absence of B7-1 and B7-2. The observed differences raise the possibility that there are other activating receptors for B7-1 and B7-2, but these have not been identified yet.

Engagement of CTLA-4 by B7 molecules inhibits T-cell cytokine production and cell-cycle progression. Based on *in vitro* studies using anti-CTLA-4 mAbs, signaling through CTLA-4 can block CD28-dependent IL-2 production, IL-2 receptor expression, and cell-cycle progression of activated T cells leading to arrest in the G_0/G_1 phase (45,46,47 and 48). Unlike CD28, CTLA-4 does not influence the induction of cell survival factors such as bcl-xL (49). Stimulation of CTLA-4–deficient T cells skews differentiation toward a Th2 phenotype (50). Thus, in the B7:CD28/CTLA-4 pathway, signaling through CD28 and CTLA-4 has opposing effects on Th cell differentiation: B7:CD28 interactions promote Th2 differentiation, whereas B7:CTLA-4 interactions limit the extent of Th2 differentiation. The definitive proof of the physiologic importance of CTLA-4 came from the finding that knock-out mice lacking this protein develop severe lymphadenopathy and splenomegaly and die of a multiorgan disease associated with infiltrates of activated T cells and macrophages (51). The disease has many features of an autoimmune disorder, but neither the target antigen nor the specificity of the pathogenic T cells is established.

Much less is known about the more recently discovered B7RP-1/B7h:ICOS pathway. *In vitro* studies have shown that engagement of this pathway triggers T-cell responses that are distinct from the responses to B7:CD28 interactions. For instance, binding of B7RP-1 to ICOS induces T-cell secretion of the cytokine, IL-10, which mainly inhibits the functions of macrophages and other APCs and terminates T-cell responses (23). These findings suggest a model in which different costimulatory ligand-receptor pairs function at different phases of T-cell–mediated immune responses. The activation of T cells may be initiated by B7-2:CD28 interactions and amplified by B7-1:CD28. As the activated T cells express ICOS, this receptor may induce the production of regulatory cytokines that dampen the response. Finally, activated T cells may express CTLA-4, which terminates further T-cell activation. Although this model is interesting, it is not formally proved. In fact, defining the relationships between these multiple ligand-receptor pairs and the way in which their activities are orchestrated during physiologic immune responses are among the major challenges in the field.

BIOCHEMISTRY OF COSTIMULATION: SIGNAL TRANSDUCTION BY CD28 AND CTLA-4

Despite considerable effort, little is known about the mechanisms by which CD28 and CTLA-4 transduce activating and inhibitory signals, respectively. In all species examined, the CD28 receptor has a high degree of structural homology; however, fundamental questions about CD28 signaling are unresolved. According to one model, CD28 ligation enhances biochemical signals triggered by the TCR, which include the activation of phosphatidylinositol-3-kinase and the Ras pathway. Consistent with this hypothesis are the findings that CD28 coaggregates with the TCR/CD3 complex when T cells bind to APCs displaying antigen (52). Furthermore, engagement of CD28 enhances the time for which the TCR remains in membrane microdomains at the site of contact with APCs. An alternative hypothesis is that CD28 delivers signals that are distinct from and independent of TCR-mediated signaling, and the two sets of signals are integrated inside the cells. The IL-2 transcription factor, cJun, is activated after CD28 engagement, but whether this transcription factor is required for CD28-mediated T-cell costimulation is not established. Furthermore, these hypotheses are not mutually exclusive, because CD28 may increase TCR signaling and may also trigger independent signals. Studies have shown that cross-linking of the TCR and CD28 results in the activation of the transcription factors NFAT (nuclear factor of activated T cells), activation protein-1 (AP-1), and nuclear factor kappa B (NF-kB)/rel (53,54) (Fig. 9.3). By contrast, stimulation of the TCR alone may be sufficient for the activation of NFAT (55,56).

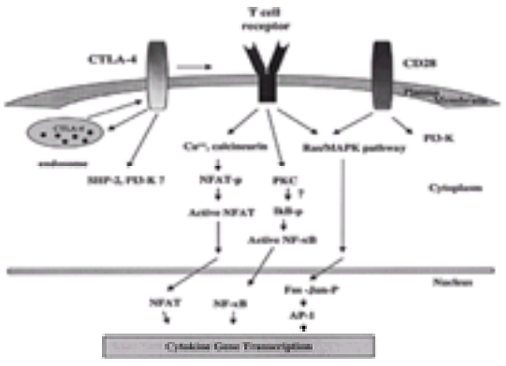

Figure 9.3. T-cell receptor (TCR) and costimulatory molecule signal transduction pathways. The *arrows* between the signaling molecules do not necessarily reflect direct interactions.

Even less is known about the biochemistry of CTLA-4–mediated inhibition. CTLA-4 has a short cytoplasmic tail that is 100% conserved between species and contains two tyrosine-containing motifs. The first tyrosine motif, YVKM, plays a pivotal role in the tightly controlled cellular localization of CTLA-4 as well as in the interaction with key signaling molecules (57,58 and 59). The nonphosphorylated YVKM can associate with a clathrin-associated adapter complex AP-2, which is responsible for continuous translocation of CTLA-4 to and from the cell surface (58,60). Phosphorylation of CTLA-4 releases binding to AP-2 and results in increased cell-surface expression. In a phosphorylated state, CTLA-4 can interact with phosphatidylinositol-3-kinase or the tyrosine phosphatase, SHP-2 (60,61,62 and 63). One of the mechanisms by which CTLA-4 may disrupt TCR-mediated signals is by dephosphorylating key signaling proteins (63). Engagement of CTLA-4 may activate SHP-2, resulting in removal of critical phosphate residues from molecules downstream of CD28 or the TCR. Both the target substrates of SHP-2 and the functional consequences of this interaction are not yet known. Some results suggest that the inhibitory activity of CTLA-4 is related to its ability to antagonize CD28-mediated activation by sequestering signaling molecules such as phosphatidylinositol-3-kinase away from CD28. Other studies indicate that CTLA-4 may bind to proteins of the TCR/CD3 complex and may directly alter TCR signaling. Evidence indicates an interaction of CTLA-4 with the CD3-z chain of the TCR complex intracellularly, a finding suggesting that CTLA-4 interferes with early TCR-signaling events (61). However, studies have suggested that tyrosine phosphorylation of the CTLA-4 cytoplasmic tail may not be required for inhibitory signaling (64,65 and 66). Further studies are needed to examine how these findings relate to B7 engagement of CTLA-4.

Finally, investigators have suggested that when B7 on APCs binds to CD28 or CTLA-4 on T cells, the signaling is bidirectional; that is, the APCs also receive some signals. However, there is no evidence that B7 molecules can function as signal transducers, and it appears likely that they only serve as ligands for the CD28/CTLA-4 signaling receptors.

CD40:CD40 LIGAND PATHWAY OF COSTIMULATION

The CD40 molecule is expressed on macrophages, dendritic cells, B lymphocytes, and many other cell populations (67,68). CD40 ligand (CD154) is expressed on activated T lymphocytes (69). The principal function of this ligand-receptor pair is in the effector phases of cell-mediated and humoral immune responses (Fig. 9.4). Activated $CD4^+$ Th cells use their CD40L to bind to the CD40 receptor. This interaction activates macrophages to kill phagocytosed microbes and stimulates B lymphocytes to proliferate and differentiate into antibody-producing cells. Deficiency of CD40 or CD40L results in profound defects in cell-mediated and humoral immunity. Hyper-IgM syndrome dramatically illustrates the essential role for CD40 signaling in humans. Hyper-IgM syndrome results from mutations in the X-linked CD40L gene (70). Loss of CD40L function manifests as defects in germinal center formation, isotype class switching, and cell-mediated immunity (71). The compromise of cell-mediated immunity leads to death of these patients by infectious agents.

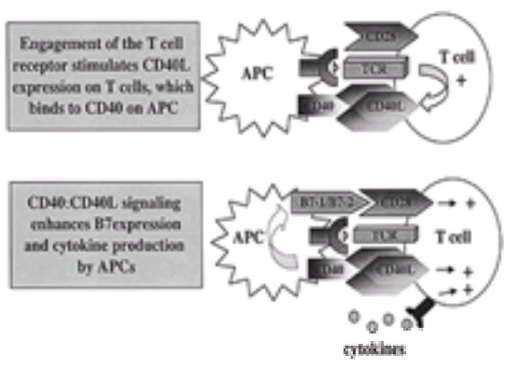

Figure 9.4. CD40:CD40L interactions. The recognition of antigen by T cells (in the presence or absence of costimulators) leads to upregulation of CD40L on T cells. B7:CD28 interactions also enhance CD40L expression. CD40L binds to CD40 on antigen-presenting cells (APCs) and stimulates the expression of B7-1 and B7-2. Cytokines produced by the APCs initiate T-cell differentiation and effector function.

The findings that CD40L-deficient T cells respond poorly to antigens and CD40L antagonists inhibit T-cell activation have led to the hypothesis that this ligand-receptor pair also serves to costimulate T cells. The likely mechanism of this costimulatory effect is that CD40:CD40L interactions activate APCs to become more potent APCs by increasing the longevity of peptide-MHC complexes. CD40L on antigen-stimulated T cells binds to CD40 on dendritic cells and macrophages. These professional APCs respond by increasing their expression of B7-1 and B7-2 and by secreting IL-12, a powerful inducer of Th1 differentiation (72,73,74 and 75). As a result, the APCs stimulate more T cells to respond to the antigen and to develop into Th1 cells. *In vivo* CD40L:CD40 interactions serve to amplify and sustain T-cell responses, whereas B7:CD28 interactions are critical for initiating these responses (76). There is little evidence that CD40L itself delivers a signal to the T cell that directly enhances T-cell responses.

IMMUNOLOGIC TOLERANCE

Tolerance to self-antigens may be induced in the generative lymphoid organs, when immature lymphocytes recognize self-antigens, or in peripheral tissues. The former is called central tolerance. Central tolerance is an integral component of lymphocyte maturation. It is also called negative selection and is discussed in Chapter 5.

Peripheral tolerance is induced when mature lymphocytes recognize antigens under particular conditions. Interest in the mechanisms of peripheral tolerance has been increasing, for several reasons. First, in animal models of autoimmune diseases in which the pathogenesis is understood, the abnormalities giving rise to autoimmunity appear to usually disrupt peripheral tolerance. Second, if we understand how self-antigens induce peripheral tolerance in mature lymphocytes, we may be able to exploit this knowledge to develop methods for shutting off unwanted immune responses. Costimulatory molecules are potentially involved in each of the mechanisms of maintaining peripheral tolerance. In the following section, we describe the mechanisms of peripheral tolerance in mature T lymphocytes, particularly $CD4^+$ T cells, with

an emphasis on the role of the B7:CD28/CTLA-4 pathway.

Peripheral T-Lymphocyte Tolerance

Three principal mechanisms of peripheral tolerance involving mature $CD4^+$ T cells are recognized (Fig. 9.5). When T cells recognize antigen with deficient or no costimulation, the T cells become functionally inactivated. This phenomenon is called clonal anergy, because it affects the antigen-specific clones of lymphocytes. When mature T lymphocytes are repeatedly stimulated by an antigen, the T cells undergo apoptosis, a process called activation-induced cell death (AICD). Self-antigens may induce anergy and AICD in specific T cells because self-antigens are normally displayed to the immune system without costimulation (the condition that induces anergy), and self-antigens are persistent and can repeatedly stimulate lymphocytes (expected to result in AICD). The third mechanism of peripheral tolerance is suppression, in which some T lymphocytes develop into cell populations that secrete immunosuppressive cytokines and terminate further lymphocyte activation.

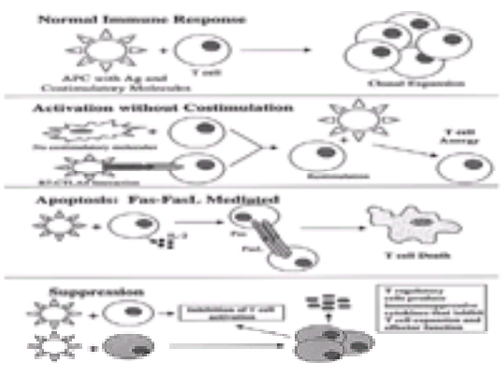

Figure 9.5. Mechanisms of peripheral T-cell tolerance. Model of T-cell:antigen-presenting cell interactions that are required for a normal immune response versus an immune response that leads to anergy induction.

Clonal Anergy

Clonal anergy was first described in cloned lines of $CD4^+$ T cells (2,77). Culture of these clones with antigen presented by APCs lacking costimulators induced a state of long-lived unresponsiveness. It has proved difficult to demonstrate the same phenomenon in normal T cells, because T cells deprived of costimulation die by programmed cell death. Several studies suggest that in normal T cells, anergy is induced when these lymphocytes use the inhibitory CTLA-4 receptor to recognize B7 molecules on APCs (78,79). This function of CTLA-4 may explain the autoimmune phenotype of the CTLA-4 knock-out mouse (51). However, many questions remain unanswered. For instance, how do T cells choose between using CD28 or CTLA-4 to interact with B7 molecules on APCs? Why is CTLA-4 the predominant receptor used when self-antigens are recognized by T cells?

Multiple strategies have emerged for investigating the induction of T-cell anergy *in vivo.* Designing an experimental system to analyze self-tolerance is challenging given the difficulty of identifying self-antigens and quantitating the levels of self-antigens. As a result, the initial studies of T-cell anergy *in vivo* compared administration of immunogenic versus tolerogenic forms of a model foreign antigen (80). Immunogenic antigen is a low dose of antigen administered subcutaneously with an adjuvant, which upregulates costimulators on host APCs at the site of injection and in regional lymphoid tissues. By contrast, tolerogenic antigen is a large dose of aqueous antigen administered systemically. Distinct effects were observed when immunogenic and tolerogenic forms of antigen were administered to naive animals. Immunogenic antigens induce T-cell expansion and differentiation *in vivo,* whereas tolerogenic antigen inhibits the ability of the T cells to respond to subsequent antigen challenge.

Our understanding of tolerance induction *in vivo* has been greatly advanced through the use of transgenic technology. An adoptive transfer approach for investigating immune responses and tolerance *in vivo* was developed by Jenkins and colleagues (81). To track a small population of antigen-specific T cells, T cells from transgenic mice specific for the ovalbumin (OVA) peptide were transferred into normal, syngeneic recipients. The transgenic T cells were allowed to home to the lymphoid tissues, and mice were either left untreated (naive) or given an immunogenic (primed) or tolerogenic (tolerized) form of OVA. At the peak of the immune response, T cells were recovered from the recipients and were stained with an antibody that recognizes the transgenic TCR, to detect the *in vivo* expansion of the T cells. To perform functional asssays, TCR transgenic T cells were isolated and cultured *ex vivo* with APCs and OVA. Under these conditions, both naive and primed T cells responded to antigen, with primed T cells showing a more vigorous response. In marked contrast, tolerized T cells failed to respond to antigen. This adoptive transfer approach has been modified to examine the individual contributions of key immunoregulatory molecules and costimulators on tolerance induction *in vivo.* By treating adoptive transfer recipients with inhibitory antibodies or by breeding TCR transgenics with the desired knock-out mice, the effect of specific genes on the development of tolerance in the periphery can be assessed. For example, using this approach, a novel role for CTLA-4 was reported in which B7:CTLA-4 interactions provided a tolerizing signal to T cells at the time of antigen recognition (78). Adoptive transfer of TCR transgenic T cells and administration of anti–CTLA-4 mAbs to mice given a tolerogenic form of antigen resulted in the development of antigen-reactive T cells and cytokine production, findings suggesting that tolerance induction may require B7:CTLA-4 interactions. These results, together with the fatal lymphoproliferative disease in the CTLA-$4^{-/-}$ mice, suggest a critical role for CTLA-4 in tolerance induction.

Transgenic approaches also have provided a way to test the hypothesis that peripheral tolerance results from antigen presentation in the absence of costimulation. If this hypothesis is valid, then overexpression of a costimulator should activate self-reactive T cells, resulting in autoimmunity. Transgenic mice overexpressing B7-1 in pancreatic islet b cells demonstrate that B7-1 expression alone is not sufficient to break *in vivo* peripheral tolerance to islet cell or keratinocyte antigens (82). Spontaneous inflammatory changes arise when B7-1 overexpression is combined with additional predisposing factors. Interbreeding of transgenic mice overexpressing B7-1 in pancreatic b cells with transgenic mice expressing tumor necrosis factor-a (TNF-a), which develop nondestructive insulitis, resulted in double transgenic mice that spontaneously developed immune-mediated b cell destruction and diabetes (83).

Although the use of transgenic technology has provided great insights into our understanding of the immune response *in vivo,* the scope of these systems is limited for several reasons, including limitation of the lymphocyte repertoire by expressing one transgenic receptor, expression levels of the transgenic receptors that are higher than physiologic expression, and constitutive expression of the transgenic receptor throughout thymic development and in the periphery. In some transgenic models, the self-antigen is ignored and does not induce anergy. The reasons that encounter of some antigens leads to anergy and others result in ignorance are unclear. It is important to consider these factors when interpreting results of studies using transgenic T cells.

Although *in vitro* and *in vivo* studies have investigated the induction of anergy, the molecular events that occur after the development of T-cell anergy are poorly understood. The failure to produce IL-2 is a key characteristic of anergic T cells. *In vitro* studies have shown that the expression of IL-2 mRNA and protein secretion were markedly reduced in anergic T-cell clones (84). This profound defect in IL-2 production has led to intense study of the signaling pathways that eventually result in IL-2 transcription. Engagement of the TCR leads to phosphorylation of the g and z chains in the associated CD3 complex (85). This phosphorylation event triggers a cascade of events that results in downstream activation of $p21^{ras}$. $p21^{ras}$ activation is important because it regulates the activation of several kinases, including MEK (MAPKK) and MAP/ERK, which are critical to the induction of AP-1 (86,87). AP-1 is a transcription factor that is required for activation of the IL-2 promoter (88).

Some studies have provided mechanistic insights into the downstream signaling events that occur after anergizing signals. The pattern of CD3 phosphorylation is different in anergic versus primed T cells (89). Decreases in $p21^{ras}$ activity have been seen. Because $p21^{ras}$ affects the activation of several downstream signaling pathways, which, in turn, regulate IL-2 gene transcription, this block in $p21^{ras}$ activity could be critical for induction of tolerance. Adding further support to this hypothesis is the observation that the decrease in $p21^{ras}$ activity correlated with a similar decline in MAPK activity (90). Because the MAPK pathway regulates Fos and Jun, which are the transcription factors that activate AP-1, the decreased MAPK activity could ultimately cause a decrease in IL-2 transcription (91). In anergized T cells, the level of AP-1 binding to the IL-2 promoter was shown to be markedly reduced, as compared with primed T cells (88). This effect was observed 3 hours after stimulation, which is the peak of AP-1 binding after stimulation *in vitro.* The levels of other transcription factors, such as NFAT and NF-kB were similar in normal and anergized T cells. Thus, the reduction of IL-2 in anergized T cells was attributed to the lack of AP-1 binding to the IL-2 promoter. In summary, although the molecular mechanisms responsible for the blockade of IL-2 production are not fully understood in anergized T cells, more recent work has shown that IL-2 production is controlled by several pathways. Studies of anergic cells have shown decreases in $p21^{ras}$ MAPK, and AP-1, activity all of which correlate with decreased IL-2 transcription.

Activation-Induced Cell Death

Repeated activation of $CD4^+$ T cells leads to coexpression of the death receptor Fas (CD95) and its ligand, FasL. AICD occurs as a result of active signaling to

lymphocytes through the Fas:FasL pathway (92,93 and 94). Fas is part of the TNF receptor (TNFR) family, which includes the costimulatory molecule, CD40 (95). The cytoplasmic domain of Fas features a conserved “death domain,” which serves as a docking site for signaling molecules. FasL is a homotrimeric protein that is a member of the TNF family. The structure of FasL allows for effective cross-linking of Fas on the surface of the same and neighboring cells. Both Fas and FasL are expressed on the surface of activated T cells and are upregulated after repeated stimulation.

Studies have begun to elucidate the biochemical events that occur during AICD. Cross-linking of Fas by FasL binding stimulates the interaction with the adaptor protein Fas-associated death domain (FADD). Binding of FasL to Fas activates a signaling cascade that results in activation of the initiator caspase, caspase-8, followed by activation of several effector caspases that ultimately lead to apoptosis. Some evidence suggests that caspase-8 affects mitochondrial membrane permeability, which stimulates apoptosis. The mitochondrial pathway of apoptosis is active primarily in cells that are deprived of survival signals. This form of apoptosis, which has been called passive cell death or death by neglect, is associated with caspase-9 activation, does not involve death receptors, and is prevented by antiapoptotic members of the Bcl family (96).

A surprising result has been that IL-2, the prototypic T-cell growth factor, actually potentiates Fas-mediated AICD. Thus, antigen in the presence of IL-2 initiates T-cell responses, but excessive amounts or persistence of antigen and IL-2 may terminate the response by triggering AICD. Failure of this death pathway may also contribute to the autoimmunity seen in knock-out mice lacking IL-2 or the a or b chain of the IL-2R (97,98). Thus, IL-2 may be a critical negative feedback inhibitor of lymphocyte responses, and other cytokines can substitute for IL-2 as a T-cell growth factor.

The importance of the Fas:FasL pathway in maintaining self-tolerance is illustrated by the autoimmune disease of Fas or FasL-mutant mice (lpr and gld) and in children with Fas mutations (autoimmune and lymphoproliferative syndrome, ALPS). The lpr and gld strains of mice develop progressive severe lymphadenopathy, systemic lupus erythematosus–like autoantibodies, and hypergammaglobulinemia (99). The rare human disease, ALPS, is caused by mutations in Fas or downstream caspases (100,101 and 102). In a murine model of multiple sclerosis, it was shown that FasL expression is critical in the initiation and recovery stages of disease (103). Thus, apoptosis through the Fas:FasL pathway may provide a critical means of destroying self-reactive T cells that are exposed to high levels of self-protein in the periphery. Paralleling these results, transgenic T cells stimulated with specific peptide undergo T-cell activation followed by AICD (104).

T-Cell–Mediated Suppression

Suppressor T cells are a population of regulatory cells that inhibit the development of self-reactive T cells. Since the 1960s, studies have suggested that tolerance to self-antigens can occur by an active process in which a population of suppressor T cells inhibits the clonal expansion and effector functions of potentially autoreactive T cells. The administration of protein antigens under conditions that induce tolerance, such as oral administration, often leads to the activation of T cells that produce immunosuppressive cytokines such as transforming growth factor-b (TGF-b) and IL-10 (105,106). These results raised the possibility that cytokine-mediated lymphocyte suppression contributes to the maintenance of tolerance. Evidence of the importance of suppression in self-tolerance has come from many studies showing that naive T cells transferred into lymphopenic mice induce multisystem autoimmune disease, but cotransfer of activated T cells protects from such disease. The interpretation of these results is that the activated T-cell population contains some cells that have encountered self-antigens and developed into regulatory cells, and in their absence potentially pathogenic autoreactive lymphocytes can function unchecked. The mechanism of action of the regulatory T cells is unknown. Studies indicate that some of these regulatory cells express CTLA-4, a finding suggesting that CTLA-4 may limit the activation of regulatory T-cell responses.

Several specialized subsets of suppressor T cells have been identified and are under investigation. Production of IL-10 and TGF-b appears to be particularly important in this immunoregulation. The relationship between these cytokines and suppressor T cells is not yet defined. A role for $CD4^+$ $CD45RB^{lo}$ T cells in suppression comes from a murine model of inflammatory bowel disease (107). Transfer of sorted $CD45RB^{hi}$ $CD4^+$ T cells from normal mice into *scid* or lymphopenic recipients lead to disease. When the combination of $CD45RB^{hi}$ and $CD45RB^{lo}$ subsets from normal mice were transferred into scid recipients, inflammatory bowel disease was prevented (108). These findings demonstrated the ability of the $CD45RB^{lo}$ $CD4^+$ T-cell population to neutralize the clonal expansion of the potentially disease-causing $CD45^{hi}$ $CD4^+$ T cells. Subsequent studies in this model system have pointed to critical roles for TGF-b and IL-10 in regulating the inflammatory reponse toward intestinal antigens (109,110).

A population of $CD4^+$ $CD25^+$ T cells also has identified as suppressor cells (111,112). In response to stimulation through the TCR, $CD4^+$ $CD25^+$ T cells, but not $CD4^+$ $CD25^-$ T cells, are unresponsive (113). Addition of $CD4^+$ $CD25^+$ T cells to the culture of $CD4^+$ $CD25^-$ T cells suppressed proliferation and IL-2 production. When $CD4^+$ $CD25^+$ T cells were isolated from TCR transgenics, similar effects were observed; however, the effect was not antigen specific. The lack of the $CD25^+$ suppressor T cells in CD25-deficient mice may be one reason that these mice develop autoimmunity. Taken together, these studies suggest that distinct populations of T cells secrete regulatory cytokines involved in T-cell suppression.

POTENTIAL THERAPEUTIC APPLICATIONS OF COSTIMULATION AND TOLERANCE

Because the B7:CD28/CTLA-4 and CD40:CD40L costimulatory pathways deliver signals that are required for T-cell activation, there has been great interest in manipulating these pathways for therapy. Costimulatory blockade could inhibit undesired T-cell responses occurring during autoimmunity, transplant rejection, or allergy, and enhanced costimulation could promote T-cell responses for tumor and vaccine immunity. Clinical trials that involve manipulation of these pathways are in progress.

Antitumor Immunity

Progress in our understanding of the antitumor immune response has led to novel strategies for developing T-cell–mediated cancer immunotherapies. The challenge in designing effective immunotherapy is to enhance the host immune response to tumors. In general, tumors are poor inducers of immunity as a result of inappropriate expression of MHC molecules, weakly immunogenic antigens, or inadequate levels of costimulatory or adhesion molecules (114). Although the long-term goal of identifying dominant tumor antigens for common cancers is important, the antitumor therapies would be more effective if they were augmented in a context that enhanced the T-cell response. One approach to increase the T-cell response is by expressing B7 costimulatory molecules on tumor cells. Genetic modification of tumors to express B7-1 or B7-2 enhances the host antitumor immune response (115,116 and 117). The rejection of tumors is accelerated by the administration of anti–CTLA-4 Abs in animal models (118). Thus, *in vivo* blockade of B7:CTLA-4 interactions with antibodies to CTLA-4 can result in the rejection of tumors, including preestablished tumors, presumably by enhancing T-cell activation (119). Therefore, there is great interest in designing immunotherapies that enhance the antitumor response either by stimulating expression of B7 or by blocking B7:CTLA-4 interactions.

Transplantation

Based on the premise that tolerance can result from antigen recognition in the absence of effective costimulation, numerous attempts have been made to prevent rejection of organ allografts by inhibiting B7:CD28/CTLA-4 or CD40:CD40L interactions *in vivo.* The efficacy of costimulatory molecule blockade in delaying graft rejection was reported first in a murine cardiac allograft model (120). When recipients of MHC-mismatched cardiac allografts were treated with CTLA-4Ig at the time of transplantation, graft rejection was delayed as late as 60 days. Later studies demonstrated that coblockade of the CD40:CD40L and B7:CD28 pathway with antagonists prevented the rejection of vascularized cardiac allografts and promoted the long-term survival of allogeneic skin grafts (121). The findings from the murine models were extended to a primate model. Treatment of primates with CTLA-4Ig and anti-CD40L mAbs was shown to prevent and reverse acute transplant rejection of MHC-mismatched renal allografts (122). As a consequence of these promising results, clinical trials are under way using costimulatory molecule antagonists to prevent transplant rejection.

Graft-versus-Host Disease

Graft-versus-host disease (GVHD) occurs when transplanted T cells mount a vigorous immune response against host tissue alloantigens. The development of GVHD is a major obstacle to successful bone marrow transplantation. Blockade of the B7:CD28/CTLA-4 and CD40:CD40L pathways has been a key strategy in the prevention of GVHD. Studies in animal models have shown that treating recipients of mismatched donor bone marrow with anti-CD40L antibodies or CTLA-4Ig prevents the expansion and cytolytic activity of the donor T cells characteristic of acute GVHD (123,124). Findings from the first clinical trial to adapt this approach to bone marrow recipients have been published (125). The strategy was to induce tolerance in donor T cells to host alloantigens *in vitro* before bone marrow transplantation. To induce tolerance, peripheral blood lymphocytes were collected from the bone marrow recipients before irradiation. The recipient lymphocytes were irradiated and were used to stimulate the donor bone marrow mismatched for one HLA haplotype, and the cells were cultured with CTLA-4Ig. After this treatment, donor bone marrow was transferred into patients who were monitored for signs of GVHD. Although this initial clinical trial was small, five patients exhibited remission several months after the bone marrow transplantation, whereas three patients exhibited acute signs of GVHD. These results are impressive, considering that other forms of treatment had failed in the patients in this study. Future clinical trials will be necessary to assess the potential benefits of this type of immunotherapy.

Autoimmunity

By using animal models, the role of costimulatory molecules in the development of autoimmunity has been an area of intense investigation. In a murine model of

multiple sclerosis, EAE, blocking B7-2 interactions increased the incidence of Th1-mediated disease, whereas similarly blocking B7-1 interactions ameliorated disease (126). Later studies demonstrated that B7-1/B7-2 deficient mice were highly resistant to EAE during the induction and effector phase of the response (127,128). Given the importance of CD40:CD40L interactions in upregulating B7-1 and B7-2, the CD40:CD40L pathway has also been investigated in EAE. Treatment with anti-CD40L mAbs either at the time of induction or after initial onset of disease resulted in dramatic reduction in EAE severity (129). Combined, these studies indicate that CD40 and B7 molecules have a critical role in the development and maintenance of EAE. Similar results have been observed in other models of autoimmunity. Studies have shown that CD40:CD40L blockade inhibits the development of collagen-induced arthritis (123). In systemic lupus erythematosus models, protective effects were observed after blockade of CD40 and CD28 pathways (130). Patients with this disease were shown elevated levels of CD40L and dysregulated expression of B7-1 and B7-2 (131). Thus, the B7:CD28 and CD40:CD154 pathways have complementary and required roles in T-cell differentiation and influence the development of autoimmunity.

In several models of autoimmunity, CTLA-4 has been shown to downregulate T-cell responses during the course of an autoimmune disease. Treatment with anti–CTLA-4 mAbs exacerbated EAE when therapy followed adoptive transfer of primed T cells (132,133 and 134). Similar effects were observed in a TCR transgenic model of diabetes in which administration of anti–CTLA-4 mAbs accelerated the development of diabetes (135). The onset of diabetes was only affected when the anti–CTLA-4 Ab was given before the onset of insulitis. These results suggest a critical role for CTLA-4 at the initiation of the autoimmune response as well as the downregulation of the ongoing response.

In light of the role of CTLA-4 in the development of autoimmunity in experimental systems and the phenotype of the CTLA-4–deficient mouse, there has been great interest in assessing whether CTLA-4 contributes to human autoimmunity. Several polymorphisms have been identified within the human CTLA-4 gene and have proven informative in genetic linkage studies (136). Polymorphisms in the CTLA-4 gene have been genetically linked to several human autoimmune diseases, which include insulin-dependent diabetes mellitus, Graves disease, Hashimoto's thyroiditis, rheumatoid arthritis, and other disorders (137,138,139,140,141 and 142). The crucial unresolved issue is whether the predisposing gene is actually CTLA-4 or another closely linked gene. This is particularly critical because other genes (e.g., CD28) expressed by T cells and other immune cells are closely linked to the CTLA-4 locus. Further studies are needed to determine whether these polymorphisms either result in altered expression of the CTLA-4 gene or modify CTLA-4 protein function.

A promising phase I clinical trial using CTLA-4Ig for the treatment of the T cell-mediated autoimmune skin disease, psoriasis vulgaris, was published recently (143). This is the first report in which blocking T-cell costimulatory activity has altered the course of disease in humans. In this study, patients with psoriasis vulgaris were treated with CTLA-4Ig, and 46% of the patients exhibited a 50% or greater improvement in clinical disease. Greater effects were observed in a subset of patients given a higher dose of CTLA-4Ig. The findings of this clinical trial provide great promise for the treatment of psoriasis vulgaris as well as other T-cell–mediated autoimmune diseases.

Allergy

Allergic reactions develop when a sensitized individual responds to innocuous foreign proteins. Although the triggers of an allergic reaction are harmless, effector T cells and the associated inflammation result in significant disease. The allergic response is characterized by elevations in IL-4 and other Th2 cytokines, serum immunoglobulin G1 (IgG1) and IgE levels, eosinophils, and mast cells (34). Interestingly, the same effector populations aid in the immunologic attack against parasites. The question whether it is possible to block the differentiation of a naive Th cell into a Th2 effector cell has been studied in animal models. In a murine model of asthma, administration of CTLA-4Ig before antigen sensitization or before reexposure to antigen blocked asthma (144). Likewise, levels of IL-4, serum IgG1 and IgE, and pulmonary eosinophilia were markedly reduced. Although CTLA-4Ig treatment has been shown to be effective in some models of allergy, some evidence suggests that Th2 cells may be more resistant to tolerance induction than Th1 cells. In a model of contact dermatitis, both anti-CD40L and CTLA-4Ig treatment were required to block the immune response to a Th2 allergen, whereas CTLA-4Ig alone was sufficient to inhibit the response to a Th1 allergen (145). Despite successful intervention by costimulatory molecule blockade, it is unclear whether this type of treatment will be effective in patients with long-term exposure to allergens. Effective immunotherapy for allergy may require a strategy that prevents naive, effector, and memory T cells from responding on subsequent exposure to the allergen. To date, there are no clinical trials for allergy using costimulatory molecule blockade as a strategy.

CONCLUSIONS

Many factors contribute to the decision of immunity versus tolerance. Costimulatory molecules play a key role in the activation and differentiation of T cells. After TCR engagement, CD28 delivers stimulatory signals to the T cells that can prevent the development of anergy by promoting T-cell activation and survival. By contrast, CTLA-4 delivers downregulatory signals to T cells by inhibiting proliferation and IL-2 production and can promote the induction of anergy. The costimulatory effects of the B7:CD28/CTLA-4 pathway depend on stimulation, at least in part, through the CD40:CD40L pathway. Engagement of the CD40 receptor results in APC activation, upregulation of B7-1 and B7-2, cytokine production, and cell survival. The activation of the APC, in turn, affects Th cell differentiation. The profound effects of disrupting the B7:CD28/CTLA-4 pathway and the CD40:CD40L pathway have been demonstrated in several models of autoimmunity, infection, and transplantation.

Several mechanisms are recognized for maintaining T-cell tolerance in the periphery. T cells that receive inadequate costimulation may be rendered anergic or unresponsive. Repeated T-cell stimulation induces apoptosis, which can be mediated through the Fas:FasL pathway. Finally, tolerance induction can be mediated by the development of suppressor T cells, which can alter T-cell differentiation and can cause immune deviation. Understanding which pathways are critical to the induction of tolerance is key to developing rational strategies of therapeutic intervention.

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10 CYTOKINES AND TYPE 1 IMMUNITY

J. David Farrar, Ph.D., and Kenneth M. Murphy, M.D., Ph.D.

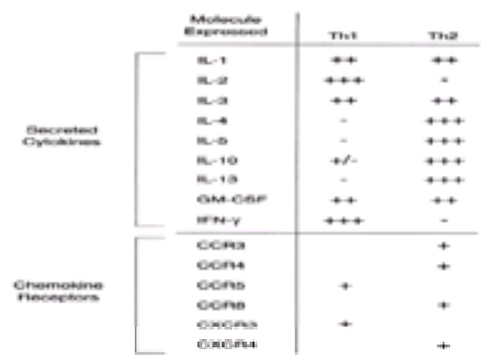
The *immune response* elicited against a particular pathogen can be categorized as either cell mediated (involving phagocytes or cytolytic cells) or humoral (involving antibody), and the $CD4^+$ T helper (Th) cell is the primary cell type responsible for orchestrating both of these responses. The capacity of Th cells to direct such responses is exerted by selective secretion of unique cytokines. For example, Th secretion of interferon-g (IFN-g) induces a cell-mediated response by activating macrophages to produce inflammatory cytokines, whereas Th secretion of interleukin-4 (IL-4) elicits a humoral response by inducing B-cell proliferation and Ig production. In 1986, Mosmann and Coffman discovered that $CD4^+$ Th cells could be classified into two subsets (designated Th1 and Th2) according to their cytokine secretion profiles (1). Th1 cells were shown to secrete IL-2 and IFN-g, and Th2 cells secreted IL-4 and IL-5. Moreover, the development of these two subsets proceeds through a mutually exclusive process such that a single pathogen favors the development of either the Th1 or the Th2 phenotype. This remarkable discovery explained how a single cell type could direct two such dissimilar immune responses.

Since the initial discovery of Th1 and Th2 cells, it has become clear that the resolution of many disease states relies almost exclusively on the development of the "appropriate" Th phenotype. For example, in the murine model of *Leishmania major* infection, IFN-g secretion by Th1 cells is required for both limiting the disease to the site of infection and eradicating the organism from resident infected macrophages (2). If, however, a Th2 response is generated to *L. major* infection, the organism will not be contained and, instead, will become systemic, ultimately causing the animal to succumb to the disease. Another important disease state associated with aberrant Th responses is autoimmunity. Autoreactive Th1 cells have been shown to be involved in such diseases as arthritis (Chapter 37 and Chapter 38) and diabetes (Chapter 48), whereas Th2 cells mediate allergy (Chapter 61) and asthma (Chapter 65). Thus, understanding the mechanism governing the development of these two distinct Th phenotypes is critical for disease diagnosis and prognosis and for designing therapeutic interventions. In this chapter, we explore the basic cellular and molecular mechanisms that link the innate immune response with the instructive process of Th1 development. Further, we examine how the type I response results in adaptive cell-mediated immunity.

CHARACTERISTICS OF TH SUBSETS

Patterns of Cytokine Expression

Early *in vitro* studies classified antigen-specific Th clones into Th1 and Th2 subsets based solely on their cytokine secretion profiles (3). Th1 cells exclusively produced IL-2 and IFN-g, whereas Th2 cells produced IL-4, IL-5, IL-10, and IL-13 (Fig. 10.1). Later, it was recognized that IL-10 expression was not always restricted to Th2 cells, and in some cases, particularly in human $CD4^+$ T cells, Th1 cells could produce both IFN-g and IL-10 (4). In addition, expression of IL-1, IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) was shared between Th1 and Th2 subsets. The Th1 and Th2 phenotypes were not restricted to *in vitro*–derived T-cell clones. Th subsets have been characterized from *in vitro*–derived bulk cultures and from *in vivo*–derived peripheral blood and biopsy samples. Additionally, Th subsets have been identified in a variety of species ranging from mice to humans (5).



	Molecule Expressed	Th1	Th2
Secreted Cytokines	IL-1	++	++
	IL-2	+++	-
	IL-3	++	++
	IL-4	-	+++
	IL-5	-	+++
	IL-10	+/-	+++
	IL-13	-	+++
	GM-CSF	++	++
	IFN-γ	+++	-
Chemokine Receptors	CCR3		+
	CCR4		+
	CCR5	+	
	CCR8		+
	CXCR5	+	
	CXCR4		+

Figure 10.1. Cytokine secretion and chemokine receptor expression profiles of Th1 and Th2 cells. **Top**: Relative levels of secreted cytokines are expressed as + (expressed) or – (not expressed). **Bottom**: Chemokine receptor expression is correlated with Th1 and Th2 subsets, but their expression cannot be considered as a useful marker for the identification of Th1 or Th2 cells.

The exclusive nature of cytokine expression fits well with the functional capabilities of these two subsets. For example, the Th1 subset, by virtue of IFN-g secretion, orchestrates a protective cell-mediated response to both intracellular and extracellular bacterial pathogens (6). In this case, IFN-g is required for the oxidative burst and delayed-type hypersensitivity (DTH; discussed later). This type of response would be ineffectual to pathogens such as helminths because these large organisms are resistant to phagocytosis and intracellular oxidative bursts. Thus, a protective type II response is mediated by Th2 cells in response to helminth parasites (7). In this situation, Th2 secretion of IL-4 elicits B-cell production of helminth antigen-specific immunoglobulin E (IgE) and secreted IgA, and IL-5 induces the recruitment and activation of eosinophils to sites of infection (Chapter 11). Clearly, host-pathogen interactions have provided a necessary selective pressure for the evolution of Th subsets.

Expression of Chemokine Receptors

Chemokines are a family of low-molecular-weight (8- to 14-kd) proteins produced by many cell types. Their function is mainly in chemoattraction of leukocytes, and chemokine activity is mediated through a family of seven transmembrane-spanning G-protein receptors (see Chapter 16). Initially, $CD4^+$ T-cell subsets could only be distinguished based on their unique cytokine secretion patterns, and efforts to identify differentially expressed cell surface markers largely failed. More recently, several chemokine receptors have been identified as selectively expressed between Th1 and Th2 cells (8), and their expression pattern is listed in Fig. 10.1. However, the expression pattern of chemokine receptors is not as phenotype restricted as that of cytokines because only a subpopulation of each Th subset expresses a given chemokine receptor. Thus, chemokine receptors cannot be used as reliable surface markers for the identification of Th1 or Th2 cells. It is interesting to speculate on the function played by each chemokine receptor, particularly those receptors whose expression is relatively phenotype specific (9). However, no such function has been ascribed to chemokine receptors and Th subsets with regard to either tissue-specific migration or cell activation.

TH1 DEVELOPMENT

Cytokine Receptors and Signaling

The hallmark of an adaptive $CD4^+$ T-cell response is the expansion of antigen-specific cells concomitant with the differentiation of the expanding clonal populations into distinct cytokine-secreting subsets. Various signals, delivered through the action of cell-surface receptors, are required for the Th developmental process to occur (10,11). The T-cell receptor (TCR) and cytokine receptors together play the most critical role in this process. TCR recognition of peptide-major histocompatibility complex (MHC) on antigen-presenting cells (APCs) leads to cellular activation and proliferation, provided the appropriate coreceptors are present. Initiation of cell division through the TCR is a critical component for both cell division and cytokine gene expression. However, the TCR signal, rather than directly mediating Th polarization, subtly influences the initial cytokine milieu that acts directly to mediate Th development (12). Thus, the signals delivered by cytokines are the critical factors governing the differentiation of Th subsets. In this section, we explore the molecular basis by which cytokines signal through their receptors and the consequence of those signals on Th1 development.

In general, the effects of the cytokines discussed later, with the exception of IL-18, are mediated through a heterodimeric complex consisting of two receptor subunits (diagrammed in Fig. 10.2A) (13). Each chain of the receptor is constitutively associated with the Janus family of tyrosine kinases referred to as JAK kinases (14,15,16 and 17). On receptor activation by cytokines, the associated JAK kinases become phosphorylated and, in turn, phosphorylate critical tyrosine residues within the cytoplasmic domains of both receptor subunits (Fig. 10.2B). Tyrosine phosphorylation of the receptor tails acts to recruit members of the signal transducer and activator of transcription (STAT) family (Fig. 10.2C). STAT molecules harbor several domains important for functions such as receptor binding, dimerization, and gene transactivation. The Src-homology-2 (SH2) domain, located near the C terminus of all STAT molecules, is responsible for both receptor binding and STAT dimerization. SH2 domains are characterized by their ability to bind phosphotyrosine residues, and their specificity of binding is determined by the amino acid sequence located adjacent to the phosphotyrosine residue within the receptor tails. The specific phosphotyrosine-containing sequences within the cytoplasmic domains of cytokine receptors dictate which unique STAT family member is recruited to a particular receptor. On docking to the receptor tails, STATs become phosphorylated by the action of the associated JAK kinases and subsequently form homodimers and heterodimers with other STAT family members. Dimerization of STATs is also accomplished through phosphotyrosine/SH2 domain interactions. These activated STAT dimers translocate to the nucleus and participate in transcriptional regulation of cytokine-inducible genes.

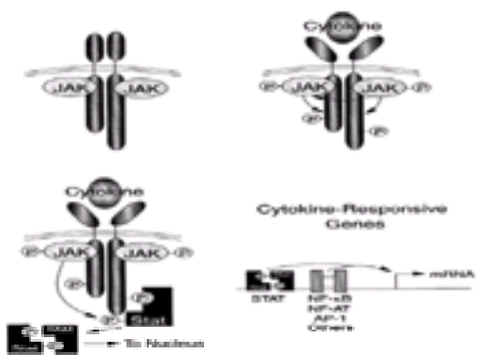


Figure 10.2. Cytokine receptor signaling mechanisms and STAT activation (see text for details).

Interleukin-12

IL-12 is a heterodimeric molecule consisting of p35 and p40 subunits and is secreted predominantly by activated professional APCs such as dendritic cells, activated macrophages, and Langerhans cells (18). Initially, IL-12 was characterized as a factor that promoted IFN-g production by natural killer (NK) cells (19 and 20). IL-12 was later shown to be responsible for promoting Th1 development when it was secreted by macrophages in response to antigens derived from heat-killed *Listeria monocytogenes* (21). Since the initial discovery of this cytokine, the essential role played by IL-12 in $CD4^+$ Th1 development was repeatedly demonstrated by gene ablation studies including the IL-12- (22), IL-12 receptor (IL-12R)b1- (23), and the Stat4-deficient (24,25) mice. In each of these murine gene knock-out models, deficient for specific components of the IL-12 signaling cascade, Th1 development is severely impaired when analyzed both *in vivo* and *in vitro.*

The IL-12R is composed of two b-type subunits (26,27). The b1 subunit is constitutively expressed on $CD4^+$ T cells, whereas the b2 subunit, required for signaling, is strictly regulated during the earliest phases of Th development (28,29). Stimulation through the IL-12R leads to the recruitment and activation of Stat1, Stat3, and, most important, Stat4 in both mouse and human T cells (30,31). The activation of Stat4 is the obligatory gateway through which Th1 differentiation proceeds. Stat4 was shown to bind directly to a unique phosphotyrosine-containing sequence within the IL-12Rb2 subunit (32), and in the absence of b2 expression, Stat4 fails to be activated in response to IL-12.

The expression of the IL-12Rb2 component is the basis for IL-12 responsiveness in $CD4^+$ T cells, and both genetic and environmental factors influence the expression of the b2 subunit during early Th priming. As mentioned earlier, certain infectious disease models have been shown to rely on the development of an appropriate Th response for the resolution of the disease. For example, studies of inbred strains of mice revealed a genetic component to resistance to *L. major* infections (2). In this murine model, BALB/c mice infected with *L. major* were unable to control the growth of the organism, whereas C57BL/6 and B10.D2 animals were able to control the infection and clear the organism. The clearance of *L. major* strictly depends on the development of a Th1 response that both C57BL/6 and B10.D2 mouse strains are able to mount.

An important insight to the genetic component of Th1 development was revealed when the difference for Th1 development was demonstrated *in vitro* between BALB/c and B10.D2 $CD4^+$ T cells. B10.D2, but not BALB/c, T cells were able to acquire and maintain the ability to respond to IL-12 during the early phases of priming (33). Thus, under neutral priming conditions (in which all endogenous cytokines are allowed to be expressed), B10.D2 cells acquired the ability to respond to IL-12, but BALB/c cells lost such responsiveness as determined by their loss of Stat4 activation in response to IL-12 and lack of IFN-g production after a third stimulation (34). This genetic difference in IL-12 responsiveness was explained by the ability of B10.D2 cells, but not BALB/c cells, to upregulate and maintain the expression of the IL-12Rb2 subunit required for IL-12 signaling (35). By intercross breeding of BALB/c and B10.D2 strains, the genetic locus controlling the ability of B10.D2 cells to acquire IL-12 responsiveness was mapped to murine chromosome 11 (36). This locus was designated *Tpm1* (T-cell phenotype modifier 1) and has been more finely mapped to include the resident genes *IL-4, IL-13, Tcf1*, and *ITK* (37). The specific genetic component and the mechanism by which *Tpm-1* regulates IL-12 responsiveness remain to be discovered.

Tpm1 seems to exert effects on IL-12 responsiveness by an intracellular pathway and is dominantly inherited. However, *Tpm-1* is not the only locus controlling IL-12 responsiveness or *L. major* resistance. For example, a region of murine chromosome 15 also influences IL-12 responsiveness, but this genetic locus seems to exert extracellular effects, that is, secreted factors, and is inherited in a recessive manner (37,38). Other genetic loci have been identified that have direct effects on resistance to *L. major* infections including loci encoding genes responsible for the oxidative burst as well as MHC genes (39,40 and 41). Finally, genetic components regulating human host-pathogen interactions have been linked to *Mycobacterium leprae, L. donovani,* and *M. tuberculosis* (42), although it has not been determined whether these loci directly influence Th development.

Interferon-g

IFN-g is a 21- to 24-kd monomeric protein and binds a heterodimeric receptor composed of a and b subunits. Signaling through the IFN-gR leads to Stat1 activation (Fig. 10.3), and the induction of Stat1-responsive genes such as IFN regulatory factor-1 (IRF-1) and MHC class II transactivator protein (CIITA) (6). IFN-g is the hallmark of the Th1 subset, its presence is not required for Th1 development, and $CD4^+$ IFN-g–producing cells can develop in the presence of neutralizing a-IFN-g antibodies, as well as in Stat1-deficient (43) and IFN-gR–deficient (44) mice. Although IFN-g does not directly promote Th1 development, IFN-g plays a pivotal role in positively regulating the expression of the IL-12Rb2 subunit.

Cytokine Receptor	JAKs Associated	STATs Activated	Others
IL-4R (α & Cγ)	Jak1/Jak3	Stat6	IRS-1 & 2
IL-10R (α & β)	Jak1/Tyk2	Stats 1,3 and 5	
IL-12R (β1 & β2)	Jak2/Tyk2	Stats 1, 3 and 4	
IL-18R	–	–	NF-κB IRAK MyD88
IFN-γR (α & β)	Jak1/Jak2	Stat1	
IFN-α/βR (R1 & R2)	Jak1/Tyk2	Stats 1, 2 and 3 (Stat4 Only in human)	

Figure 10.3. Cytokine receptors and JAK/STAT association (see text for details).

The analysis of *in vitro* Th development has been greatly advanced with the use of transgenic TCR mice. In this system, most of the $CD4^+$ T cells within these animals express the identical TCR with a known antigen specificity. Thus, *in vitro* T-cell culture systems were established with these T cells to study the effects of soluble factors in Th development. Data from these studies demonstrated that the IL-12Rb2 subunit is not expressed in naive $CD4^+$ T cells; however, after primary stimulation with either IL-12 or IL-4, IL-12Rb2 mRNA is markedly induced in Th1 and is weakly expressed in Th2 cells 3 days after stimulation (28). Yet, this expression was transient in Th2 cells because the b2 chain was completely extinguished by 5 days after stimulation. Loss of IL-12Rb2 expression correlated precisely with the loss of IL-12-induced Stat4 activation in Th2 cells. Conversely, b2 mRNA expression was dramatically increased and maintained in IL-12–treated Th1 cultures up to 9 days after stimulation, a finding suggesting that the b2 subunit was stably expressed in Th1 cells but not in Th2 cells. The effect of IL-12 was not direct because neutralizing antibodies to IFN-g inhibited IL-12Rb2 mRNA expression. Furthermore, b2 expression was also increased when T cells were primed in the presence of both IL-12 and IL-4, and the induction of b2 could only be inhibited when the activity of IFN-g was blocked during the early phase of T-cell priming. These results suggested that IFN-g acted directly and dominantly over IL-4 to induce the expression of the IL-12Rb2 subunit.

Interferon-a/b

Type 1 IFNs comprise a large class of highly related molecules. IFN-a is a family of individual monomeric proteins encoded by more than 20 unique genes, whereas IFN-b is a single protein encoded by a single gene (45). Additionally, IFN-a/b is expressed by many cell types and is induced by viral infection through the cytoplasmic detection of double-stranded RNA molecules (46). Although many isoforms of type I IFNs exist, only one IFN-a/b receptor (IFNAR) complex has been identified. Remarkably, all IFN-a/b isoforms tested can bind and activate the IFNAR in a species-specific manner.

Early studies of human $CD4^+$ Th development suggested that IFN-a/b could act directly to induce Th1 development (47). In contrast, IFN-a could not act either directly or indirectly to influence Th1 development in mice (48). Furthermore, both IFN-a and IFN-b induced the development of human Th1 cells, even when endogenous IL-12 was neutralized, a finding suggesting that IFN-a/b was acting in a direct manner (29). Thus, the activity of type I IFNs to induce IFN-g expression is selectively lost in mice, but it is likely not unique to human T cells.

The ability of IFN-a to induce Th1 development in human T cells was particularly puzzling until it was shown that IFN-a could recruit and activate Stat4 in response to IFN-a/b in human, but not murine, $CD4^+$ T cells (Fig. 10.3) (49,50). Although no known human disorders have been attributed to Stat4 deficiencies, the lack of Th1 development in Stat4-deficient mice implicates the involvement of Stat4 in Th1 development in human T cells as well. Thus, Stat4 activation in response to IFN-a/b in human, but not murine, T cells explains the species-specific ability of type 1 IFNs to induce Th1 development only in the human system.

The ability of the human IFNAR to activate Stat4 affords human $CD4^+$ T cells specific advantages regarding diseases. First, human T cells proceed through Th1 development in the absence of both IFN-g and IL-12 because both the IFN-aR1 and R2 receptor subunits are constitutively expressed on virtually all cells. Thus, virally infected cells directly influence Th1 development by the production of IFN-a. Two unique cases of IL-12 unresponsiveness, caused by mutation in the IL-12R, have been reported in humans (51,52). These patients had severe susceptibilities to bacterial infections, yet their antiviral responses were relatively intact. One possible explanation is that bacterially induced IL-12 production has no effect in driving Th1 development because their T cells failed to respond to IL-12. However, viral immunity was maintained in these patients because IFN-a alone (produced by infected cells) may support Th1 development, thus allowing for a productive antiviral response.

Interleukin-18

IL-18 is structurally related to the IL-1 family of cytokine molecules and is secreted by a variety of cell types, most notably by activated macrophages. IL-18 was first identified as an activity that magnified IFN-g production by Th1 cells (53), and like IL-1, IL-18 is synthesized as an inactive precursor and is cleaved to an active form by caspase 1 before secretion (54). The receptor for IL-18 is also structurally related to the IL-1R and activates the downstream signaling components IRAK (IL-1 receptor-associated kinase) and MyD88 (Fig. 10.3) (55,56). Furthermore, stimulation of the IL-18R pathway leads to the activation of NF-kB family members and possibly AP-1.

T cells display restricted and regulated expression of the IL-18R; however, neither naive $CD4^+$ nor $CD8^+$ T cells express any measurable cell-surface IL-18 binding activity (57,58). Within 48 hours of TCR stimulation, $CD4^+$ and $CD8^+$ cells demonstrated increased IL-18R expression. Th2 cells do not express IL-18R, and it is not clear whether the expression of the IL-18R absolutely requires a signal from IL-12 (because Th2 cells do not express IL-12Rb2) or whether Th2 cells have downregulated the expression of the IL-18R as a result of other factors responsible for differentiation.

Initial reports suggested that IL-18 could influence Th1 development independently of IL-12 activation. Indeed, both the IL-18-deficient (59) and the Myd88-deficient (56) animals showed decreased Th1 responses *in vivo* to bacterial pathogens such as *Propionibacterium acnes.* However, the diminished Th1 response in IL-18–deficient mice was not as severe as demonstrated for the IL-12–deficient or Stat-4–deficient animals. Studies demonstrated that IL-18, alone, could not direct the development of Th1 cells *in vitro* (55). Rather, IL-18 synergized with IL-12 to induce high and sustained levels of IFN-g secretion by Th1 cells and Th1 clones. Furthermore, stimulation with IL-12 and IL-18 in the absence of TCR activation led to remarkably high IFN-g secretion by Th1 cells.

Thus, two separate signals lead to IFN-g production by Th1 cells (60). First, the signal delivered by the TCR induces IFN-g mRNA in Th1 cells in the absence of either IL-12 or IL-18. The second signal delivered by combined treatment of Th1 cells with IL-12 and IL-18 leads to sustained IFN-g secretion when compared with TCR stimulation. These two separate signals (TCR and IL-12/IL-18) seem to act independently of each other but have similar effect on Th1 cells by positively regulating IFN-g gene expression. The factors responsive to these two pathways that positively regulate the IFN-g gene have yet to be identified.

Interleukin-10

IL-10 is secreted as a 17- to 20-kd protein and binds a heterodimeric receptor composed of two distinct subunits (61). The high-affinity binding a chain is paired with a low-affinity b chain required for signaling and activates Stats 1, 3, and 5 (Fig. 10.3) (62,63). Although IL-10 is predominantly secreted by the Th2 subset, it is expressed by other cell types such as mast cells, macrophages, keratinocytes, and many neoplastic T- and B-cell derived lymphomas. IL-10 was first characterized as a factor that suppressed cytokine production by Th1 cells and has now become recognized as a powerful inhibitor of both *in vitro* and *in vivo* Th1 development.

Although IL-10 binds a typical class II cytokine receptor and is linked to the JAK/STAT signaling pathway (64), its molecular mechanism of activity is unique compared with the actions of other T-cell–derived cytokines. First, IL-10 does not inhibit IFN-g production or Th1 development by direct actions on T cells. Rather, IL-10 acts indirectly by inhibiting cytokine production from activated macrophages (65,66). Second, the mechanism of cytokine inhibition seems to be direct on cytokine secretion and not through a global inhibition of IFN-g signaling because many of the actions of IFN-g on macrophages remain intact after treatment with IL-10, such as the upregulation of IRF-1 and class II expression. Finally, the direct molecular target for IL-10 inhibition of both tumor necrosis factor-a (TNF-a) and IL-12 is controversial. One target for IL-10 inhibition has been proposed to be the direct transcriptional inhibition of IL-12p35, IL-12p40, and TNF-a mRNAs (67). In contrast, other studies have suggested that IL-10 blocks IL-12 and TNF-a production at the posttranscriptional level (68,69). This is likely accomplished by decreasing mRNA stability of IL-12 and TNF-a through specific AU-rich sequences encoded in the 3' untranslated regions of both IL-12 and TNF-a mRNAs.

Instructive Developmental Signals

Th1 development appears to proceed through at least two phases, which we refer to as capacitation and differentiation (Fig. 10.4) (70). Because naive CD^+ T cells do

not express any appreciable levels of the IL-12Rb2 subunit, these precursors (Thp) do not have the capacity to respond to IL-12 immediately on T-cell activation. Rather, naive Thp cells must acquire the ability to respond to IL-12 by inducing cell-surface expression of the IL-12Rb2 chain, a process that depends on IFN-g and Stat1. This first phase of development is capacitation and is necessary for a Thp to become fully responsive to IL-12. However, in the absence of IL-12, these capacitated cells still do not have the ability to become fully IFN-g–producing cells. If a strong IL-4 signal is delivered, rather than IFN-g, during the early phases of capacitation, the expression of the IL-12Rb2 subunit is extinguished, thereby blocking Th1 development. Thus, the differentiation phase, proceeding to a full potential to produce IFN-g, requires the signal from the IL-12R and is a Stat4-dependent process. Once a $CD4^+$ T cell passes through the Stat4-dependent differentiation phase, it has the capacity to produce IFN-g on subsequent restimulation. Moreover, a fully differentiated Th1 cell can secrete IFN-g in response to TCR signaling and does not require additional IL-12 stimulation, and this is referred to as factor-independent phenotype stability.

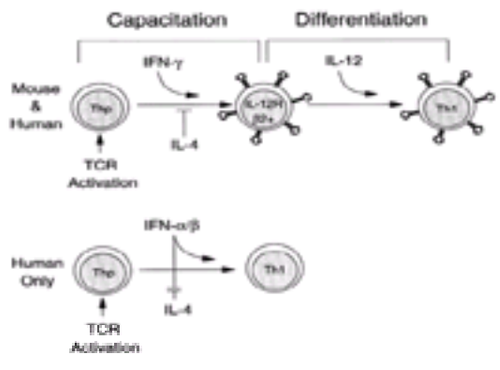


Figure 10.4. Th1 development occurs in two stages: capacitation and differentiation (see text for details).

As discussed earlier, human $CD4^+$ T cells have an inherent second pathway for Th1 development through the action of type I IFNs (Fig. 10.4). Because IFNARs are constitutively expressed, IFN-a/b can act directly on naive Thp cells to induce Th1 differentiation. Moreover, data have demonstrated that IFN-a/b acts dominantly over the effects of IL-4 and can block any negative regulatory mechanism imposed by IL-4, thereby circumventing the need for a capacitation phase of Th1 development in human $CD4^+$ Th cells.

IN VIVO TYPE 1 RESPONSES

Induction of Th1 Differentiation

The initiation of a type 1 immune response begins with Th1 development within secondary lymphoid organs, that is, spleen and lymph nodes (Fig. 10.5A). Naive $CD4^+$ Thp cells are instructed toward Th1 development through their interactions with activated macrophages that have immigrated into secondary lymphoid organs from distal sites of infection. Bacterial pathogens are potent stimuli for macrophage activation. Through the action of several pattern recognition receptors, such as CD14 (71) and Toll-like receptors (72), bacterial products elicit proinflammatory cytokine production as well as migration into spleen and lymph nodes. Additionally, IFN-g, secreted by innate immune cells such as NK cells, acts to upregulate MHC class II expression and antigen presentation by activated macrophages (73). Once in the spleen or lymph node, these activated macrophages initiate $CD4^+$ T-cell proliferation through antigen (MHC II/TCR) and coreceptor (B7/CD28) interactions as well as autocrine IL-2 production by activated T cells. However, Th1 differentiation (described earlier) requires signaling through the IL-12R, and experimentally, this process can be blocked *in vivo* by administering neutralizing IL-12 antibodies (inhibiting IL-12 activity) or exogenous IL-10 treatment (inhibiting IL-12 production) (74). After differentiation, Th1 cells orchestrate a type 1 response both within the secondary lymphoid organ and directly at sites of infection.

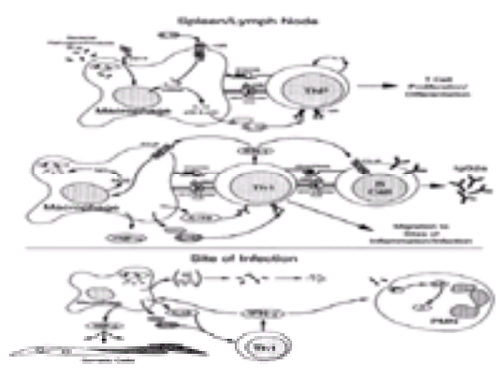


Figure 10.5. *In vivo* Th1 development and type I responses. **A**: Induction of Th1 differentiation. **B**: Initiation of the type I response in secondary lymphoid organs. **C**: Th1 effector functions and delayed-type hypersensitivity.

Th1 Effector Functions and Delayed-Type Hypersensitivity

A fully differentiated Th1 cell arising from this differentiation process has the capacity to secrete high levels of IFN-g in the absence of further IL-12 signaling. Within secondary lymphoid organs, IFN-g, produced by Th1 cells, eliminates bacterial pathogens from within infected macrophages (Fig. 10.5B). This activity is accomplished through signals delivered by the IFN-g receptor leading to macrophage expression of the enzyme inducible nitric oxide synthase (iNOS) (75). Expression of iNOS leads to an intracellular oxidative burst by the production of nitric oxide (NO) from the reaction with L-arginine and molecular oxygen. L-arginine is the only nitrogen donor to this reaction, and the rate-limiting enzyme in L-arginine synthesis, argininosuccinate-synthase, is also induced by IFN-g. The precise mechanism by which NO exerts intracelluar bacteriocidal activity is not known, but many bacterial and viral pathogens are targets for NO-mediated destruction.

The second arm of the type 1 immune response is the induction of specific antibody production mediated by IFN-g. Within secondary lymphoid organs, Th1 cells provide B-cell help through interactions involving TCR/MHC class II and costimulatory molecules, namely CD40/CD40L (Fig. 10.5B) (76,77). In conjunction with these signals, IFN-g induces Ig production and class switching to the IgG2a isotype (78). Among all Ig isotypes produced by B cells, IgG2a is uniquely suited to participate in cell-mediated immunity. IgG2a is poor at inducing complement opsonization because it does not contain sequences required for binding of the initiating complement cascade protein C1q (see Chapter 26). Rather, IgG2a is involved in antibody-dependent cellular cytotoxicity through interactions with FcgRII receptors expressed on the surface of cytolytic cells such as NK cells and polymorphonuclear lymphocytes (PMN, neutrophils).

On differentiation, mature antigen-specific Th1 cells emigrate from secondary lymphoid organs and colonize into sites of inflammation and infection by processes discussed in Chapter 15 and Chapter 16. Th1 cells extravasate into infected tissues, where they undergo further stimulation through interactions with resident activated tissue macrophages (Fig. 10.5C). As mentioned earlier, activated macrophages produce high levels of IL-12 and IL-18, which, in concert, result in sustained high levels of IFN-g production by Th1 cells. IFN-g is the central mediator of the DTH reaction within infected tissues, and the hallmarks of DTH are the oxidative burst and infiltration of PMNs within these tissues (79). In response to IFN-g stimulation, macrophages mediate an extracellular oxidative burst. In addition to NO production, macrophages also secrete superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2). Synthesis of O_2^- is catalyzed by reduced nicotinamide adenine dinucleotide (NADPH)–dependent oxidase. When combined, these reactive intermediates provide greater target specificity for the eradication of many types of bacterial pathogens than can be accomplished by the production of NO alone.

A second important soluble mediator of DTH is TNF-a and is secreted by activated macrophages (80,81). TNF receptors are ubiquitously expressed and lead to a variety of responses, the most important of which for DTH is apoptosis (82). Apoptosis is a mechanism that is beneficial to the resolution of disease because somatic cells infected with intracellular pathogens do not have the capacity to eradicate the organism. Thus, induction of apoptosis of infected somatic cells serves to eliminate the pathogen through the action of phagocytic cells, which process and eliminate both somatic cell and bacterial cell debris.

In addition to activated macrophages, PMNs also play a fundamental role in DTH and the eradication of bacterial pathogens. IFN-g has pleiotropic effects on

neutrophils. First, IFN-g acts as a chemoattractant for neutrophil migration into sites of inflammation. The sheer numbers of neutrophils recruited to infected sites, in addition to vascular hypertrophy, partially accounts for the swelling phenomenon observed in inflamed tissues. Second, IFN-g induces phagocytosis in PMNs that have immigrated into infected tissues. In addition, phagocytosis is also accompanied by an intracellular oxidative burst and is an important mechanism for the elimination of bacterial pathogens.

The resolution of DTH probably depends on a decrease in the concentration of local bacterial pathogens and by-products that initiated the response. However, if left unchecked in cases in which pathogens are slow to be eliminated, DTH will not be resolved and will form a granulomatous reaction characterized by increased swelling and hardening of the inflamed area. Fibrosis and vasculitis accompany granuloma formation, and in cases of chronic DTH, morphologic changes also occur in the resident macrophage population. These chronic DTH macrophages have increased cytoplasm and intracellular organelles and can resemble epithelial tissue. Because fibrosis is a normal aspect of wound healing, it can be injurious to the host during chronic DTH reaction, particularly in organs where fibrosis interferes with normal organ function, such as the lung.

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11 CYTOKINE REGULATION OF TYPE 2 IMMUNITY

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A NOTE ON TERMINOLOGY

The terms used to classify cytokines are confusing. The terms “Th1” and “Th2” properly refer to $CD4^+$ T cells that produce distinct sets of cytokines. Although the cytokines produced by these cell types were originally referred to as “Th1 cytokines” or “Th2 cytokines,” respectively, subsequent discoveries that they can also be produced by cell types other than $CD4^+$ T cells led immunologists instead to refer to “Th1-associated” or “Th2-associated cytokines.” For the sake of convenience, the terms “type 1” and “type 2” were later adopted to refer to the cytokines that are produced by Th1 and Th2 cells, respectively, and to the patterns of inflammation produced by these sets of cytokines. Unfortunately, other investigators adopted the terms “type I cytokine” and “type II cytokine” to refer to cytokines defined by particular structure rather than particular function or cellular origin. Perversely, all the cytokines that fall into the type 2 functional classification are structurally type I cytokines, and the quintessential functional type 1 cytokine, interferon-g (IFN-g), is structurally a type II cytokine. To complicate matters further, variants of individual cytokine receptors are referred to as type I or type II, such as the type I or type II interleukin-4 (IL-4) receptor. Because this chapter focuses on cytokine function rather than structure, I use the terms “type 1” and “type 2” to refer to the functional classification.

GENERAL PRINCIPLES

The modern era of cytokine immunology started with Mosmann and Coffman's 1986 report that $CD4^+$ T cells cloned after repeated *in vitro* stimulation can be divided into two groups that produce different sets of cytokines (1). One group, designated Th1, produced IL-2 and IFN-g, but not B-cell stimulatory factor 1 (BSF-1, later renamed IL-4), whereas a second group, designated Th2, produced IL-4, but neither IL-2 nor IFN-g. Some cytokines, including IL-3 and tumor necrosis factor-a (TNF-a), were produced by both Th1 and Th2 cells.

The importance of the Th1/Th2 dichotomy was demonstrated during the next 2 to 3 years by the observation that the cytokine responses associated with Th1 or Th2 cells (termed type 1 and type 2 cytokine responses, respectively) are associated with disease resolution or disease progression and death, respectively, in mice infected with a protozoan parasite, *Leishmania major* (2,3). Production of the Th1-associated cytokine, IFN-g, was shown to be necessary to induce an enzyme, inducible nitric oxide synthetase, that is required for macrophage killing of *L. major,* whereas the Th2-associated cytokine, IL-4, inhibited production of this enzyme and allowed parasite proliferation and dissemination (4).

These seminal observations have guided studies of cytokine biology for the past 14 years. Additional cytokines have been added to both the Th1 subset (lymphotoxin, also called TNF-b) and the Th2 subset (IL-5, IL-9, and IL-13) (5,6). Investigators have come to understand that although the Th1/Th2 dichotomy is useful for characterizing immune-mediated disorders and responses to pathogens, fully polarized Th1 or Th2 cells that produce the complete set of Th1-associated or Th2-associated cytokines, respectively, without producing any cytokines of the opposite set, are more the exception than the rule (7,8 and 9). *In vivo* immune responses, which reflect cytokine production by cell populations, rather than by individual cells, can also be fully polarized or anywhere in between. Within this broader interpretation, the Th1/Th2 concept has been shown to be just as applicable to humans as it is to the mouse (10,11,12 and 13).

$CD4^+$ T cells are not the only cell type that can produce type 1 or type 2 cytokines or the only cell type that can produce polarized selections of cytokines. $CD8^+$ T cells, which were initially thought to secrete only type 1 cytokines (14), were also determined to be capable of secreting type 2 cytokines (15); furthermore, the conditions that stimulate $CD4^+$ or $CD8^+$ T cells to differentiate into type 1 or type 2 cytokine-secreting cells were determined to be similar (16). Natural killer (NK) cells were found to be an important source of the type 1 cytokine, IFN-g, although they can also secrete type 2 cytokines under some conditions (17,18); basophils and mast cells were shown to secrete a selection of predominantly type 2 cytokines, including IL-4 (19,20,21 and 22), and eosinophils were reported to secrete IL-4 and IL-5 (23,24). Determination of the relative importance of different cell types to a particular cytokine response remains an important area of ongoing research.

Much has been learned about the mechanisms by which type 1 and type 2 cytokines produce their effects. Cytokine receptors have been identified, along with some of their associated signaling molecules and cellular distributions. In some instances, it has been possible to track signaling pathways all the way from receptor ligation to the biologic effects that characterize type 1 or type 2 cytokine responses. In additional cases, biologic effects have been characterized sufficiently to reveal how selective production of a particular set of cytokines leads to a characteristic immune and inflammatory response, even though the route from receptor ligation to biologic effect remains incompletely mapped.

Much has also been learned about the cellular physiology and molecular biology of the process by which naive T cells differentiate into cytokine-secreting effector cells. Conditions that favor differentiation to Th1 or Th2 have become understood, and transcription factors that directly induce type 1 or type 2 cytokine production and their binding sites in cytokine gene promoters and enhancers have been identified, although the critical question of what makes some antigens allergenic remains unanswered.

This chapter addresses these issues with regard to the type 2 cytokines. Particular attention is given to the *in vivo* biologic effects of type 2 cytokines that promote immune and inflammatory responses that protect or damage the host in the process of dealing with real or perceived pathogens.

PHYSICAL CHARACTERISTICS OF TYPE 2 CYTOKINES

Characterization of biologic activities in the supernatants of murine Th2 cells and of the mRNA expressed by these cells identified three cytokines in addition to IL-4 that are often produced by $CD4^+$ T cells that secrete IL-4 but not IFN-g. These cytokines, IL-5, IL-9, and IL-13, are related not only in that they can all be produced by the same cell population, but also by the chromosomal location of their genes [IL-4, IL-5, IL-9, and IL-13, along with the IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) genes, are located in region q31-33 of chromosome 5 in humans and in a syntenic region of chromosome 11 in the mouse, except IL-9, which is located on mouse chromosome 13] (25,26), by their sharing of receptors and signaling pathways (27), and by their functional properties (all promote allergic reactions and all promote host protection against gastrointestinal nematodes). In addition, all the type 2 cytokines are members of the short-chain subset of the structural type I cytokine family: each has four a-helical bundles containing approximately 15 amino acids per bundle. Furthermore, all the type 2 cytokines bind to

receptors that are type I membrane-spanning glycoproteins, which include (a) fibronectin type III modules in an extracellular domain that has four conserved cysteines, (b) a membrane-proximal tryptophan-serine-X-tryptophan-serine sequence, and (c) a cytoplasmic domain that includes a membrane-proximal box 1/box 2 region in which box 1 is proline-rich and involved in the initiation of signaling (27). Some physical properties of the type 2 cytokines are shown in Table 11.1.

Cytokine	Chromosomal location (Human/Mouse)	Polypeptide Size	Major Cell Source	Structural Features
IL-4	5/11	20-kd monomer	T cell, mast cell/basophil	Short chain 4 α helices
IL-5	5/11	20-kd homodimer	T cell, mast cell/basophil, eosinophil	Short chain 4 α helices
IL-9	5/13	14-kd monomer	T cell, mast cell/basophil	Short chain 4 α helices
IL-13	5/11	14-kd monomer	T cell, mast cell/basophil, NK cell	Short chain 4 α helices

IL, interleukin; NK, natural killer.

TABLE 11.1. Type 2 Cytokines

An additional cytokine, IL-10, is often described as a type 2 cytokine because it is found in culture supernatants of activated type 2 cells (28), and it can suppress the generation of type 1 cytokine responses by inhibiting antigen presentation and IL-12 production (28,29). I prefer to classify IL-10 separately as an antiinflammatory cytokine, because (a) it can inhibit both type 1 and type 2 cytokine-induced inflammation and responses (29,30), (b) its *in vivo* production does not correlate well with production of IL-4, IL-5, IL-9, and IL-13 (31,32), (c) it is induced by an inflammatory cytokine, IL-12, that inhibits all other type 2 cytokine responses (33), (d) its receptor has a structure that resembles that of the IFN receptors rather than the receptors for the type 2 cytokines (27), and (e) the location of the IL-10 gene is outside the type 2 cytokine gene cluster, on chromosome 1 (in both mice and humans) (34).

COORDINATE PRODUCTION OF TYPE 2 CYTOKINES

An early version of the original Th1/Th2 concept suggested that CD4$^+$ T cells would produce either the complete set of type 1 cytokines or the complete set of type 2 cytokines (1,5). Further experimentation demonstrated a more complex reality. First, on initial stimulation, CD4$^+$ T cells often produce IL-2 in the absence of other type 1 or type 2 cytokines (35,36). On further stimulation, T cells can differentiate into cells that secrete a mixture or type 1 and type 2 cytokines. T cells at this stage have been referred to as Th0 cells (7,37,38). Although it has been argued that all differentiating CD4$^+$ T cells pass through a Th0 stage (38), *in vivo* studies have only established this for a few antigens and immunization protocols; other *in vivo* studies have failed to demonstrate a stage of mixed type 1 and type 2 cytokine production to immunogens that eventually induce a strongly polarized type 1 or type 2 cytokine response (31,32,39). Regardless of the differentiation route taken by CD4$^+$ T cells before they become Th1 or Th2 cells, it is now generally agreed that the Th1/Th2 concept should only be applied to mature, fully differentiated cells.

Second, as noted earlier, even fully differentiated, mature CD4$^+$ T cells do not necessarily produce all type 1 or all type 2 cytokines. Instead, individual T cells may produce a selection of some type 1 or type 2 cytokines or even a mixture of type 1 or type 2 cytokines. Even when they are viewed as a population rather than as individual cells, *in vivo* responses often include production of some, but not all, cytokines of one type or a combination of type 1 and type 2 cytokines. Examples are the production of large amounts of IL-4 without production of IL-5 during *in vivo* responses to some protein antigens (40) and production of IL-5 and IFN-g during respiratory syncytial virus infection (41).

Third, even during the relatively “pure” type 2 cytokine responses made by mice infected with some gastrointestinal nematode parasites, not all type 2 cytokines are made simultaneously; production of IL-5 and IL-9 precedes production of IL-4, which precedes production of IL-13 (31).

Fourth, studies of cells in particular locations, such as the small intestine, have demonstrated CD4$^+$ T-cell populations that produce combinations of cytokines, such as IL-10, and transforming growth factor-b (TGF-b) that may promote gut immune responses, such as immunoglobulin A (IgA) production, but inhibit inflammatory and immune responses at other locations (42). Nomenclatures have been devised that emphasize the unique qualities of these additional cell populations and their contributions to the immune system at the expense of an understanding that cytokines are, to a considerable extent, individually regulated.

Fifth, the same stimuli may have different effects on cytokine production in different individuals; individual genotype and previous immunologic experience both affect the selection and quantity of cytokines produced. This variability is of practical and theoretic importance. For example, although asthma is associated with increased production of type 2 cytokines, the roles played by IL-4, IL-5, IL-9, and IL-13 in disease may vary in different patients with this disease, and even the type 1 cytokine, IFN-g, appears to have a role in asthma pathogenesis (43). Consequently, therapy directed against a particular cytokine may be more efficacious in some patients than in others.

RECEPTORS FOR TYPE 2 CYTOKINES

The general characteristics of type 2 cytokine receptors are described earlier (27) (Table 11.2). Each receptor consists of two polypeptide chains; some of these are shared among receptors for different cytokines. There are two receptors for IL-4 (the type I and type II IL-4 receptors); the type II IL-4 receptor is also the sole known receptor for IL-13 (44). Both IL-4 receptors contain IL-4 receptor (R) a chain, which, in the type I IL-4R, is paired with cytokine receptor common g chain (g_c) and in the type II IL-4R is paired with IL-13Ra_1 chain (the g_c earns its name by participating in the IL-2, IL-7, IL-9, and IL-15 receptors, as well as the type 1 IL-4R) (27,44). IL-4 is bound predominantly by IL-4Ra chain in both the type I and type II IL-4Rs, whereas the type 2 IL-4R binds IL-13 predominantly through an interaction with IL-13Ra_1 chain. The IL-9 receptor resembles the type I IL-4R in that it contains g_c and an a chain that binds IL-9 (45). In contrast, the IL-5R, in addition to its unique IL-5Ra chain, contains the cytokine receptor common b chain (b_c), that is also a constituent of the IL-3 and GM-CSF receptors (46,47).

Cytokine	Membrane Receptor	Secreted			
	Polypeptides	Polypeptides	JAKs	Stats	Additional Signaling Molecules
IL-4	IL-4Rα/γ_c (type I)	sIL-4Rα	1,3	6	IRS-1, IRS-2, IRS-3
	IL-4Rα/IL-13Rα$_1$ (type II)		1,2, Tyk2	6	
IL-5	IL-5Rα/β_c	sIL-5Rα	1,2	5a,5b	CrkL, Btk, Lyn, Fyn, Raf-1
IL-9	IL-9Rα/γ_c		1,3	3,5a,5b	IRS-1, IRS-2
IL-13	IL-4Rα/IL-13Rα$_1$	sIL-13Rα$_2$	1,2, Tyk2	6	IRS-1, IRS-2

IL, interleukin.

TABLE 11.2. Receptors and Signaling Molecules for Type 2 Cytokines

Type 2 cytokine receptor chains exist in secreted as well as transmembrane forms. Alternately spliced versions of IL-4Ra and IL-5Ra mRNA encode secreted proteins that bind IL-4 or IL-5, respectively, without signaling (48). These soluble “receptors” may act as cytokine antagonists that prevent bound cytokine from ever reaching receptors and signaling cells (48). Alternatively, because complexes of cytokine and cytokine binding protein have a longer *in vivo* half-life than the free cytokine and can dissociate to release active free cytokine, cytokine binding proteins may increase the duration of a cytokine effect when a relatively large amount of a cytokine is released over a short period (49).

In the case of IL-13, a more complex situation occurs in which a second IL-13 binding molecule, IL-13Ra_2, is produced in both cell membrane and secreted forms (44).

IL-13Ra$_2$ binds IL-13 more avidly than IL-13Ra$_1$, but it is not known to be part of a signaling receptor. Consequently, IL-13Ra$_2$ may be a particularly effective inhibitor of IL-13.

TYPE 2 CYTOKINE SIGNALING

Each receptor for a type 2 cytokine signals through at least two pathways; one that involves JAK-Stat signaling and a second that involves activation of a more ubiquitous tyrosine kinase, such as phosphatidylinositol-3-kinase (PI3K) (27). In each case, signaling is initiated by linking of the two receptor chains by the cytokine. This linkage allows transphosphorylation of tyrosine residues on Janus kinases (JAKs) associated with box 1/box 2 sites on the cytokine receptors. The activated JAKs also phosphorylate tyrosine residues on cytoplasmic domains of the cytokine receptors; these residues become docking sites for signal tranducers of activation of transcription (Stats), which contain phosphotyrosine-binding SH2 domains. Phosphorylation of Stat tyrosine residues by JAKs causes the Stats to dissociate from cytokine receptor docking sites, to dimerize, and to translocate to cell nuclei, where they can stimulate the transcription of specific genes by binding to elements in the promoter regions of these genes.

Phosphorylated tyrosine residues on cytoplasmic domains of the cytokine receptors also act as docking sites for adaptor proteins, such as insulin receptor substrate-1 (IRS-1) and IRS-2 (50). Once phosphorylated by JAKs, these proteins bind and allow the tyrosine phosphorylation and activation of PI3K, which initiates signaling through the Ras–membrane-associated protein (MAP) kinase cascade, leading to assembly of the transcription factor, AP-1 (51).

As shown in Table 11.2, only four JAKs have been identified (JAK1, JAK2, JAK3, and Tyk2). Each JAK can associate with several different cytokine receptor polypeptides. For example, JAK1 and JAK3 are involved in signaling through all the g$_c$-associated cytokine receptors (JAK3 associates with g$_c$; JAK1 associates with the cytokine receptor a chains), and JAK1 is also involved in signaling through the IFNa/b, IFN-g, IL-10, granulocyte colony-stimulating factor (G-CSF), the gp130 family, and possibly the b$_c$ family cytokine receptors (27). Some Stats are more cytokine specific (Table 11.2). For example, Stat6 is activated only by IL-4 and IL-13 (it docks to phosphotryosine residues on the cytoplasmic domain of IL-4Ra) (52). Studies of Stat6-deficient mice demonstrate that Stat6 is responsible for most effects of IL-4 and IL-13 on cell differentiation and contributes, directly or indirectly, to IL-4 stimulation of proliferation and IL-4 enhancement of B-cell survival (53,54 and 55). Similarly, Stat4 is activated only by IL-12 in mice and is required for IL-12 stimulation of type 1 cytokine expression and inhibition of type 2 cytokine expression (56). In contrast, Stat5a activation and Stat5b activation are stimulated by multiple cytokines in addition to IL-5 and IL-9 (27.,57,58 and 59). This difference in Stat specificity suggests that specificity may be attained by different mechanisms. Some Stats (Stat4, Stat6) may be activated by receptors that have a broad distribution on different cell types; specificity for these Stats is attained through their activation by one or a small number of cytokine receptors. Stats, such as Stat5a and Stat5b, which are activated by multiple cytokines, allow for specificity of cytokine effect because of the limited cellular distribution of receptors for each cytokine that activates those Stats. For example, IL-5 and GM-CSF both activate Stat5a and Stat5b, but they stimulate proliferation and differentiation of different cell types because IL-5 and the GM-CSF receptor a chains are expressed by different cells types (60).

EFFECTS OF TYPE 2 CYTOKINES

Type 2 cytokines generally promote noncytotoxic antibody production and allergic inflammation, whereas type 1 cytokines generally promote antibody-mediated and cell-mediated cytotoxic responses through their effects on several different cell types. Only the effects of the type 2 cytokines on specific cell types are discussed here.

Effects on T Cells

IL-4 is regarded as the master type 2 cytokine because it has the unique ability to stimulate naive CD4$^+$ T cells to differentiate into type 2 cytokine-secreting cells (61,62,63 and 64). Neutralization of IL-4, blocking of IL-4Ra, or a genetic deficiency of IL-4, IL-4Ra, or Stat6 is often (but not always) associated with diminished type 2 cytokine production (54,55 and 56,65,66,67 and 68). IL-4 and IL-13 can also enhance type 2 cytokine production less directly, by inhibiting production or effects of cytokines, such as IL-12 and IFN-g, that suppress T-cell differentiation into type 2 cytokine-secreting cells (44). This view is supported by observations that IL-4 suppresses T-cell expression of the IL-12 receptor b$_2$ chain (69) and that type 2 cytokine production is decreased in IL-13–deficient mice and is increased in IL-13 transgenic mice (70,71), even though T cells lack IL-13R (type II IL-4R) (44).

The possibility that another type 2 cytokine, IL-9, also regulates type 2 cytokine production has been controversial. Although some T cells proliferate in response to IL-9 (6), and thus must express an IL-9 receptor, and although anti–IL-9 antibody has been reported to suppress the Th2 response in mice infected with the gastrointestinal nematode *Trichuris muris* (72), T-cell differentiation and cytokine production appear to be normal in IL-9–deficient mice (73).

Effects on Antigen-Presenting Cells

IL-4 promotes antigen presentation by B cells and dendritic cells by stimulating increases in class II major histocompatibility complex (MHC) expression and expression of the costimulatory molecules CD80 and CD86 (74,75,76,77 and 78). IL-13 may have similar effects, except mouse B cells lack IL-13 receptors (44,79). However, because other cytokines, such as IFN-g, and noncytokine stimuli also promote antigen presentation (80,81), defects in antigen presentation are not generally profound in IL-4 or IL-4Ra–deficient mice.

IL-4 (and possibly, IL-13), can also affect type 1–type 2 cytokine balance through their effects on antigen presentation. IL-4 may promote type 2 cytokine responses by suppressing dendritic cell production of IL-12 or by suppressing T-cell or NK cell expression of IL-12 receptor b$_2$ (69,82). In contrast, IL-4 stimulates the differentiation of CD8$^+$ dendritic cells that promote T-cell differentiation into Th1 cells by producing IL-12 (83,84). Thus, although the overall effect of IL-4 and IL-13 is enhancement of type 2 cytokine responses, these cytokines also have effects on antigen-presenting cells (APCs) that promote T-cell activation in general and limit Th2 polarization.

Effects on B Cells

As a general rule, type 2 cytokines promote B-cell activation, proliferation, and differentiation. IL-4 regulates B-cell receptor expression and function: it increases B-cell expression of class II MHC antigens (74), the T-cell costimulatory molecule, CD86 (B7-2) (75,76), the receptor for cell-mediated T-cell help, CD40 (85), and the low-affinity IgE receptor, CD23 (86), whereas it suppresses IFN-g induction of the low-affinity IgG receptor, FcgRII (87), which activates inhibitory tyrosine and inositol phosphatases (88). Because CD23 participates in antigen presentation by focusing IgE-bound antigen onto B cells (89), and signaling through the B-cell form of FcgRII inhibits B-cell activation (90), these IL-4–induced changes promote the ability of B cells to become activated by antigen, to present antigen to CD4$^+$ T cells, and to be stimulated by contact with activated CD4$^+$ T cells. In addition, IL-4 enhances the survival of unstimulated and antigen-stimulated B cells *in vivo* and *in vitro* (91,92 and 93), and it causes a net migration of circulating B cells to the spleen (94). Once B cells have become activated, IL-4 influences the isotype of Ig secreted by differentiating B cells and promotes isotype switching to IgG1 in the mouse, IgG4 in humans, and IgE in both species (95). IL-4 also inhibits secretion of the cytotoxic antibody isotypes, IgG2a and IgG3 in the mouse; however, no equivalent effect has been shown for humans.

Mice and humans also differ in their response to the IL-4–related cytokine, IL-13. Human, but not mouse, B cells have been shown to express IL-13Ra$_1$ chain, and this allows them to respond to this cytokine. Consequently, IL-13 generally has the same effects as IL-4 on human B cells, but it seems able to stimulate mouse B cells only under special circumstances (44).

Although relatively little is known about how the type 2 cytokine, IL-9, stimulates B cells, it generally appears to enhance the effects of IL-4; it increases isotype switching to IgE, particularly when IL-4 is present in limiting quantity (96). The effects of IL-5 on B-cell responses, although marked in several *in vitro* culture systems, appear *in vivo* to be limited to stimulation of proliferation and clonal expansion of the B1 B-cell subset (97,98), which constitutes a large percentage of peritoneal B cells and produces largely T-independent antibodies to bacterial antigens (99).

Proinflammatory Effects

The effects of type 2 cytokines on inflammatory cells reinforce their stimulation of allergic responses and inhibition of cytotoxic responses. Type 2 cytokines, including IL-4, IL-9, and IL-13, attract neutrophils and eosinophils to the site of cytokine production, most likely by stimulating the production of several CC and CXC chemokines and by acting with TNF-a to enhance the expression of adhesion molecules, including vascular cell adhesion molecule-1 (100,101,102,103,104 and 105). IL-5 can also contribute to migration of eosinophils to an inflammatory focus (106), and, more important, it stimulates eosinophil production, survival, and activation (107). The importance of IL-4 and IL-13 for the development of localized eosinophilia is shown by the greatly decreased number of pulmonary eosinophils in mice treated with IL-4Ra monoclonal antibody (mAb) (which inhibits IL-4 or IL-13 responses) after pulmonary immunization (108), whereas the importance of IL-5 in eosinophil responses is shown by the large decrease in eosinophil numbers in all organs, including blood, that is observed in IL-5–deficient mice (97).

IL-4 and IL-9 also contribute, along with IL-3 and c-*kit* ligand, to the stimulation of mast cell proliferation and differentiation. Systemic IL-4 treatment is associated with increased numbers of intestinal mast cells (109). Pulmonary overproduction of IL-9 (but not IL-4) is associated with increased numbers of mast cells in the lungs (110).

IL-9 appears to be more important for early pulmonary mast cell responses to antigens that localize in the lungs than for responses that develop after repeated immunization; the former are suppressed, whereas the latter are normal in IL-9–deficient mice (73). IL-9 may be less important (and IL-4 more important) for the intestinal mast cell response than for the pulmonary mast cell response; an intestinal mast cell response can be induced by treating normal mice with IL-4 and is normal or near normal in worm-infected IL-9–deficient mice, but it is suppressed when worm-infected mice are treated with IL-4 antagonists (73,109,111). IL-13 stimulation has not been associated with mastocytosis, a finding suggesting that mast cells or their precursors may lack IL-13Ra_1 chain.

All the type 2 cytokines have been associated with increased mucus production. Inhalation of IL-4, IL-5, IL-9, or IL-13, or transgenic overexpression of any of these cytokines in the lungs, causes bronchial goblet cell hyperplasia (110). *In vitro* experiments, however, have not convincingly determined whether the stimulatory effects of any of these cytokines on increased mucus production are direct or indirect. It also remains to be determined whether different type 2 cytokines have distinct effects on the chemical and physical character of the mucus that they stimulate.

Type 2 cytokines have also been associated with stimulation of fibrosis. *In vitro,* culture of fibroblasts with IL-4 stimulates increased collagen production (112,113). *In vivo,* IL-4 and IL-13 have been shown to contribute to the development of a fibrotic response to *Schistosoma mansoni* eggs; fibrosis is decreased to some extent in IL-4 antagonist–treated mice, to a greater extent in mice treated with an IL-13 antagonist, and almost absent in IL-4Ra–deficient mice, which cannot respond to either cytokine (114,115 and 116). Whether the stronger association of IL-13 than IL-4 with fibrosis and the increased gastrointestinal mucus production (70) result from differences in amounts of IL-4 and IL-13 produced or from differences in the effects of these cytokines is not known.

Both IL-4 and IL-13 also contribute to allergic reactions by increasing sensitivity to mediators, such as histamine, serotonin, and platelet-activating factor, that are released by degranulating mast cells, most likely by acting synergistically with these mediators to increase vascular permeability (117). It is unlikely that this effect is restricted to rodents; humans treated with IL-4 in an attempt to suppress tumor growth have developed vascular leak syndrome as a side effect (118). Thus, type 2 cytokines not only stimulate mastocytosis and production of the IgE antibodies that induce mast cells to release inflammatory mediators, but they also increase the responsiveness of vascular epithelial cells to these mediators.

Effects on Epithelial Cells

IL-4 and IL-13 both increase the permeability of epithelial cell monolayers *in vitro* through effects on the tight junctions that connect these cells (119,120). A similar effect on intestinal epithelial permeability has been observed in mice treated with IL-4 and in mice in which gastrointestinal nematode parasite infection has stimulated increased IL-4 and IL-13 secretion (Shea-Donohue T, Sullivan C, Finkelman FD, et al. The role of interleukin-4 in *Heligmosomoides polygyrus*–induced alterations in murine intestinal epithelial cell function. Submitted for publication). IL-4 also causes *in vivo* changes in ion absorption and secretion that result in a net loss of salt and fluid in the intestine, an effect that may contribute to the development of diarrhea when local type 2 cytokine production is high, as in mice infected with intestinal nematodes.

Effects on Smooth Muscle

Mice treated with IL-4 develop changes in intestinal smooth muscle function, with an increased contractile response. This change is most likely indirect and mast cell dependent, because it is not seen in IL-4–treated, mast cell–deficient W/W^v mice (121).

Antiinflammatory Effects

Despite these proinflammatory effects, IL-4 and IL-13 are often referred to as antiinflammatory cytokines, because they can suppress inflammatory responses that are associated with type 1 cytokine production. Some of these antiinflammatory effects result from suppression of production of cytokines (IL-12, TNF-a, IFN-g) that induce precursor T cells to differentiate into cells that kill targets through a contact-dependent mechanism (cytotoxic T lymphocytes). Additional antiinflammatory effects result from suppression of production of inflammatory cytokines, such as TNF-a and IL-12, and from suppression of macrophage responsiveness to IFN-g and TNF-a (82,122,123,124 and 125). These effects of IL-4 and IL-13 decrease the activity of enzymes that produce reactive oxygen and nitrogen intermediates that kill ingested pathogens (124,125). The importance of the antiinflammatory effects of IL-4 is illustrated by the increased inflammation and death observed in IL-4–deficient mice infected with some pathogens, such as *S. mansoni* (126) or *Borrelia burgdorferi* (127,128) (the pathogen responsible for Lyme disease).

In sum, the type 2 cytokines induce an immune and inflammatory response that is characterized by increased antibody production, especially production of IgE and noncytotoxic IgG isotypes, infiltrates rich in eosinophils and mast cells, increased mucus production, increased sensitivity of smooth muscle to contractile stimuli, and increased epithelial cell permeability, including vascular permeability. At the same time, the type 2 cytokines suppress inflammatory responses that are associated with the killing of intracellular or small extracellular organisms, including the production of complement-fixing IgG antibodies, macrophage production of nitric oxide and other reactive molecules that kill ingested organisms, and induction of cytotoxic lymphocytes.

ROLE OF TYPE 2 CYTOKINES IN PROTECTIVE IMMUNITY

Because worm infections usually induce a type 2 cytokine response (129), it was reasonable to hypothesize that these cytokines could protect the host against worms. This hypothesis has not been proven for human infections, because the interventions that would be necessary to demonstrate a protective relationship directly would be unethical. Studies of the association between serum IgE levels and the severity of reinfection of patients living in endemic areas who have been cured of *S. mansoni* infection support this hypothesis, however, by revealing an inverse correlation between serum IgE concentration, an index of IL-4 and IL-13 activity, and severity of reinfection (130,131).

Mouse studies provide direct evidence that the type 2 cytokines can be host-protective. Mouse strains that make a type 1 cytokine response to oral inoculation with *T. muris,* a whipworm that closely resembles the human parasite *T. trichiura,* develop a chronic infection, whereas worm larvae are fully expelled before they mature in mouse strains that make a predominantly type 2 cytokine response to the same parasite (132). Moreover, when mice that would normally develop a type 1 cytokine response and chronic infection were treated with anti–IFN-g antibody or with IL-4, they instead produced type 2 cytokines and rapidly expelled worm larvae, whereas treatment of mice that would normally develop a type 2 response with IL-12 or anti–IL-4Ra antibody induced a type 1 response and chronic infection (133).

Additional studies in one mouse strain, C57BL6, that develops a mixed type 1–type 2 cytokine response to *T. muris* infection, has revealed roles for IL-4, IL-13, and IL-9 in host protection. C57BL/6 mice generally expel *T. muris,* but they do so more slowly and less completely than BALB/c mice, which develop a strong type 2 cytokine response to this parasite. Inhibition of IL-4, IL-13, or IL-9 in C57BL/6 mice is sufficient to tip the balance to type 1 cytokine predominance and to prevent worm expulsion, whereas inhibition of IL-4 generally fails to prevent worm expulsion in BALB/c mice (133,134 and 135). Furthermore, inhibition of IL-4 or IL-13 fails to prevent worm expulsion in C57BL/6 mice if IFN-g is also blocked, whereas chronic infection develops if both IL-4 and IL-13 are inhibited, despite blocking of IFN-g (136). These observations suggest that effector mechanisms that lead to worm expulsion can be induced by either IL-4 or IL-13, perhaps with a boost from IL-9, but they are inhibited by IFN-g.

Studies of mice infected with either of two rodent-specific gastrointestinal nematode parasites, *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus,* or with *Trichinella spiralis,* a gastrointestinal nematode that infects many mammalian species, allow generalization of the observations made with *T. muris*–infected mice. Despite substantial differences in the life cycles of these worms, the development of protective immunity in every case depends on the presence of IL-4Ra chain, the IL-4Ra–associated signaling molecule, Stat6, and the production of IL-4 and/or IL-13 (129,137,138). Details of how these cytokines, receptors, and signaling molecules contribute to host protection, however, vary considerably from one parasite to the next. Although all elements of a stereotyped Th2 response (eosinophilia, production of IgE and IgG1, intestinal mucosal mastocytosis and goblet cell hyperplasia, and increased smooth muscle contractility and intestinal permeability) are induced by each of these parasites (31,129,138), the importance of each of these elements in host protection varies from parasite to parasite and from primary to second infection with the same parasite. Particularly instructive comparisons can be made between primary and second infections with *T. spiralis* and between primary infections with *T. spiralis* and *N. brasiliensis.*

Expulsion of *T. spiralis* adults and larvae is mast cell dependent in both primary and secondary infections. During a primary *T. spiralis* infection, mast cell degranulation is T-cell dependent, but antibody independent. In contrast, the accelerated worm expulsion that occurs during a second *T. spiralis* infection appears to require triggering of mast cell degranulation by IgE, although a smaller contribution may be made by IgG. Either IL-4 or IL-13 production is sufficient to induce normal expulsion during a primary *T. spiralis* infection, but IL-4 is required for the accelerated secondary response. This is probably because IL-4 is required in the mouse to stimulate IgE production and, possibly, because it also helps to generate and sustain T cells that rapidly produce type 2 cytokines on restimulation (138,139).

Mechanisms of host protection during primary *N. brasiliensis* and *T. spiralis* infections differ in several ways. First, *N. brasiliensis* expulsion can be induced by IL-4 in the absence of mast cells, T cells, and B cells (129,140), whereas IL-4 treatment does not induce T-cell–deficient or mast cell–deficient mice to expel *T. spiralis* (138). Second, Stat6-deficient mice make normal IL-4, IL-5, IL-13, eosinophil, and IgG1 responses to *N. brasiliensis* infection, as well as considerably increased mast cell responses, but they fail to expel this parasite even if they are treated with IL-4 or IL-13 (137). In contrast, IL-3, IL-4, IgG1, and mast cell responses are considerably reduced, IFN-g production is increased, and worm expulsion is blocked when Stat6-deficient mice are infected with *T. spiralis*; IL-4 treatment reconstitutes mast cell

responses and partially reconstitutes worm expulsion (138). Third, mice must express IL-4Ra on both bone marrow–derived and non–bone marrow–derived cells to expel *T. spiralis,* whereas selective expression on non–bone marrow–derived cells is sufficient to allow expulsion of *N. brasiliensis* (141). Finally, although either IL-4 or IL-13 production is sufficient to allow normal worm expulsion during a primary *T. spiralis* infection, IL-13 deficiency retards *N. brasiliensis* expulsion to a much greater extent than IL-4 deficiency (70,137).

These observations may be explained by the following model: expulsion of *T. spiralis* is primarily a mast cell–dependent process. Mast cell release of mediators, such as histamine, induces changes in gut physiology that lead to worm expulsion. Either IL-4 or IL-13, acting on intestinal cells through a Stat6-dependent process, increases sensitivity to mast cell–produced mediators and allows a sufficient response to cause expulsion (117). *T. spiralis*–specific IgE production is sufficiently strong and rapid during a secondary response to *T spiralis* to enhance mast cell responses, thus allowing for more rapid worm expulsion, whereas other T-cell–induced factors are the dominant cause of mast cell activation during a primary infection. Stat6 deficiency allows increased production of the type 1 cytokines that are normally produced in response to *T. spiralis* and suppression of the type 2 cytokine response; these changes suppress the mast cell response that is required for worm expulsion. Treatment with IL-4 reconstitutes the mast cell response in *T. spiralis*–infected Stat6-deficient mice, but it fails to increase the sensitivity to mast cell–produced mediators that is required for optimal worm expulsion.

In contrast, the lack of any type 1 cytokine response in *N. brasiliensis* infection appears to allow development of a normal type 2 response, even in the absence of Stat6, consistent with the view that Stat6 signaling is more important for blocking type 1 cytokine inhibition of IL-4 responses than for directly stimulating type 2 cytokine responses. As a result, *N. brasiliensis*–infected Stat6-deficient mice develop normal eosinophil responses and increased mast cell responses [Stat6 signaling has a suppressive effect on mast cell development (137)], but they fail to expel the parasite because another, still unknown effect of Stat6 signaling on the gut, possibly an increase in the quality and quantity of mucus produced (70), is required to expel this parasite. It is not known why IL-13 is more important than IL-4 for promoting *N. brasiliensis* expulsion. Because both IL-4 and IL-13 bind to the type II IL-4R and IL-4 treatment induces IL-13–deficient mice to expel *N. brasiliensis* (44,129), it seems more likely that the primary importance of IL-13 results from either increased production of this cytokine relative to IL-4 or more efficient signaling by IL-13 of the type II IL-4 receptor, rather than from a qualitative difference in IL-4 and IL-13 effects on the gut.

Demonstration of a role for IL-5 or eosinophils in host protection against worm infections is more difficult than demonstration of roles for IL-4 and IL-13. No defects in expulsion of *T. muris, T. spiralis, N. brasiliensis,* or *H. polygyrus* have been observed in IL-5–deficient mice or in mice treated with a potent IL-5 antagonist, even though these mice are strikingly deficient in circulating eosinophils (129,142,143 and 144). The observation that IL-5–deficient mice can still develop intestinal eosinophilia, which depends on the chemokine eotaxin (145), suggests that it may be premature to conclude that eosinophils have no importance in protection against these parasites. It is more likely, however, that eosinophils contribute primarily to host protection against worms when their larvae migrate through host tissues. Consistent with this view, mice that overexpress IL-5, and consequently have increased peripheral eosinophilia, destroy *N. brasiliensis* larvae shortly after they penetrate the skin (146). In addition, destruction of filarial larvae and larvae of *Strongyloides venezuelensis* and *Angiostrongylus cantonensis* is decreased in IL-5–deficient mice (147,148,149 and 150).

In addition to providing protection against helminthic parasites, type 2 cytokine-associated inflammation appears to protect against some ectoparasites, such as the tick, *Haemaphysalis longicornis.* Protection appears to involve mast cells and IgE (151). Most likely, mast cell degranulation in response to tick antigen cross-linking of IgE bound to FceRI increases vascular permeability and causes sufficient skin edema to prevent the tick from locating a host blood vessel. Consequently, properties common to ticks and other insects (perhaps dust mites and cockroaches) may have become stimuli for the generation of type 2 cytokine responses.

In sum, all elements of type 2 cytokine-associated immunity and inflammation appear to contribute to host protection against the universe of helminth and insect parasites, even though protection against any individual helminth does not seem to require the full panoply of the type 2 inflammatory response. Mammalian hosts may be unable to recognize a specific nematode or insect parasite sufficiently well to make only those responses that would protect against that parasite. Instead, they recognize a property or properties common to these parasites as a trigger for the stereotyped type 2 cytokine response that protects against many worms and some insects. It also seems possible that the grouping of type 2 cytokine genes (along with the genes for IL-3 and GM-CSF, which contribute to mastocytosis and eosinophilia) in a single chromosomal region may facilitate coordinated transcription of these cytokines (25,26 and 27).

Related reasoning can be used to provide an evolutionary rationale for the phenomenon that type 1 and type 2 cytokines not only induce distinct inflammatory responses but also suppress both production and effects of the alternate set of cytokines. Why should any stimulus that can be recognized by the host as foreign and dangerous not induce a mixed type 1–type 2 cytokine response that could protect against every pathogen by activating every inflammatory effector mechanism? A likely explanation may be that inflammatory effector mechanisms nearly always damage the host to some extent in ridding the host of the parasite. Damage can vary from mild irritation to death, as observed in bee venom–induced anaphylaxis or gram-negative bacteria-induced endotoxin shock. Thus, to the extent that a host can recognize a pathogen class and can determine which inflammatory effector mechanisms best protect against that class, the host is best served by activating only those effector mechanisms that provide protection and suppressing those that are nonprotective. Mammalian hosts appear predominantly to recognize two pathogen classes: those, such as worms and ticks, against which only type 2 cytokine-induced inflammation provides protection and those, such as viruses and intracellular bacteria and protozoa, against which only type 1 cytokine-induced inflammation provides protection. By having the appropriate cytokine response suppress the inappropriate alternative cytokine response, the host limits damage to itself without limiting its ability to damage the parasite.

ROLE OF TYPE 2 CYTOKINES IN ATOPIC DISEASES

The host's ability to restrict a cytokine-induced immune response to a situation in which it is host protective is far from perfect. The same type 2 cytokine-induced immune and inflammatory responses that protect the host against parasites (IgE, eosinophilia, mastocytosis, chemokine secretion, adhesion molecule expression, mucus production, and increased vascular and epithelial permeability) cause symptoms in allergic disorders. Human studies provide correlative evidence that supports the association between type 2 cytokines and asthma. The elevated total and allergen-specific serum IgE levels that are found in patients with anaphylaxis, asthma, allergic rhinitis, and atopic dermatitis (152,153,154 and 155) suggest increased production of the cytokines, IL-4, IL-13, and IL-9, that stimulate IgE production, whereas the increased numbers of eosinophils in blood, skin, nasal polyps, and lungs suggest increased production of IL-5. Evidence that treatment with an anti-IgE mAb ameliorates symptoms in patients with asthma and allergic rhinitis (156,157) and that anti–IL-5 mAb treatment decreases eosinophilia in asthmatic patients (158) further implicates these cytokines in the pathogenesis of these diseases.

Biopsy evidence also associates type 2 cytokines with human allergy. Biopsies of skin from patients with atopic dermatitis, nasal polyps from patients with allergic rhinitis, and bronchial tissue from patients with atopic asthma demonstrate increased type 2 cytokine mRNA levels and increased numbers of cells that secrete IL-4, IL-5, IL-9, and IL-13 (T cells and mast cells or basophils) in these tissues (159,160,161,162 and 163). Studies of cytokine levels in bronchoalveolar lavage fluid, cytokine production by lymphocytes from conjunctival, lung, or nasal biopsies, or bronchoalveolar or nasal lavage demonstrate consistent results (164,165,166 and 167). Population studies also support a connection between type 2 cytokines and allergy by demonstrating associations between asthma and a gene or genes that map to the type 2 cytokine locus (region 31–33 of chromosome 5) (168) and between asthma and alleles of the IL-4, IL-9, IL-4Ra, and the IL-13 receptor genes (169,170,171 and 172).

Direct evidence for involvement of type 2 cytokines in allergic disorders is provided by studies with cytokine transgenic mice. Eosinophilia is observed in transgenic mice that systemically overexpress IL-5 (173), mastocytosis is observed in transgenic mice that systemically overexpress IL-4 or IL-9 (174,175), high serum IgE levels are seen in mice that systemically overexpress IL-4 or IL-13 (176,177), and IL-4 transgenic mice develop a disease that closely resembles vernal conjunctivitis (176). Studies of cytokine gene knock-out mice show consistent results: IL-5–deficient mice fail to develop normal eosinophil responses (97), IL-4–deficient mice lack serum IgE (66), both IL-4– and IL-13–deficient mice tend to make type 1 cytokine-biased immune responses (66,70), and the development of mastocytosis and goblet cell hyperplasia is delayed in appropriately stimulated IL-9–deficient mice (73).

Animal models have also been used to investigate the roles of type 2 cytokines in specific allergic diseases, such as systemic anaphylaxis and asthma. Studies with mouse models of anaphylaxis reveal two pathways that can lead to anaphylactic shock: one in which antigen cross-linking of IgE bound to FceRI leads to mast cell release of histamine and platelet activating factor that cause vascular collapse (177a) and a second in which vascular collapse is induced predominantly by platelet activating factor released when macrophage FcgRIII is cross-linked by IgG-antigen complexes (177b). As may be expected, IgE-mast cell–mediated anaphylaxis, but not IgG-macrophage–mediated anaphylaxis, is IL-4 dependent (177b,177c). IL-4 and IL-13, however, also contribute to anaphylaxis through a second mechanism: the severity of anaphylaxis is considerably increased if mice are treated with IL-4 or IL-13 shortly before challenge with antigen, anti-IgE antibody, histamine, platelet-activating factor, or serotonin. At least part of this effect appears to result from synergistic enhancement of vascular permeability by IL-4 and IL-13 and vasoactive mediators (117). Because IgE-antigen interactions induce release of type 2 cytokines by basophils as well as the release of vasoactive mediators by mast cells, this synergy between the effects of type 2 cytokines and vasoactive mediators may well contribute to both the severity of systemic anaphylaxis and the efficacy of IgE-mediated host protection against gastrointestinal nematodes.

Transgenic mice that selectively overexpress a type 2 cytokine in their lungs have helped to establish the importance of these cytokines in the pathogenesis of asthma. Bronchial goblet cell hyperplasia and pulmonary inflammatory infiltrates that include eosinophils are observed in mice that selectively overexpress IL-4, IL-5, IL-9, or IL-13 in their lungs (110). In addition, increased responsiveness to methacholine, a physiologic marker for asthma, has been observed in IL-5, IL-9, and IL-13 lung transgenic mice (110), and impressive bronchial mastocytosis has been observed in IL-9 lung transgenic mice (110). Remodeling changes characteristic of chronic asthma (shedding of bronchial epithelium, subepithelial fibrosis, smooth muscle hyperplasia) have been observed to a limited extent in some of these mice, but they

have been more convincing in transgenic mice that overexpress inflammatory cytokines, such as IL-11, in their lungs (110).

Mouse models have also been used to evaluate the effects of inhibiting one or more type 2 cytokines on the development of asthma in appropriately immunized animals. Mice “primed” with ovalbumin adsorbed to alum adjuvant, then exposed to aerosolized ovalbumin, develop an eosinophil-rich acute perivascular and peribronchial inflammatory response, bronchial goblet cell hyperplasia, increased vascular permeability, and increased responsiveness to methacholine (178,179,180 and 181). Similar changes occur in mice immunized nasally with strong allergens, such as *Ascaris* pseudocelomic fluid, dust mite extracts, or *Aspergillus* extracts, even in the absence of adjuvant and systemic priming (182,183). Allergen induction of pulmonary eosinophilia is greatly reduced, in most studies, in mice deficient in IL-4 or in wild-type mice treated with an IL-4 or an IL-4Ra antagonist (108,178). Development of goblet cell hyperplasia has also been reduced by IL-4 deficiency or IL-4 antagonist treatment (108,184,185 and 186). IL-5 deficiency and IL-5 antagonists even more strongly suppress the development of pulmonary eosinophilia, and virtually no eosinophils are observed in immunized mice that lack both IL-5 and the chemokine eotaxin; however, IL-5 deficiency does not suppress bronchial goblet cell hyperplasia (178,179 and 180,187,188). IL-13 antagonists suppress pulmonary eosinophilia and goblet cell hyperplasia to less of an extent than IL-4 antagonists, but they enhance suppression when added to IL-4 antagonists (189,190 and 191). Similar, strong suppression is observed in mice deficient in IL-4Ra or Stat6 (184,192, and Finkelman FD, unpublished data). IL-9 deficiency does not prevent the development of pulmonary eosinophilia but is associated with delayed development of goblet cell hyperplasia and pulmonary mastocytosis (73). Treatment with IL-4, IL-5, IL-13, and IL-4Ra antagonists can have strong suppressive effects even if it is initiated after pulmonary inflammatory responses have already developed (108,178,179,189,190).

Because IL-4, unlike the other type 2 cytokines, stimulates the differentiation of naive $CD4^+$ T cells into Th2 cells, some inhibitory effects of IL-4 antagonists on allergic pulmonary inflammation may reflect inhibition of Th2 differentiation, rather than direct inhibition of inflammation. For example, IL-4 antagonists may block goblet cell hyperplasia by preventing the differentiation of T cells into cells that secrete IL-4, IL-5, IL-9, and IL-13 (110,185), all of which have been reported to stimulate goblet cell hyperplasia *in vivo.* Because survival and type 2 cytokine secretion by fully differentiated Th2 cells have been reported to be IL-4 independent (193), IL-4 antagonists may be expected to be unable to suppress established allergic responses that are driven by IL-5, IL-9, or IL-13. Contrary to this expectation, both allergic inflammation and type 2 cytokine secretion that develop in IL-4–deficient mice that have inhaled both allergen and IL-4 resolve when IL-4 administration is discontinued, even if allergen administration is continued (182). Thus, in an *in vivo* setting, an effective IL-4 antagonist may well inhibit even an ongoing allergic response.

The relationship between type 2 cytokines and the development of hyperresponsiveness to cholinergic agonists is more complicated. IL-4 or IL-5 antagonists suppress allergen-induced pulmonary hyperresponsiveness in some, but not all, models (178,179 and 180); an IL-13 antagonist has been reported to suppress hyperresponsiveness in some situations in which it was not suppressed by IL-4 antagonists (189,190), and a deficiency of both IL-5 and eotaxin has been reported to prevent the development of hyperresponsiveness that is not prevented by a deficiency in either cytokine alone (188). Differences in immunization procedure, mouse strain, dose and preparation of cytokine antagonist used, and method for characterizing the bronchospastic response to cholinergic stimulation make it difficult to draw a simple conclusion from these studies. Probably, it is safest to summarize all the animal model studies of asthma by concluding that each type 2 cytokine is a reasonable target for therapy, that different patients (like different animal models) may benefit most by suppression of different cytokines, and that only therapeutic trials of antagonists in defined patient populations will reveal their therapeutic efficacy. With this reservation, my own view is that effective blocking of IL-4Ra, which would inhibit the direct effects of both IL-4 and IL-13 and could suppress T-cell production of IL-5 and IL-9, would be the most effective cytokine-related single-agent inhibitor of allergy. In patients with asthma, trials of IL-4, IL-5, and IL-4Ra antagonists that are currently in progress, and planned trials of IL-4 and IL-9 antagonists, should test this view.

ROLE OF TYPE 2 CYTOKINES IN OTHER DISEASES

Although type 2 cytokines have most convincingly been implicated in the pathogenesis of atopic disorders, they may also be involved in the pathogenesis of other inflammatory disorders. Increased IL-4 production has been reported in some patients with systemic lupus erythematosus (194), and lupuslike disease in some mouse models has been ameliorated by deleting the IL-4 gene (195). In contrast to allergy, however, no evidence indicates an imbalance between type 1 and type 2 cytokine production in lupus; increased production of IFN-g has also been detected in lupus, and deletion of the IFN-g gene in mouse models also inhibits disease development (195,196). IL-4 may be more closely involved in the pathogenesis of another autoimmune disorder, systemic sclerosis. Cells that secrete IL-4, but not IFN-g, are overrepresented in the skin of patients with this disease, IL-4 promotes fibroblast synthesis of collagen, and anti–IL-4 mAb prevents development of skin fibrosis in the “tight skin mouse” model of scleroderma (112,197,198 and 199).

Type 2 cytokines may also be important inhibitors of inflammatory diseases. Patients with type 1 diabetes mellitus or multiple sclerosis, NOD mice, which spontaneously develop diabetes, and immunized mice that develop the experimental allergic encephalitis model of multiple sclerosis all have type 1 cytokine predominance in their inflammatory lesions (200,201 and 202). Furthermore, the mouse models of both diseases can be prevented by treatment with IL-4 (203,204). Consequently, there is some reason to fear that treatment of atopic patients with type 2 cytokine inhibitors could increase the risk of development of diabetes, multiple sclerosis, or other type 1 cytokine-related diseases. Paradoxically, although decreased IL-4 production permits development of diabetes in the NOD mouse, IL-4Ra deficiency prevents disease development in this animal model (205), possibly because of contributions made by IL-4 and IL-13 to the differentiation of APCs.

INDUCTION AND REGULATION OF TYPE 2 CYTOKINE PRODUCTION

Evidence that type 2 cytokines protect the host against an important class of pathogens, but induce immunopathology that can threaten the host's well-being and survival, indicates the importance of mechanisms that regulate type 2 cytokine production. Mechanisms that regulate the differentiation of naive T cells into cytokine-secreting cells include the cytokine milieu of the differentiating T cell, antigen dose, T-cell receptor (TCR) affinity, for the MHC II–peptide complex, T-cell costimulation, site of immunization, and characteristics of the APC. These mechanisms are not mutually exclusive but are likely interdependent.

Cytokine Milieu

In vitro studies demonstrate that cytokine milieu can determine whether naive $CD4^+$ T cells differentiate into polarized Th1 or Th2 cells. Th1 differentiation is promoted by IL-12 through a Stat4-dependent mechanism (56,206,207), enhanced, in at least some situations, by IFN-g and IFN-a/b (208), and inhibited by IL-4, through a Stat6-dependent mechanism (209); Th2 differentiation is promoted by IL-4–induced Stat6 signaling (53,54 and 55), and it is inhibited by IL-12 and the IFNs (33,210,211 and 212). Similar results are observed regardless of whether normal, naive T cells are activated by anti-CD3 mAb and APCs and or TCR-transgenic T cells are activated by APCs pulsed with the relevant antigen or peptide. In the absence of APCs or APC-related costimuli, naive, resting $CD4^+$ T cells stimulated with anti-CD3 in the presence of antibodies to IL-4 or IL-4Ra, IFN-g, and IL-12 produce little IL-4 (213,214 and 215), particularly if T cells are restimulated after allowing them to return to a resting state. Although this finding is consistent with the possibility that IL-4 is required to prime for an IL-4 response, more recent studies suggest that combined CD3 and CD28 signaling induces even naive $CD4^+$ T cells to produce IL-4, which then promotes increased IL-4 production and Th2 stabilization by signaling through Stat6 (68,216,217,218,219,220 and 221).

In vivo studies also indicate that IL-4 signaling is important for type 2 cytokine production, particularly if type 2 cytokine production is measured by restimulating T cells *ex vivo* (66). Such studies support the view that production of IL-4 early in an immune response by NK T cells (222), basophils (21,22), or mast cells (19) may be important for generation of a type 2 cytokine response. However, stimuli such as *N. brasiliensis* or anti-IgD antibody, which induce a strong type 2 cytokine response in normal mice, still do so in NK T cell–deficient mice (223,224), as well as in chimeric mice in which a functional IL-4 gene is only expressed by T cells (225). Moreover, the same stimuli induce a relatively normal IL-4 response even in IL-4Ra–deficient mice, which cannot respond to IL-4, and in Stat6-deficient mice, which lack the signaling mechanism by which IL-4 influences T-cell differentiation (68,221). Studies with Stat6-deficient mice suggest that Stat6 signaling may contribute more to the survival of type 2 cytokine-secreting cells or to the stabilization of epigenetic changes in the IL-4 promoter and enhancer that allow rapid type 2 cytokine production when memory cells are restimulated than to induction of an initial IL-4 response (68). *In vivo* studies fully support the importance of IL-12 and the IFNs as inhibitors of type 2 cytokine production (33,210,211); however, $CD4^+$ T cells do not always differentiate to Th2 cells in IL-12– or Stat4-deficient mice (215,226,227), and studies have not yet addressed whether all T cells differentiate into IL-4 secreting cells in the combined absence of IL-12, IFN-g, and IFN-a/b. Thus, it remains unclear whether naive $CD4^+$ T cells differentiate by default into Th2 cells if they are stimulated by antigen and APCs in the absence of any stimuli that promote Th1 differentiation.

Investigators have identified another cytokine, IL-18, that can suppress Th2 differentiation. However, although IL-18 acts synergistically with IL-12 to stimulate IFN-g production and to inhibit IL-4 production, in the absence of IL-12, IL-18 can enhance the production of IL-4 and IL-13 (228,229,230 and 231). Although IL-18 can thus amplify either Th1 or Th2 responses, initial studies in IL-18–deficient mice indicate that this cytokine is not required to induce polarized Th1 or Th2 responses but that the IL-18–deficient mouse is more type 2 cytokine-biased than wild-type mice (232 and Nakanishi K, unpublished data).

Antigen Dose and Affinity

Studies performed long before type 1 and type 2 cytokines were identified demonstrated that antigen dose can influence the characteristics of an immune response: low-dose immunization favored the development of a cytotoxic T-cell response, whereas high-dose immunization favored production of large amounts of antibody (233). Because the former response is consistent with type 1 cytokine dominance, whereas the latter is more characteristic of a type 2 cytokine response, *in vivo* studies were performed to determine directly whether antigen dose could influence T-cell differentiation. Indeed, in mice inoculated with *L. major* or *T. muris,* relatively small inocula favored a Th1 response, whereas larger inocula favored type 2 cytokine production (234,235). However, both these infectious disease models induce

responses that are finely balanced between type 1 and type 2 cytokine production, so any intervention that favors one or the other response even slightly may tip the balance. No evidence indicates that low doses of *N. brasiliensis,* or any other potent inducer of type 2 immunity, will stimulate a type 1 cytokine response or that high doses of *Listeria monocytogenes,* or any other potent inducer of type 1 immunity, will stimulate a type 2 cytokine response. Furthermore, *in vitro* studies that have used antigen or peptide to stimulate TCR transgenic T cells have yielded more complex results; in one study, both low and high peptide doses induced a predominantly type 2 response, whereas intermediate doses stimulated a predominantly type 1 response (236).

Studies of the relationship between TCR affinity for the MHC class II–peptide complex and T-cell differentiation patterns demonstrate that high-affinity interactions induce type 1 cytokine responses *in vitro* and *in vivo,* whereas lower-affinity interactions induce type 2 responses (237,238 and 239). Altering peptides to raise or lower the affinity of the interaction between MHC class II–peptide and TCR has a consistent effect (237,238,239 and 240). These results at first seem inconsistent with the *in vivo* studies of antigen concentration; both increased affinity and increased antigen concentration should increase the number of MHC class II–peptide-TCR interactions between the APC and T cell and the stability of the APC–T-cell interaction. An alternate explanation, that high antigen concentrations are required to allow "low-affinity" T cells to become activated and produce type 2 cytokines, is discussed later in this chapter.

Costimulation

Th2, but not Th1, responses require ligation of CD4 on the responding T cell. Both the increased interaction strength between T cell and APC that is provided by the CD4–MHC class II interaction and signaling mediated by the cytoplasmic domain of the CD4 molecule appear to contribute to the generation of a type 2 cytokine response (241,242). Costimulation of $CD4^+$ T cells by B7 (CD80/CD86) ligation of CD28 is also a requirement for Th2, but not Th1, differentiation in most cases (216,217 and 218), and increasing the ratio of CD28:TCR signaling may shift responses from Th1 to Th2 (242,243). Mechanisms for the CD28 effect on cytokine production are unknown. Th2 responses to some gastrointestinal nematode parasites are at least partially CD28 independent (244), a finding suggesting that these parasites either activate other host signaling pathways that promote Th2 differentiation or directly signal T cells in a way that promotes the Th2 response.

Once naive T cells have differentiated into effector and memory cells, CD28 costimulation appears to contribute to maintenance of both type 1 and type 2 responses, but it may be more important for maintenance of the type 1 response (245,246). In contrast to CD28 costimulation, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 costimulation, under some circumstances, appears to suppress Th2 cytokine production (247).

Antigen-Presenting Cells

Although antigen-presenting B cells, dendritic cells, and macrophages can induce either a Th1 or a Th2 response, given the appropriate cytokine milieu (248), antigen presentation by B cells appears to favor type 2 cytokine responses, whereas antigen presentation by dendritic cells or macrophages appears to favor type 1 responses (249,250,251,252 and 253). This may reflect greater production of IL-12 and IFN-a/b by dendritic cells and macrophages than by B cells. Alternatively, B cells may produce a molecule (still unknown) that promotes Th2 differentiation.

Site of Immunization

Administration of antigen by an inhaled route is more likely to induce a type 2 cytokine response than intravenous or intraperitoneal administration of the same antigen (254,255). The mechanism for this effect is not known, but it may reflect differences in APC population or cytokine milieu at the different immunization sites.

Attempt at Synthesis

The selection of molecules representative of a particular class of pathogen, such as lipopolysaccharide, as triggers for APC secretion of IL-12 and IFN-a/b, which regulate T-cell type 1–type 2 cytokine production, provides an elegant and direct way to induce naive T cells to produce cytokines that best control a particular infection. It is more difficult, however, to understand the evolutionary logic of allowing antigen concentration, TCR affinity, and costimuli to regulate T-cell production of type 1 or type 2 cytokines. Possibly, the small inoculum that typically initiates a bacterial, viral, or protozoal infection has been selected evolutionarily as a signal to secrete type 1 cytokines, which best defend against most of these pathogens, whereas the large quantity of enzymes and other proteins produced by even a few invasive nematode parasites has been interpreted as a signal for the type 2 cytokine response that generally protects against these parasites. Because only T cells whose TCRs bind peptide-MHC with high affinity are likely to be sufficiently stimulated to differentiate into cytokine-secreting cells when peptide concentration is low, high-affinity binding to the TCR may have been selected as a trigger for a type 1 cytokine response. In contrast, at higher antigen concentration, the density of MHC class II–peptide on APCs should be sufficient to also activate T cells whose TCRs bind MHC class II–peptide with lower affinity; these T cells will be induced to secrete type 2 cytokines. Because more T cells are likely to bind a given MHC class II–peptide complex with low than with high affinity, the predominant cytokine response should shift from type 1 to type 2 as antigen concentration increases and lower-affinity T cells are stimulated. This would be true even if high antigen concentrations enhanced type 1 cytokine production by the relatively small numbers of T cells that bind peptide–MHC class II with high affinity. By the same logic, CD4 and CD28 costimulation may have evolved as a selective requirement for Th2 differentiation because high-affinity MHC–peptide interactions with the TCR do not require costimuli to induce naive T cells to differentiate into effector cells, whereas low-affinity interactions require the additional stimuli to activate naive T cells.

One limitation of this hypothesis is that pathogenic virus, bacteria, and protozoa often proliferate to produce a large antigen load before the immune system is fully activated. This increase in antigen load would stimulate a type 2 cytokine response were it not for the ability of pattern recognition molecules on APCs and NK cells to recognize molecules associated with each of these infectious agents as triggers for the IL-12, IFN-a/b, or IFN-g responses that suppress type 2 immunity and enhance the type 1 response. In contrast, the large antigen loads that are associated with even early nematode infections and the inability of these parasites to activate pattern recognition molecules to induce production of Th1-promoting cytokines (or their production of molecules that actively stimulate Th2 differentiation through an undiscovered pathway) allow predominantly low-affinity T cells, which produce Th2 responses, to be activated without any inhibition by IL-12 or the IFNs. Although this hypothesis ties the different regulators of $CD4^+$ T-cell differentiation together in a way that makes some biologic sense, it is highly speculative and requires experimental verification.

TRANSCRIPTIONAL REGULATION OF TYPE 2 CYTOKINE PRODUCTION

Three excellent reviews of transcriptional regulation of type 1 and type 2 cytokine production have been published (256,257 and 258). Transcriptional regulation of IL-4 production has been particularly well studied because IL-4 is such an important regulator of type 2 cytokine production. Regulatory elements in a promoter immediately 5' of the IL-4 gene and in a distal 3' enhancer of this gene bind the transcription factors GATA-3 and c-Maf, which are selectively expressed in cells that secrete type 2 cytokines, Stat6 (which, in T cells, requires IL-4 for activation), and transcription factors, including NFATc, AP-1, and NIP45, that lack tissue specificity.

The zinc finger protein, GATA-3, is required for the development of T cells and the nervous system and hematopoietic systems, as well as for Th2 differentiation (259). Several studies support its importance as an inducer of type 2 immunity. A dominant negative, inducible mutant form of GATA-3 inhibits Th2 differentiation (260), whereas transgenic overexpression of GATA-3 causes increased production of all type 2 cytokines and stimulates even fully differentiated Th1 cells to produce some type 2 cytokines (259,261). GATA-3 directly transactivates the proximal IL-5 promoter (262,263), but it probably activates IL-4 transcription by binding to a GATA-3 dimer in a 3' enhancer region of the IL-4 gene (264). GATA-3 upregulation of its own transcription may provide a mechanism for stabilizing type 2 cytokine production (261,265). Although IL-4 induces GATA-3 transcription through a Stat6-dependent mechanism, GATA-3 can also be transcribed in the absence of IL-4 and ectopic expression of GATA-3 stimulates IL-4 production even in Stat6- and IL-4Ra–deficient mice (261). Taken together, these observations suggest that GATA-3 is the central, Th2-promoting transcription factor. However, because no system has been devised that completely blocks GATA-3, it is premature to conclude that GATA-3 is an absolute requirement for any type 2 cytokine production.

In addition to stimulating its own transcription, GATA-3 induces transcription of c-Maf, a basic region–leucine zipper factor that binds to a MARE site in the proximal IL-4 promoter (261,266). c-Maf strongly enhances IL-4 production, although c-Maf–deficient T cells can be stimulated to produce some IL-4 (267), and studies have not yet been performed to determine whether strong stimuli of Th2 differentiation can induce Th2 responses in c-Maf–deficient mice *in vivo.* Unlike GATA3, c-Maf has no direct transcription-promoting effect on type 2 cytokines other than IL-4 (267).

Although Stat6 signaling is not required for the generation of a primary type 2 cytokine response, it clearly can enhance the magnitude of the response, perhaps by inducing GATA-3 transcription (261). Because the importance of Stat6 is particularly evident in responses to stimuli that also induce IFN-g production, Stat6 may contribute to type 2 cytokine production by suppressing the inhibitory effects of IL-12 (possibly by decreasing $IL\text{-}12Rb_2$ expression on T cells) and IFN-g. In addition, Stat6 signaling appears to be particularly important for the generation and survival of $CD4^+$ T cells that make rapid type 2 cytokine responses on restimulation with antigen or anti-CD3 antibody (68).

Although members of the nuclear factor of activated T cells (NFAT) family lack tissue specificity, they are important regulators of type 2 cytokine production. The absence of NFATc1 in lymphoid cells results in reduced IL-4 production, whereas the absence of NFATc2 leads to increased type 2 cytokine production and the combined absence of NFATc2 and NFATc3 promotes a particularly strong type 2 cytokine bias (268). Investigators have suggested that all these NFAT family members activate IL-4 gene transcription during the early phase of an IL-4 response, whereas NFATc2 and NFATc3, but not NFATc1, are important in limiting IL-4

gene transcription later in the response (268). Because the NFAT transcription factors bind to the IL-4 promoter and enhancer regions in Th2 cells, but not in naive T cells or Th1 cells, it has also been suggested that Th2-specific factors, such as GATA-3, are required *in vivo* to make these regions NFAT accessible (264). This possibility is consistent with the observation that transfection of T cells with GATA-3 rapidly decreases nucleotide methylation and increases accessibility (as measured by DNAse hypersensitivity) of promoter and enhancer regions of the IL-4/IL-13 locus (264).

Type 2 cytokine gene transcription is negatively, as well as positively, regulated. The IL-12–activated transcription factor, Stat4, suppresses the differentiation of naive $CD4^+$ T cells into Th2 cells (269). The transcription factor Bcl-6, which overlaps with Stat6 in its nucleotide sequence binding specificity, inhibits type 2 responses; increased type 2 cytokine production is found in Bcl-6–deficient mice and even in mice deficient in both Bcl-6 and Stat6 (270). A member of the T box transcription factor family, T-bet, stimulates IFN-g transcription and can suppress type 2 cyotkine production even in fully polarized Th2 cells, although suppression does not involve a direct effect on the proximal IL-4 promoter (271). Thus, both the differentiation of naive T cells into type 2 cytokine-secreting cells and the continuing production of type 2 cytokines by polarized Th2 cells are likely to reflect the balance of several stimulatory and suppressive transcription factors as well as the accessibility of relevant promoter and enhancer regions to these factors, rather than the effect of a single factor.

Although these observations greatly extend understanding of how type 2 cytokine responses are regulated, several important issues require further clarification. Current observations fail to explain independent regulation of the individual type 2 cytokines (if GATA-3 is required for an IL-4 response and directly transactivates the IL-5 gene, why do some stimuli induce IL-4, but not IL-5 transcription?). They also fail to explain why immunogens that induce normal primary type 2 cytokine responses *in vivo* in Stat6 mice fail to induce normal secondary type 2 cytokine responses in these mice. Perhaps Stat6 signaling is required *in vivo* to maintain the viability of differentiated Th2 cells or to maintain GATA-3 transcription when TCR stimulation becomes limiting. A third area that remains mechanistically confusing is regulation of cytokine expression at the level of the individual alleles of a cytokine gene. Only a single IL-4 allele is transcribed in most polarized Th2 cells that have been generated *in vitro* (272). If polarized Th2 cells are rested, then restimulated, they usually again activate a single IL-4 allele; however, the same allele is not necessarily the one activated. Th2 cells derived from a clone in which one allele is preferentially activated preferentially activate the same allele to the same extent after restimulation (273). This finding suggests that certain mechanisms regulate the probability of activation of a particular IL-4 allele in a single cell, but chance has a role in the final event that induces activation (i.e., allele activation is a crap shoot with subtly loaded dice).

TH2-SELECTIVE RECEPTORS

Both naive T-cell differentiation and the function of polarized, differentiated T cells may depend on receptors that are expressed differently by Th1 and Th2 cells. IL-4–secreting cells lose expression of the b_2 chain of the IL-12R and thus lose susceptibility to the inhibitory effects of IL-12 on type 2 cytokine production (69). This loss, however, is neither absolute nor necessary for Th2 stability: Th2 cells can be induced to reexpress IL-12Rb_2 by exposure to IFN-g, and polarized Th2 cells that express a transgenic IL-12R remain insensitive to IL-12 effects on type 2 cytokine production (69). IL-4–secreting cells, unlike Th1 cells, continue to express IFN-gRb chain and thus retain sensitivity to the inhibitory effects of this cytokine on cell growth. IFN-gRb is lost, however, by Th2 cells that survive IFN-g treatment (274,275). Although the IL-18R has been reported to be Th1-specific, observations that IL-18 can enhance IL-4 and IL-13 production make it likely that this specificity is also less than absolute (231,276).

Chemokine receptors are also differentially expressed, with Th2 cells more likely than Th1 cells to express CCR3, CCR4, and CCR8 and less likely to express CXCR3 and CCR5 (277,278). These differences, and the preferential expression of P-selectin ligand-1 by Th1 cells (279,280), suggest that differential chemokine and selectin expression can determine whether predominantly type 1 or type 2 cytokine-secreting cells are attracted to an inflammatory site. Because chemokines can activate, as well as attract, lymphocytes (281), it is likely that chemokines can contribute to the polarization of a cytokine response. In this regard, CCR1 and MCP-1 have both been reported to enhance Th2 responses (282,283 and 284).

Additional molecules that are preferentially expressed by Th2 cells include the IL-1–like molecule T1/ST2, which may contribute to type 2 cytokine production (285,286), and inducible costimulatory molecule, a CD28 family molecule that may be important for the reactivation of Th2 memory (287,288 and 289).

CHALLENGES FOR THE FUTURE

Research during the past 14 years has defined the type 1 and type 2 patterns of T-cell cytokine production and has provided considerable information about how these responses are regulated, how they control infection, and how they contribute to immune pathology. This increased appreciation of the importance of these cytokines has encouraged further studies, which, during the next 20 years, should further elucidate important basic issues and lead to the development of therapeutics that modify type 2 production and effects. Basic research should determine the mechanisms by which allergens and worms selectively induce the type 2 cytokine response; should determine whether the type 2 cytokine response is actively induced or defaults from a lack of cytokines that stimulate the type 1 response, should identify the mast cell–independent mechanisms by which type 2 cytokines protect against gastrointestinal nematode parasites, should identify the distinct contributions of IL-4 and IL-13 to type 2 immunity, should determine the signaling and transcriptional mechanisms by which IL-9 enhances type 2 inflammation and immunity, should identify the mechanisms that create stable Th2 memory and the stimuli that suppress established type 2 responses, and should elucidate the interactions among transcription factors that allow tandem or independent expression of different type 2 cytokines. Translational research should develop cytokine agonists and antagonists that improve control of infectious diseases (particularly through improved vaccines) and treatment of allergic and autoimmune disorders. Most first-generation therapeutics will probably be proteins, including soluble cytokine receptors and anticytokine antibodies that neutralize specific cytokines and anticytokine receptor antibodies and mutant cytokines that block cytokine receptors. Considerable effort will be required to identify possible untoward effects and to establish the safest, most convenient, and most efficacious means of protein delivery. As specific signaling mechanisms become better understood and the precise molecular interactions among cytokines, receptors, and signaling molecules are elucidated, it seems likely that small molecule agonists and antagonists will be produced, along with small molecules that stimulate or suppress the production of specific cytokines. At every stage of development, difficult decisions will have to be made about the safety, efficacy, and cost of type 2 cytokine-related therapeutics relative to alternative therapies for the same diseases. Despite uncertainties, it seems safe to predict that the dramatic advances in type 2 cytokine biology that have occurred during the past 14 years and will continue during the next 20 years will translate into equally dramatic improvements in the treatment of allergy and infection.

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12 APOPTOSIS AND THE IMMUNE RESPONSE

Douglas R. Green, Ph.D

The process of apoptotic cell death affects essentially all cell types in all multicellular animals; therefore, it should not be surprising that it has roles in the immune system. In this case, however, the functions of apoptosis may extend further than in other systems because many apoptotic defects (through spontaneous or designed mutations) appear to impact predominantly on the immune system. I believe there are at least three reasons for this effect:

First, apoptosis plays a major role in the control of cell number, and in most systems, considerable flexibility exists in how tightly cell number can be regulated. In two cases, however, a failure in the apoptotic machinery of a single cell and its progeny can lead to the patient's death: (a) when a cell has transformed and will become a cancer and (b) when a lymphocyte responds to autologous antigen and will cause a devastating autoimmune disease. Because efficient functioning of apoptotic pathways prevents these occurrences, these diseases are rare (considering the many opportunities for these problems to arise in any patient). Second, apoptosis is an important effector mechanism in the immune system. Cytotoxic lymphocytes mediate their effects through the induction of apoptosis in target cells, and many cytokines function to either induce or inhibit apoptosis.

Third, cell death in any cell has an impact on the immune system through the consequences of the clearance of the dead cells. Cells that die via necrosis appear to simulate inflammatory responses, whereas apoptotic cells are rapidly cleared without associated inflammation. As will discussed herein, the phagocytosis of apoptotic cells is not “silent” but impacts on immunologic function through effects on the phagocytes.

This chapter begins with a brief overview of the machinery of apoptosis in vertebrates, and these probably apply to all cell types (not only to cells of the immune system). After the overview is a consideration of many of the ways these mechanisms function in the cells of the immune system.

MACHINERIES OF DEATH

Fate of an Apoptotic Cell

Apoptotic cell death is defined by the morphologic appearance of the dying cell (and this remains the definition, despite our current understanding of the biochemistry of the process). The cell undergoing apoptosis will display some or all of the following features (Fig. 12.1): blebbing, chromatin condensation, nuclear fragmentation, loss of adhesion and rounding (in adherent cells), and cell shrinkage. The DNA cleaves and can take the form of high-molecular-weight fragments or fragmentation into an oligonucleosomal “ladder” (Fig. 12.1). It is important to note that ladder formation or its absence *per se* is not proof that apoptosis has or has not occurred; *bona fide* apoptosis can occur in the absence of a DNA ladder, and laddering can occur in necrotic cells (1). The *in situ* DNA nick and labeling (TUNEL) assay, which detects double-strand DNA breaks in cells, also should be evaluated cautiously.

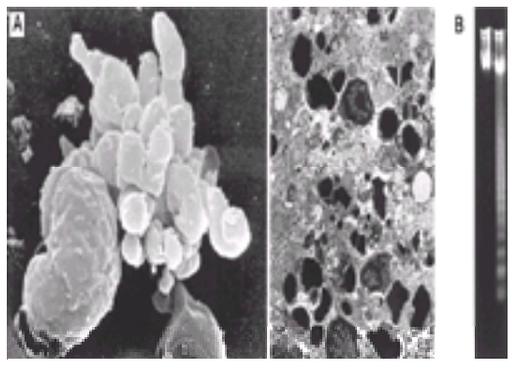

Figure 12.1. Apoptotic changes. T cells undergoing apoptosis *in vitro* and in the thymus after activation. **A:** Blebbing **(left)** and nuclear condensation **(right)**. **B:** Oligonucleosomal DNA fragmentation in apoptotic T cells is apparent in the **right lane** (**left lane**, DNA from untreated, viable cells).

Arguably, the most important change in an apoptotic cell is the change that leads to its phagocytosis. If a cell is eaten, it is essentially already dead, whether or not any other changes associated with apoptosis occur. During apoptosis, cells alter their plasma membrane so that they are rapidly taken up by both “professional” (macrophages, dendritic cells) and “nonprofessional” (e.g., epithelial cells) phagocytes. Although several receptors on phagocytes have been implicated in this clearance process (2,3,4 and 5), only one change in the apoptotic cell has been identified (although we know there are others): the externalization of phosphatidylserine (PS). Normally, PS is sequestered to the inner leaflet of the plasma membrane, and during apoptosis it equilibrates to both inner and outer leaflets. This can be most easily detected by the use of annexin V-FITC, which specifically binds PS (6), but care must be taken in interpreting results because any disruption of the plasma membrane will expose PS. A receptor for PS on phagocytes recently was identified as a major player in the recognition of apoptotic cells (7).

Executioner Caspases and Their Substrates: The Orchestration of Death

In most cases, the apoptotic phenomena described above are a result of the cleavage of specific substrates by caspases, a collection of cysteine proteinases that become active during apoptosis. Several different caspases are constitutively present in cells, and these reside in the cytosol in an inactive form (8). The most prevalent of these, caspase-3, is ultimately responsible for most of the effects, although it is supported by two others, caspases-6 and -7. These three caspases are often referred to as the *executioner caspases* to indicate their role in coordinating the death of the cell.

When the executioner caspases become active during apoptosis, however, this does not in itself kill the cell (i.e., these are not digestive enzymes). It is the cleavage of specific substrates that results in the changes we see as apoptosis.

For example, high- and low-molecular-weight DNA fragmentation is caused by the action of caspase-3 on a complex of a nuclease (CAD/DFF40) and its inhibitor (iCAD/DFF45) (9,10). Caspase-3 cleaves the inhibitor, allowing the nuclease to cut the chromatin (it appears that the amount of activated nuclease determines whether DNA fragmentation will proceed to a ladder) (Fig. 12.2). Following DNA cleavage, the chromatin condenses, but this change is due largely to another caspase substrate, called acinus (11).

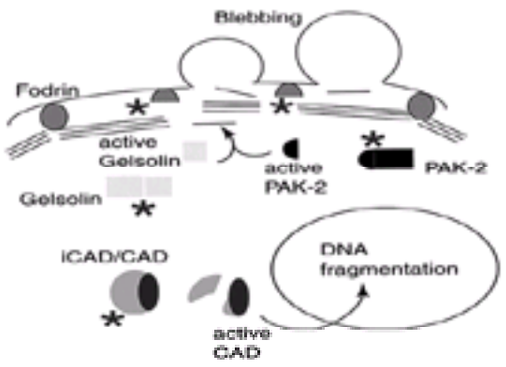

Figure 12.2. Caspase substrates cause apoptotic changes. Caspase cleavage (*) of molecules in the cell triggers the changes seen as apoptosis. CAD nuclease is activated by caspase cleavage of its chaperone inhibitor, iCAD; gelsolin and PAK-2 are activated by caspase cleavage to remove the regulatory domains of these enzymes; fodrin activity is destroyed by caspase cleavage. Many other substrates are known, and several have probable functions in apoptosis.

Blebbing is orchestrated via the cleavage of gelsolin (12) and p21-activated kinase-2 (13,14) to activate these enzymes and probably through cleavage of fodrin (15) to dissociate the plasma membrane from the cytoskeleton. The result is probably an effect of microfilament tension and local release because depolymerization of actin prevents blebbing (16). Interestingly, in some cells undergoing apoptosis, blebbing appears to occur independently of caspase activity (17), but in others, it is caspase dependent.

The externalization of PS during apoptosis is usually caspase dependent (18), although the precise mechanisms have not been elucidated. In some cells, PS externalization appears to be caspase independent (19), although, again, the mechanism is unknown. PS externalization in platelets does not involve caspases (20).

Many other caspase substrates are known in the cell, although their functions are often unclear or unproved. Similarly, many of the effects shown in Fig. 12.1 are caspase dependent (i.e., caspase inhibitors block them), but the mechanisms have not been elucidated.

Cruel Cuts: Activating the Caspases

In general, caspases are activated when the single-chain proform is cleaved to remove the prodomain and separate the large and small subunits (Fig. 12.3). The active caspase is a tetramer of two large and two small subunits, with two active sites.

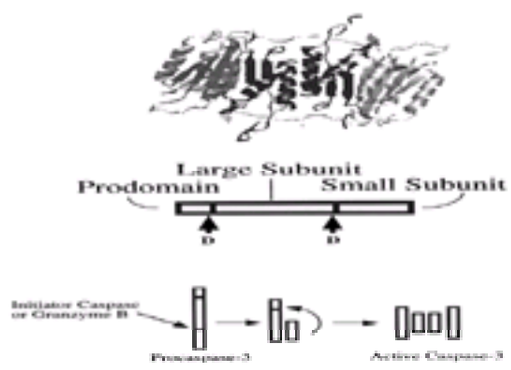

Figure 12.3. Cleavage of caspases activates them. **A:** Structure of mature caspase-3. **B:** Organization of the procaspase-3 polypeptide. *Large arrows* indicate aspartic acid cleavage sites for generation of the mature, active enzyme. **C:** Activation of procaspase-3 by cleavage.

With one interesting exception, the best enzymes for converting pro- to active caspases are other caspases. The exception is a serine protease, granzyme B that plays a major role in the killing of target cells by cytotoxic lymphocytes (discussed in more detail in the section entitled Apoptosis in the Immune System). Granzyme B can directly cleave and activate caspases, including caspase-3 (21,22), and thereby has the potential to bypass most of the regulatory processes discussed herein.

The ability of caspases to activate other caspases leads to the idea of a caspase cascade, with other caspases first becoming active and in turn activating executioner caspases (which can activate all remaining caspases as well as cleaving key substrates). These “upstream” caspases sometimes are referred to as *initiator caspases* to indicate their roles in trigger apoptosis by activating the executioners. The discussions here focus on two of the initiator caspases, caspase-8 and caspase-9, which have quite different functions in triggering apoptosis. Both of these caspases, however, have in common an excellent ability to cleave and activate executioner caspases. So the problem becomes, “how are the initiator caspases activated in the first place?”

One clue to the difference between initiator and executioner caspases is the nature of the prodomains. The executioner caspases have extremely short prodomains, whereas those of the initiators are longer. The latter contain protein–protein interaction motifs that give us further insights into how they function. These interaction motifs bind to adapter molecules that permit the autoactivation of the initiator caspases. For the two initiator caspases discussed here, this happens in different ways. Caspase-8 interacts with its adapter molecule via a motif called the death effector domain (DED); caspase-9 interacts with a different adapter molecule via a caspase recruitment domain (CARD) (23,24). Interestingly, however, the protein interaction motifs in these two caspases, although unrelated at the level of primary sequence, are structurally similar (Fig. 12.4), a similarity they also share with another domain, the death domain (DD), the function of which is discussed later (DDs are not present in caspase prodomains).

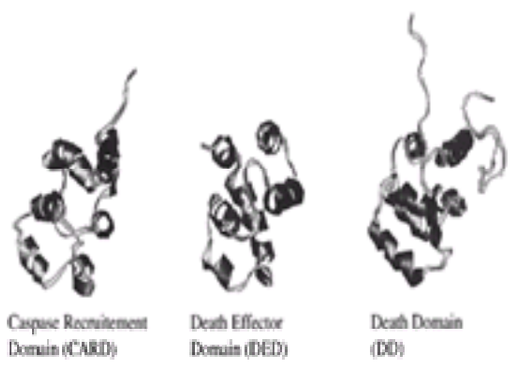

Figure 12.4. Protein–protein interaction domains with major roles in apoptosis.

In addition to caspases, cells also contain natural inhibitors of the caspases. These inhibitors of apoptosis proteins (IAPs) were first identified in baculovirus but subsequently were found in human cells (25). IAPs can inhibit caspases directly and also may promote their degradation via ubiquitination. Not all IAP molecules function specifically in apoptosis, but it is likely that at least some of them play an integral role in the process.

Here we consider two general types of signaling pathways leading from a triggering event to activation of an initiator caspase (and from there to apoptotic death). The

first involves specific binding of certain cytokines to a class of cytokine receptors. The second depends on the participation of mitochondria.

Death Ligands, Death Receptors

The tumor necrosis factor (TNF) receptor family includes molecules that provide essential signals for inflammation, lymphocyte activation, and the genesis of secondary lymphoid organs (26,27 and 28). A subset of these receptors provides another function: the induction of apoptosis. These “death receptors” include TNFR1, Fas (CD95), DR3/WSL, and the TRAIL/Apo-2L receptors (TRAIL-R1/DR4, TRAIL-R2/DR5). Other members of this family also trigger apoptosis, although the mechanisms are less well understood.

When any of these death receptors are bound by their ligands (TNF or lymphotoxin, Fas-ligand (FasL), the ligand for DR3[1] or TRAIL/Apo-2L, respectively) apoptosis can occur as a consequence. The Fas-FasL interaction is perhaps the simplest scenario to understand the process. Ligation of Fas (by FasL or stimulatory antibodies) causes the rapid formation of an intracellular signaling complex. The intracellular region of Fas (and all the other death receptors) contains a death domain (DD) (Fig. 12.4). Ligation of Fas causes recruitment to the DD of another protein, FADD (28), which also contains a DD, and these bind via a DD–DD interaction. FADD also contains a DED, and this in turn recruits procaspase-8, which binds via its DED (Fig. 12.5).

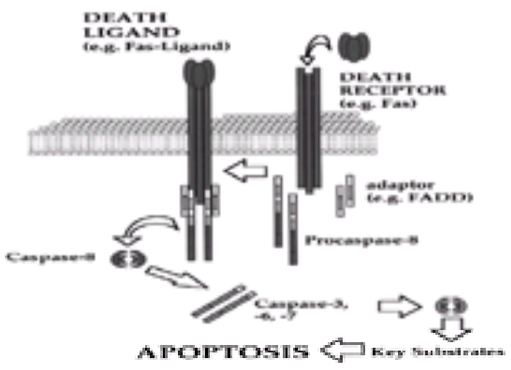

Figure 12.5. Signaling from a death receptor. The activation of caspase-8 by ligation of Fas is illustrated. Ligated Fas recruits FADD to the intracellular region, which in turn recruits procaspase-8. The caspase transactivates and the mature caspase now can cleave and activate procaspase-3, leading to apoptosis.

Bringing two or more procaspase-8 molecules together is sufficient to activate them because the procaspase-8 molecules have a low level of activity, and in close proximity, two procaspase-8 molecules can process each other to the mature, active forms. The active caspase-8 now triggers the apoptotic caspase cascade. DR3 also binds FADD and requires this adapter for induction of apoptosis (30,31). It is likely that DR3 signals similarly to Fas. In contrast, TRAIL receptor signaling for apoptosis can occur in the absence of FADD (31), although the receptors do appear to bind this adapter (32).

Signaling from TNFR1 is made more complicated by the need for another adapter protein, TRADD. TRADD binds ligated TNFR1 via DD–DD interaction, and then TRADD recruits FADD. FADD now recruits caspase-8, leading to its activation.

Ligation of a death receptor does not necessarily lead to caspase-8 activation and death. TNFR1 also can activate NF-kB. In cells that are resistant to TNFR1-mediated apoptosis, inhibition of this transcription factor can sensitize the cells to this form of death (33). One way in which NF-kB works is to induce expression of two TNFR1-binding proteins (TRAF-1 and -2) and an IAP (c-IAP), which is recruited to the receptor by the other proteins and inhibits the apoptotic signaling (34). Most likely, several other antiapoptotic functions of NF-kB exist.

Interestingly, NF-kB has no effect on Fas-mediated apoptosis (35), which is certainly regulated as well, however, because freshly activated T cells are resistant to this form of apoptosis despite expressing Fas and the necesssary signaling molecules (36). A molecule with a number of different names, often referred to *as c-FLIP* (37), can block Fas-mediated apoptosis. c-FLIP resembles procaspse-8, but without the active proteinase site, and can be recruited to the ligated Fas-signaling complex. Interleukin-2 (IL-2) can downregulate c-FLIP expression (38), which might explain why IL-2 can sensitize activated T cells to Fas over time. Other studies, however, have shown that c-FLIP is not recruited to the signaling complex in resistant T cells (36), and therefore other regulators may be important.

Mitochondria and the Functions of the Bcl-2 Family

Bcl-2 is among the best known antiapoptotic proteins (39), and its discovery as an oncogene in follicular lymphomas has helped to shape our thinking about the roles of apoptosis in preventing oncogenesis. It is one of a family of proteins that include antiapoptotic (Bcl-2, Bcl-xL) and pro-apoptotic (Bax, Bak) molecules. In addition, numerous proteins that share limited homology but probably similar structure are also in this family (e.g., Bid, Bad, Bim); these are called *BH3-only* proteins because of four Bcl-2 homology regions, and these share only the third.

Bcl-2 and Bcl-xL reside on the mitochondrial outer membrane, and the proapoptotic family members are either cytosolic or present on the mitochondrial membrane as well. Although they also are found elsewhere (endoplasmic reticulum, nuclear envelope), their major effect appears to be on the mitochondria.

A stress-induced apoptotic pathway, which also can function in response to other inducing signals, triggers apoptosis by the induction of oligomerization of cytosolic Bax or Bak and localization to the mitochondrial outer membrane (40,41). Although it is not known precisely how this occurs in all forms of apoptosis, one factor that can cause Bax oligomerization is a sudden increase in cytosolic pH (42), which was seen in the case of IL-7 withdrawal.

The translocation of Bax or Bak then can induce a dramatic event in the mitochondria. These organelles then release the proteins in the space between their inner and outer membranes, including one key protein: cytochrome c. Cytochrome c is encoded by a nuclear gene, but when it is imported into the mitochondria, it is coupled with a heme group to become holocytochrome c, and it is only this form that functions to induce caspase activation (43).

The antiapoptotic proteins Bcl-2 and Bcl-xL work to prevent this cytochrome c release from mitochondria and thereby preserve cell survival (43,44). Studies using cell-free systems provide strong support for the idea that this is the major site of action of these proteins in preventing apoptosis.

When cells release cytochrome c, they do so suddenly and completely and apparently from all the mitochondria in the cell (this has been established for only one cell line, but it may be generally true) (45). Following cytochrome c release, caspases are activated and the cells undergo apoptosis.

Apoptosis occurs through the formation of an “apoptosome,” which is dependent on both cytochrome c from the mitochondria and either adenosine triphosphate (ATP) or dATP in the cell Fig. 12.6). Cytosol contains a protein called apoptotic protease activating factor-1 (APAF-1) (46,47), and cytochrome c binds and induces it to oligomerize. This, then, recruits an initiator caspase: procaspase-9 (47,48). Unlike other caspases, procaspase-9 does not appear to be activated simply by cleavage but instead must be bound to APAAF-1 to be active (49,50). These proteins interact via CARD–CARD interactions. The apoptosome now recruits procaspase-3, which is cleaved and activated by the active caspase-9 and released to mediate apoptosis.

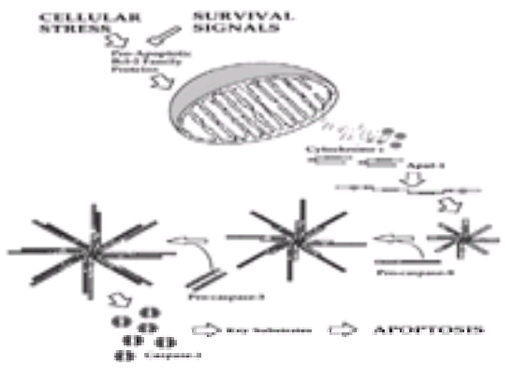

Figure 12.6. Induction of apoptosis via the mitochondrial pathway. Cellular stress induces proapoptotic Bcl-2 family members to translocate to from the cytosol to the mitochondria, where they induce the release of cytochrome c. Cytochrome c catalyzes the oligomerization of APAF-1, which recruits and promotes the activation of procaspase-9. This in turn activates procaspase-3, leading to apoptosis.

Death Receptors and Mitochondria: A Bid Farewell

The mitochondrial pathway involving Bcl-2 family members, mitochondria, cytochrome c, Apaf-1, and caspase-9 is fundamentally distinct from that of the death receptors. Cells lacking caspase-8 (51) or FADD (31) do not respond to death ligands but undergo apoptosis induced by other agents, such as cellular stress; cells that lack caspase-9 (52,53) or APAF-1 (54,55) do not undergo apoptosis induced by such stressors but readily die in response to death ligands.

Controversy arises, however, when we consider the antiapoptotic effects of Bcl-2 on signaling by Fas. In some cells, Bcl-2 can interfere with Fas-mediated apoptosis, whereas in others it cannot (56). [This remains controversial; some investigators contend that Bcl-2 family members never interfere with Fas-mediated apoptosis (57)]. If Bcl-2 sometimes interferes with death mediated through Fas, is this through a new function of the antiapoptotic protein or a role for mitochondria?

Evidence suggests the latter. One of the BH3-only proteins, Bid, is cleaved by active caspase-8 (activated by ligation of the death receptors), and this then translocates to the mitochondria, where it triggers cytochrome c release (58,59 and 60), probably through interaction with Bax (61,62) or Bak. Bcl-2 can prevent this Bid-mediated effect. In cells, expression of Bid can sensitize for TNF-induced apoptosis (58). *In vitro*, the ability of caspase-8 to activate caspase-3 is greatly facilitated by the presence of Bid and mitochondria (63). These observations suggest that when caspase-8 is limiting, the action of Bid on mitochondria can determine whether a cell will undergo apoptosis (via cytochrome c release and activation of caspase-9). In support of this, Bid-deficient mice are relatively resistant to the lethal effects of anti-Fas antibody *in vivo*, and their hepatocytes do not readily undergo Fas-mediated death (64). In contrast, lymphoid cells from these mice are fully sensitive to death via Fas ligation.

I have touched on only a few of the mechanisms at work in the apoptotic process in this brief overview to begin to create perspective on how this form of death impacts on the function of the immune system, which is the next topic.

APOPTOSIS IN THE IMMUNE SYSTEM

The basic mechanisms for the process of apoptosis apply to nearly all cell types, and the cells of the immune system are not an exception. Apoptosis plays roles in effector mechanisms of target cell destruction, in controlling immune cell numbers, and in the effects of the phagocytosis of apoptotic cells on immune function. In each case, I address only those aspects of each that relate directly to apoptosis. The reader should keep this in mind because apoptosis is not the only mechanism of relevance to these phenomena and processes.

Cytotoxic Effector Mechanisms

Cytotoxic lymphocytes kill their target cells, such as infected or transformed cells, and in many cases, this is via induction of apoptosis. There are two major mechanisms for this cell death: granule-mediated and death ligand-mediated cytotoxicity, both of which work by caspase activation involving all of the major pathways discussed in the last section.

Activated cytotoxic lymphocytes (cytotoxic T cells, natural killer cells) often contain granules that are released on contact with the specific target cell. These granules contain two key mediators of cytotoxicity: perforin and a collection of serine proteases called *granzymes*. Perforin is essential for granule-mediated cytotoxic killing (65), and by itself it can induce necrotic cell death (but only at relatively high concentrations). To induce apoptosis, perforin requires additional mediators, and this is the function of the granzymes (66,67). Perforin forms pores in the target cell membrane, which led to the idea that the mediators of apoptosis pass through these pores into the cytotoxic cell. This is an oversimplified view, however.

When a cytotoxic cell releases the contents of its granules, the granzymes enter the target cell via endocytosis (this has been shown only for one key granzyme, granzyme B). The internalized granzyme is inactive until perforin also acts on the target, causing the granzyme to be released into the cytosol (68,69). A model for this sequence of events is shown in Fig. 12.7.

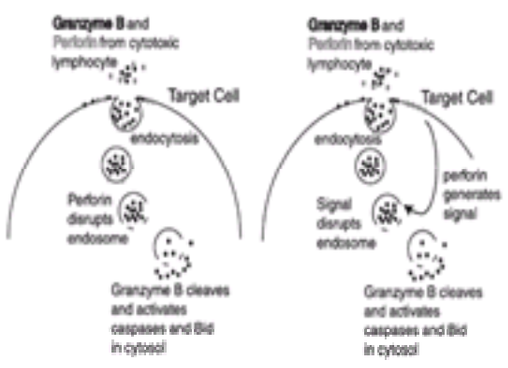

Figure 12.7. Perforin plus granzyme B induce apoptosis on engagement of target cells by cytotoxic lymphocytes. Only the target cell is shown following the release of the contents of a cytotoxic granule at the point of contact. Two possible modes of action of perforin on the target cell are shown. **A:** Perforin directly causes dissolution of the endosome containing granzyme B, either by incorporating as the endosome forms or through fusion with an endosome containing perforin. **B:** Perforin generates a signal at the plasma membrane that causes dissolution of the endosomes to release granzyme B to the cytosol. The nature of such a signal is unknown.

Once the granzymes enter the cytosol, they act to trigger apoptosis. Several granzymes are capable of doing this, but the most rapid and important one is granzyme B. As discussed in the section on basic mechanisms, granzyme B is capable of directly processing and activating several of the caspases, including caspase 3 (21,22). Granzyme B also can process and activate Bid, leading to cytochrome c release from the mitochondria (70). This appears to be important under conditions in which the amount of granzyme B introduced into the cell is low, and the involvement of Bid can amplify the process. Other granzymes also can induce apoptosis, albeit more slowly (67), but the precise mechanisms are not fully elucidated.

Cytotoxic lymphocytes also kill their targets via the expression and action of death ligands. TNF, lymphotoxin, and FasL all can be expressed by cytotoxic lymphocytes and often play roles in the death process via the ligation of their receptors on the targets and the ensuing apoptotic process.

Both granule-mediated and FasL-mediated cytotoxic mechanisms have been implicated in cytoxicity, and in some cases it has been proposed that these mechanisms have distinct functions. For example, both mechanisms have been implicated in graft-versus-host disease (71,72 and 73), but it has been suggested in one model that graft-versus-leukemia was mediated predominantly by granules, whereas FasL contributed to graft-versus-host disease (73).

Control of Cell Numbers

The elimination of selected clones of antigen-specific lymphocytes is at the core of modern immunology, embodied in the concept of clonal deletion. This occurs, at least in part, through an apoptotic process; but the role of apoptosis in the control of cell number extends beyond this principle to phenomena that include cell longevity, the response to cell damage, hormonal effects, immunologic privilege, and others.

GROWTH FACTOR DEPRIVATION

Growth factors maintain the survival of both myeloid and lymphoid cells, and this is through inhibition of apoptosis. For example, targeted disruption of the IL-7 gene

causes a loss of thymocytes, which can be prevented by transgenic expression of Bcl-2 (74,75). The ability of growth factors to maintain cell survival is due to both transcription-dependent (e.g., expression of anti-apoptotic Bcl-2 family members) and -independent events (e.g., activation of Akt/PKB). For example, neutrophils undergo spontaneous apoptosis in culture, which can be prevented by granulocyte macrophage–colony-stimulating factor (GM-CSF); this effect can be seen even by using anucleate neutrophil cytoplasts (76). Most growth factors are also survival factors, although there are exceptions (in both directions: growth without survival and survival without growth). Arguably, deprivation of growth factors may be the major inducer of apoptosis in the body (77).

ACTIVATION-INDUCED CELL DEATH

Another critical mechanism for the induction of apoptosis in the immune system is activation-induced cell death (AICD), especially via antigen receptors (78). This permits the elimination of lymphocytes capable of recognizing a specific antigen. During B- and T-cell development, AICD results in negative selection. Interestingly, although we have long suspected that negative selection of self-reactive thymocytes proceeds via activation-induced apoptosis (79,80 and 81), the precise mechanism responsible for this effect in thymocytes remains unknown. The mechanism for AICD in immature or mature B cells is similarly largely unknown.

Mature lymphocytes also can undergo AICD. In T cells, this happens if the cells are previously activated or transformed before the ligation of the antigen receptor. Under these conditions, activation does two things: It sensitizes the cells to Fas-mediated apoptosis and induces expression of the FasL (82,83,84 and 85). The FasL engages Fas, and the cells die via the Fas-mediated apoptotic pathway. In the absence of Fas or FasL, AICD can be mediated by TNF, acting through either the TNFR1 (and the death pathway discussed earlier) or via TNFR2 (which engages the apoptotic process in a manner that has not been fully elucidated) (86). AICD is probably important in the process of peripheral deletion, wherein activated T cells expand in number and then undergo depletion via apoptosis *in vivo* (87). Animals lacking Fas or FasL display defects in peripheral deletion (88,89), although TNF signaling also can be involved (90). IL-2 is thought to be important for the priming of T cells for AICD (91), and animals lacking IL-2 or IL-2 receptors can show defects in peripheral deletion (92,93) [although this is not universally the case (94)].

Effective transplantation tolerance for allogeneic grafts also appears to require a process of AICD. Pharmacologic treatments that promote AICD in host lymphocytes promote such tolerance, while those that interfere with it can prevent it (95,96). Compellingly, expression of antiapoptotic Bcl-2 proteins in the lymphocytes can interfere with the generation of long-term tolerance, strongly implicating apoptosis in the process (96). It therefore appears that elimination by apoptosis, and not simply via anergy, is required for the manifestation of this “holy grail” of transplantation biology.

IMMUNOLOGIC PRIVILEGE AND FASL

The phenomenon of immunologic privilege has been recognized for more than a century, and in the last few years we have gained some insights into how it is maintained. Sites in the body that appear to preclude cell-mediated immune responses are “privileged;” and this effect is mediated in part by the expression of FasL in the tissue. For example, when an immune response is induced by the injection of virus into the anterior chamber of the eye, infiltrating cells die by apoptosis (97). This is dependent on the expression of functional Fas on the infiltrating cells and functional FasL in the tissues of the eye (97,98). Under normal circumstances (i.e., privilege), there are no immune consequences in the eye, but in Fas- or FasL-defective animals, the failure of the cells to undergo apoptosis results in a massive, destructive inflammatory response (97).

Similarly, a role for FasL has been proposed for acceptance of allogeneic grafts from privileged tissues. Transplanted allogeneic testes were observed to be accepted if functional FasL was present (99), but this result is controversial (100). Similarly, murine allogeneic corneal grafts were accepted at a high rate (as is the case with humans) unless there were defects in FasL (graft) or Fas (recipient) (101,102 and 103). Human cornea expresses functional FasL (101), which might be relevant for the most common form of tissue transplant performed today.

Some tissues that normally do not constitutively express FasL will do so during potent immune responses, which may represent a form of “inducible immune privilege” (Fig. 12.8). Examples include the liver and small intestine, which express functional FasL only if activated T cells are present (104). This effect is at least partially responsible for peripheral deletion *in vivo* because FasL on peripheral tissues was found to be important in this phenomenon (104). It is likely that inducible privilege plays a role in recovery from autoimmune attack; autoimmune T cells that trigger transient encephelomyelitis in wild-type mice can induce persistent, lethal disease in animals that lack functional FasL (105,106). It is not unlikely that the inducible expression of FasL in cells of the nervous system (107) trigger apoptosis in the infiltrating lymphocytes and thus bring about remission.

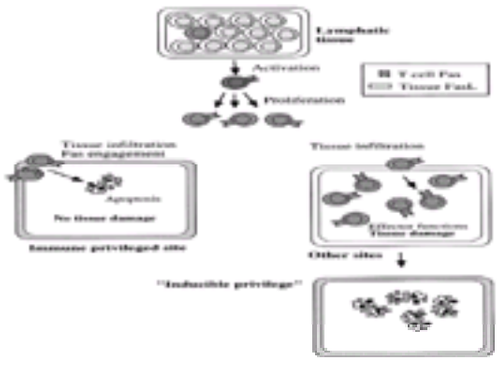

Figure 12.8. Constitutive and inducible immune privilege. Sites such as the anterior chamber of the eye have constitutive FasL and display privilege because any infiltrating cell undergoes Fas-mediated apoptosis. Other sites express FasL only if there is a strong immune response and therefore may have “inducible privilege,” limiting the numbers of activated lymphocytes and thus restricting the amount of damage the tissue sustains.

Similarly, ultraviolet (UV) irradiation of skin induces FasL expression in keratinocytes, and this causes deletion of activated lymphocytes entering the site (108). The immunosuppressive effect of UV was thus shown to be dependent on this FasL expression.

Our understanding of the role of FasL in immunologic privilege is confounded by the observation that FasL can also induce a potent granulocytosis *in vivo*. Whereas some studies have shown that FasL expression by tumors can inhibit immune responses [the “Fas counterattack” (109)], others found that enforced expression of FasL induced eradication of the tumor by neutrophils (110). Similarly, transgenic expression of FasL in pancreatic islet cells, rather than inhibiting diabetes, triggered islet cell destruction by neutrophils (100). Clearly, additional signals are required for FasL to provide tissue protection. One candidate for this second signal is transforming growth factor-b (TGF-b), which blocks the FasL-induced granulocytosis (110).

OTHER INDUCERS OF APOPTOSIS

In many cell types, DNA damage causes cell-cycle arrest and DNA repair, but in lymphocytes, the result is usually apoptosis. This is presumably an adaptation to limit mutations because, as we noted, the progeny of a single autoimmune lymphocyte can cause catastrophic damage. It also can initiate apoptosis during development, when the first antigen receptor chain (in B or T cells) rearranges, and the death process then halts on surface expression of the immature receptor and generation of survival signals (of course, this is conjecture).

The way in which DNA damage triggers apoptosis is different in different cells. Immature thymocytes require p53 to undergo this form of apoptosis (111,112), whereas mature T-cell blasts do not (113). In the latter, at least some of the death may be caused by stress-induced expression of FasL (114). It is most probable, however, that other pathways also participate. In thymocytes, DNA damage-induced apoptosis is blocked by Bcl-2 (115), and thus the mitochondrial pathway is likely to be involved. The ability of DNA damage to trigger apoptosis in cells of the immune system is important clinically (e.g., in patients undergoing radiotherapy or chemotherapy) and may explain the potent antiinflammatory effects of methotrexate, which is a potent inducer of apoptosis (116).

Cell numbers in the immune system also are controlled by hormones. One of the best-known examples is the apoptotic effect of glucocorticoids on immature T cells. Although this was one of the first models for the study of apoptosis (117), we still do not know precisely how it comes about. Bcl-2 can block this form of death, and therefore it may be mediated via the mitochondrial pathway discussed in the last part. This has not been formally demonstrated, however. Nevertheless, glucocorticoid-induced apoptosis is clearly important in a variety of scenarios, including lymphoid development, the response to stress, and in immunomodulation.

The signals triggered by glucocorticoids and those triggered by ligation of the antigen receptor leading to apoptosis in thymocytes are mutually antagonistic; if both are provided at the same time, they can cancel each other out and the cell survives (118). This idea has been integrated into a hypothesis to explain how similar signals

(engagement of the antigen receptor) can mediate cell death through negative selection and cell survival through positive selection (when the affinity for the receptor is lower than in the first case); in the latter, cell death may be countered by glucocorticoid signals. Cells that receive a strong antigen receptor signal die by AICD (negative selection), and cells that receive no antigen receptor signal die of glucocorticoid-induced apoptosis ("neglect") in this model (Fig. 12.9). Cells that receive an antigen receptor signal that is neither too weak nor too strong survive and mature (positive selection). Recent evidence has provided support for this model (119,120).

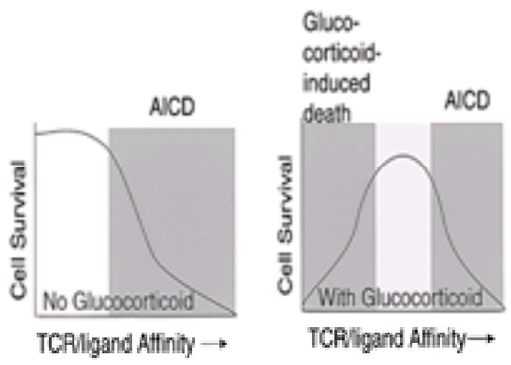

Figure 12.9. A model for positive and negative selection based on the dual inhibitory effects of the signaling pathways initiated by glucocorticoids and T-cell receptor ligation. This is based on the experiments and ideas of Ashwell and colleagues (118,119,120,121,122,123,124,125,126,127,128,129 and 130).

Impact of Apoptotic Cells on the Immune System

In addition to the various roles of apoptosis in the life and death of cells of the immune system, apoptosis has another role: Apoptotic cells are taken up by phagocytes and alter their functions.

Unlike apoptosis, necrosis induces an inflammatory response, although it remains unknown what components of a necrotic cell do this. In addition, the uptake of necrotic cells by dendritic cells can activate them to express costimulatory molecules (121). Cells that undergo secondary necrosis following apoptosis (this occurs *in vitro* when the apoptotic cells are not phagocytosed) do not stimulate dendritic cells in this way (121). Apoptosis, in contrast, was long considered "silent" with respect to inflammation and immunity. This view has undergone a number of modifications in recent years.

The uptake of apoptotic cells by macrophages or dendritic cells has a number of consequences. First, it can stimulate the production of inhibitory cytokines, such as TGFb, and prevent the production of proinflammatory cytokines, such as TNF (122,123). This has obvious benefits in situations where apoptosis occurs physiologically, but it also can have pathologic consequences. For example, trypanosomes trigger T-cell apoptosis through an unknown mechanism, and uptake of the apoptotic cells dramatically inhibits the antiparasite response by macrophages, such that the infection escalates (124). This effect could be mimicked simply by administering apoptotic cells *in vitro* or *in vivo*; the parasite gained a tremendous growth advantage through the inhibition of macrophage and other inflammatory effector mechanisms.

This antiinflammatory effect is mediated at least in part through one of the receptors for apoptotic cells on the phagocyte. Several receptors have been identified on phagocytes that play roles in the uptake of apoptotic cells: the vitronectin receptor (2), CD36 (3,4), CD14 (5), and a recently identified receptor for PS (7). The last of these is interesting for two reasons: First, many apoptotic cells externalize PS early in the apoptotic process (see section entitled The Machineries of Death); second, ligation of this receptor generates a signal that induces expression of inhibitory cytokines and inhibits production of inflammatory ones (7). Regarding the other receptors, little is known about what they recognize on apoptotic cells or how their binding might affect cellular processes.

Another effect of the uptake of apoptotic cells is the phenomenon of cross-priming. Dendritic cells can present antigens they take up via the class I major histocompatibility complex (MHC) pathway, which normally is reserved for cytosolic proteins (125). This process appears to be engaged when the antigen is taken up on or with apoptotic cells (126,127), suggesting that the phagocytic process for uptake of apoptotic cells may be different from that of other materials. Alternatively, something about the apoptotic cell may disrupt the endosome and permit access to the cytosol. Whether active caspases or other effector molecules (e.g., reactive oxygen) in the apoptotic cell are necessary for this effect is currently unknown but, if so, could provide keys to the process. The antigenic peptides present in the class I MHC molecules of dendritic cells that have engulfed apoptotic cells are now prepared to present the antigen to CD8 cells for the generation of cytotoxic effector cells. This would have obvious value in the induction of immune responses to cells infected with intracellular parasites that induce apoptosis or under conditions where innate responses trigger apoptosis in the infected cells; dendritic cells that take up the dying infected cell would now present the parasite antigens to CD8 cells.

The preceding scenario makes sense in the setting of infection but perhaps not for other situations in which apoptosis occurs, such as in normal cellular turnover. It has been suggested, though, that in such a case, the presentation of antigenic peptides (from normal self-components) would function to maintain tolerance because, in the absence of infection, there would not be costimulatory signals, such as those induced by activated CD4 cells (128).

This idea, that tolerance is maintained by cross-priming of self-antigens through dendritic cell uptake of apoptotic cells has appealing features and is consistent with some experimental evidence. For example, introduction of antigen-coupled cells into the anterior chamber of the eye induces a state of tolerance for cell-mediated immune responses (anterior chamber-associated immune deviation, ACAID), but this depends on the induction of apoptosis in the antigen-coupled cells (98), which can occur through the effects of the FasL at the site or artificially by induction of apoptosis in the cells before administration. Injection of apoptotic, antigen-coupled cells that have been phagocytosed by splenic adherent cells also induced tolerance, suggesting that the phenomenon is related to those we have been discussing. The effect also depends on the apoptotic cells expressing functional IL-10, which they do as they die, and this influences the cell that engulfs them to deviate the responses to T-helper cell (Th2) type (129). Other cells, such as keratinocytes (130), also produce IL-10 under conditions of stress that lead to apoptosis, although how this influences the immunologic effects of their engulfment is not known.

CONCLUSIONS

Apoptosis clearly has a range of roles and effects in the immune system, some of which have been discussed here. Because the effects are often dramatic (cells live or die), it is tempting to think they are the predominant ways in which the system is controlled. In some cases, I think this is true, but any system as complex as the immune system will employ many overlapping mechanisms to ensure proper function, and the control of cells by induction or prevention of apoptosis is only one of them. It is likely that we need necrotic cell death to initiate immune responses in many cases, and we need apoptotic cell death to control them. Cell death can be a healthy thing.

[1] A ligand capable of binding DR3 has been described (29) but induces apoptosis poorly. Nevertheless, DR3 can function as a death receptor; therefore, another ligand may exist.

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13 ANTIBODIES AND THEIR RECEPTORS

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ANTIBODIES

The first experiments that proved the physical existence of antibodies were performed in 1890 by von Behring and Kitasato (1), who showed that serum obtained from rabbits immunized with tetanus or diphtheria toxins could prevent disease in mice infected with such pathogens. The unknown substance present in serum that provided protection on transfer was salt-precipitated a year later by Tizzoni and Cattani and named *antitoxin* (Italian *antitossin*).

These initial observations on passive immunity soon were generalized by the work of Paul Ehrlich with plant toxins and by Jules Bordet, who demonstrated that a protective response could be generated even against whole cells (*erythrocytes*). The more inclusive term *antibody* (German *Antikörper*) thereby replaced antitoxin.

In an attempt to explain the formation of antibodies, Paul Ehrlich advanced a comprehensive theory (2), which postulated that antigens induced cell damage by binding to preexisting chemical “side chains” or receptors (antibodies) at the surface of host cells. This binding would result in a selective cell-surface depletion of those side chains specific for the antigen. To compensate for their loss, the cell would produce an excess of receptors, which then will appear in the serum as free antibodies. Although highly speculative, Ehrlich's theory was surprisingly insightful for two reasons: First, it implied that the antigen–antibody interaction would send a signal to the cell nucleus to manufacture large amounts of antibody. Second, the theory stressed the antibody's chemical nature and suggested that its specificity for antigen would be dictated by the stereochemistry of its binding site. These ideas fascinated chemists such as Arrhenius (3) and others, whose theoretical and experimental contributions established the basis of immunochemistry. This field, which dominated the first half of the twentieth century, was more concerned with the chemical nature of antigen-antibody interaction than with its biologic consequences.

The first successful attempt to identify antibody molecules was accomplished in 1939 by Tiselius and Kabat (4), who demonstrated that hyperimmunization increased the concentration of gamma globulins in serum and that this fraction contained antibody activity. Because g-globulins are large-molecular-weight proteins, it was inferred that further characterization of antibodies, now termed *immunoglobulins,* necessitated splitting them into smaller, easily handled fragments. In 1959, Porter succeeded in digesting rabbit immunoglobulin G (IgG) with limiting concentrations of the proteolytic enzyme papain. This generated two discrete fragments: a monovalent *f*ragment with *a*ntigen *b*inding activity (Fab) and a second fragment that retained the antibody's effector functions and crystallized readily into a lattice, termed Fc (5). It should be noted that the inability of the antigen specific Fab fragments to crystallize correlated with chemical heterogeneity, that is, differences in amino acid sequence, a prelude to the characterization of variable domains. Edelman and Poulik used a similar strategy, assuming that antibodies, like many other proteins, would be composed of a number of independent polypeptides held together by disulfide bounds. When methods that usually disrupt such bonds were tested, myeloma globulins were separated into two distinct chains, which, based on their sizes on starch gels, subsequently were termed *heavy* (H) and *light* (L) chains (6).

Similar studies using the protease pepsin were successful in digesting antibodies into an F(ab') fragment with bivalent antigen binding activity and several small fragments derived from the Fc portion of the molecule, the largest termed pFc' (7,8 and 9). These pioneer studies, the results of which are summarized in Fig. 13.1, were fundamental in deciphering the structure of immunoglobulins by establishing the existence of heavy and light chains and predicting that antigen-binding and antibody-effector functions were located in distinct protein domains.

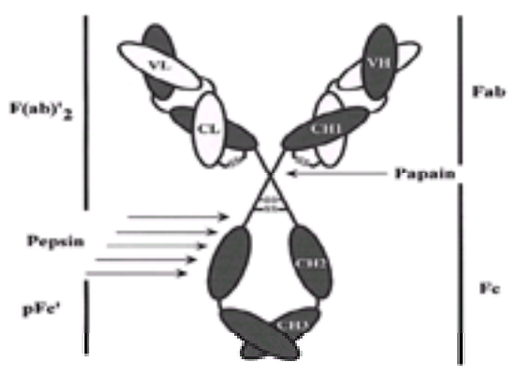

Figure 13.1. Schematic diagram of an immunoglobulin G (IgG) molecule. Immunoglobulin domains are represented as *dark ovals* for heavy chains and as *clear ovals* for light chains. *SS* indicates the interchain and intrachain disulfide bridges. Treatment of the molecule with the proteolytic enzyme papain (or trypsin) generates three fragments: a crystallizable Fc portion and two identical Fab fragments. Pepsin digestion, on the other hand, cleaves the IgG molecule below the heavy-chain interchain disulfide bonds, yielding the entire F(ab)'2 fragment and numerous smaller polypeptides from the Fc domain, the largest one being pFc'. VL and CL, variable and constant region of the light chain; VH, variable region of the heavy chain; CH1 to CH3, constant regions 1 to 3 of the heavy chain.

Sequencing studies in the 1960s subdivided light chains, and later heavy chains, into an amino terminal V (variable) region, which varied substantially in amino acid composition between different antibodies, and a C (constant) domain, whose sequence was conserved between antibodies of the same isotype (10,11). Radiographic crystallography, on the other hand, confirmed the proteolytic experiments of Porter and Edelman by showing that indeed all immunoglobulins were composed of two pairs of heavy and light chains of approximately 25 and 55 kd (12,13). The overall structure of immunoglobulins was stabilized by a series of interchain and intrachain disulfide bounds as well as strong hydrophobic interactions created at heavy- and light-chain interfaces (Fig. 13.2). Perhaps most interestingly, both chains were found to share a basic three-dimensional structure, the Ig fold (Fig. 13.2). This fold or domain consists of two antiparallel b-pleated sheets, each containing between three to four b-strands joined at their hydrophobic core by a disulfide bridge. The loops connecting the strands are not critical for generating the proper folding of the Ig domain and are therefore free to vary and contribute to the diversity required for immune responses. This assumption was sustained at the time by the comparative studies of Wu and Kabat on myeloma light-chains sequences (14). Wu and Kabat defined *variability* as the ratio between the number of different amino acids found at a given position and the frequency of the most common amino acid seen at that position (Fig. 13.3). By graphing the distribution of variability (Wu and Kabat plot) in heavy-chain V regions, three *h*yper*v*ariable domains emerged (HV1, HV2, HV3), separated by four relatively invariant or *f*ramework *r*egions (FR1, FR2, FR3, FR4). The conserved framework regions correspond to the b-strands in the Ig-folded structure, whereas the hypervariable (HV) domains constitute the loops connecting the strands. All HV loops from heavy- and light-chain V regions are brought together at the apex of the Fab fragment, generating the antigen-binding site. Because this site forms a surface complementary to the antigen, the HV regions are also known as *c*omplementarity *d*etermining *r*egions, or CDRs (15).

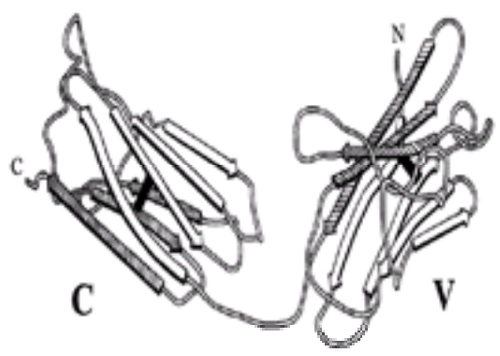

Figure 13.2. Schematic representation of the V and C domains of an immunoglobulin light chain. The b strands composing the immunoglobulin domain are represented as *arrows*. *Shading* of the arrows distinguishes between the two antiparallel b-pleated sheets, each containing three (shaded) and four (unshaded) b strands. The disulfide bridge connecting the two b sheets are depicted as *black bars*. (Adapted from Edmundson A, Ely K, Abola E, et al. Rotational allomerism and divergent evolution of domains in Ig light chains. *Biochemistry* 1975;14:3953–3961, with permission.)

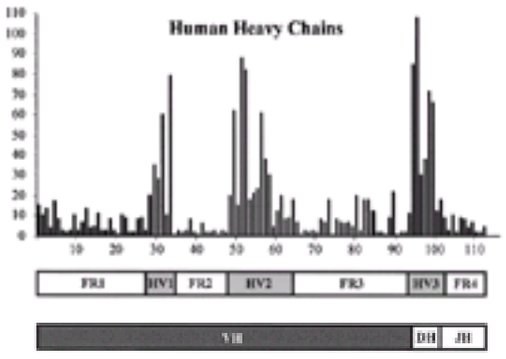

Figure 13.3. Variability plot generated by comparing human heavy-chain sequences. Y axis, degree of variability. X axis, amino acid position for the variable region of the heavy chain. From these comparisons, the framework (FR) and hypervariable (HV) domains were defined as shown. For comparative purposes, the approximate distribution of the variable (VH, *dark box*), diversity (DH, *gray box*), and joining (JH, *white box*) heavy-chain domains is aligned. Variability plot is based on Kabat EA, National Institutes of Health (U.S.) Columbia University. Sequences of proteins of immunological interest. Bethesda, MD: U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health; 1991.

GENERATION OF DIVERSITY

At the time Ehrlich proposed his comprehensive theory, scientists had only immunized experimental animals with a limited number of antigens, namely toxins. The work of Obermeyer, Pick, and Landsteiner in the second decade of the twentieth century clearly established that antibodies could be elicited against a myriad of antigens, including substances that never would be found in nature, such as haptens coupled to protein carriers. These observations established the basis of a new biologic puzzle: How could an animal generate antibodies against such a variety of synthetic substances? At the time, it seemed most logical to imagine that antigens would convey the information necessary for the synthesis of the antibody molecule. The first theory based on this assumption was forged in 1930 by Haurowitz and later was refined in 1940 by Linus Pauling (16). The *instructive or template theory*, as it became known, proposed that antigens served as templates to nascent antibody molecules, which would coil around them, creating the complementarity. The idea of instruction was widely accepted by chemists and biochemists working in the antibody problem. The theory, however, failed to explain several of the more important biologic aspects of the immune response, such as the presence of long-term memory responses and the increase in antigen specificity on repeated immunizations. Perhaps more importantly, it did not explain how immunological tolerance was acquired. These objections were the basis for a new theory conceived by the ideas of Burnet and Lederberg: the clonal selection theory (17,18). This theory revisited Ehrlich's concept that antibody recognition patterns preexisted before the introduction of antigen into the animal and proposed that diversity was generated by somatic mechanisms that randomly generated the antibody specificity in individual cells. The interaction of antibody–antigen determinant at the cell surface would result in the clonal expansion of that particular cell and the secretion of immunoglobulins. In turn, tolerance would result from the deletion of self-reactive cells early in development. An important corollary of the theory was that lymphocytes were monospecific, that is, “one cell per one antibody.” This notion was tested and corroborated by Nossal and Lederberg (Nossal JV, Lederberg J. Antibody production by single cells. *Nature* 1958;181:1419–1420.) on single-cell cultures and Edelman's work establishing Bence Jones proteins, isolated from multiple myeloma patients, as immunoglobulins carrying a single specificity (Edelman GM, Gall E. The antibody problem. *Annu Rev Biochem* 1969;38:415–466). The clonal selection theory, as recognized by Burnet himself, was a darwinian answer to the more Lamarckian instructionalistic view of the antibody problem. What the novel theory did not explain were the molecular mechanisms of the selection, which remained untouched for almost two decades.

As stated, preliminary sequencing studies recognized that light chains were composed of a V domain whose amino acid sequence varied from antibody to antibody and a conserved C domain. This raised the possibility that Ig chains were the result of a fusion of two genes, as advanced by Dreyer and Bennett (19). This hypothesis, which challenged Garrod's “one gene one polypeptide” theory (20), was validated by the work of Tonegawa and co-workers (21). The study showed that a light-chain mRNA probe containing both the V and the C regions hybridized to different DNA fragments as digested with restriction enzymes and separated by electrophoresis. Shortly after, RNA–DNA R-loop (22,23) and sequencing experiments (24) not only confirmed that V and C regions were coded by different genes but also defined a new set of genes, the *J segments*. These data shed light for the first time into the mechanism of diversity by proving that a given V gene segment was joined to a J segment through a recombination process, later termed *V(D)J recombination.* A meticulous comparison between the k J region DNA with known antibody protein sequences revealed more variability at the junction point between the V and J segments (25). This new diversity, called *junctional,* predicted that, in addition to successful rearrangements, “forbidden” or out-of-frame recombinations would occur as well.

Heavy-chain assembly turned out to be more complicated because three independent gene segments are involved: VH, D, and JH of approximately 98, 3-7, and 12-17 amino acids, respectively. The VH segment encodes a leader peptide sequence and both the CDR1 and CDR2 regions. The CDR3 is encoded by the D segment and the VH-D and D-JH junctions (Fig. 13.3). The remainder of the J segment encodes the last framework region. Conversely, the light chain is encoded by two gene components: VL, which comprises all CDRs, and the JL segment. Despite variations in their number and organization, these segments are arranged in clusters along the same chromosome in nearly all species that were studied (26).

At the heavy-chain locus, V(D)J recombination initiates random D to JH joining, followed by VH to DJH rearrangements (27). The V(D)J recombinase complex recognizes recombination signal sequences (RSSs) flanking the gene segments involved (26,28). RSSs are composed of a palindromic conserved heptamer, CACAGTG, followed by the nonamer ACAAAAACC. These elements are separated by a nonconserved spacer of either 12 ± 1 or 22 ± 1 base pairs (bp) (27), which constitute one and two complete turns of the DNA helix, presumably allowing the DNA groove of both RSSs to be aligned during the recombination process (29). In fact, recombination will occur only rarely between gene segments flanked by like spacers, a phenomenon known as the *12-23 rule* (30). This “rule” precludes aberrant recombination events, such as VH to JH, from happening (24,31).

Depending on the alignment of the gene segments on the chromosome, V(D)J recombination can occur by two mechanisms: inversions and deletions (32,33) (Fig. 13.4). When the recognition sequences are facing each other, recombination proceeds by deletion of the intervening DNA as circles containing the fused heptamers. Inversions occur when the RSSs are oriented in the same orientation. In these reactions, the heptamers and nonamers are retained in the chromosome, and no DNA is lost. It has been estimated that about 40% of Vk-genes are in opposite orientation with respect to the Jk-segments (34).

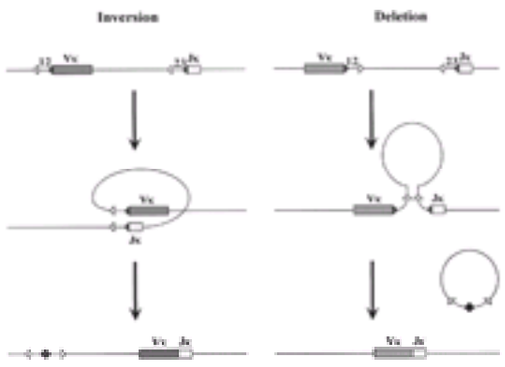

Figure 13.4. Inversional and deletional recombination of the k locus. Inversions occur when the recombination signal sequences (RSSs) of the Vk and Jk genes are in the same orientation in the chromosome (*open triangles*: nonamers; *closed triangles*, heptamers; 12 and 23, DNA spacers). When the recognition sequences are facing each other (approximately 40% of the time), the recombination proceeds by deletion of the DNA intervening sequences as circles.

Secondary recombinations at the Vk-chain gene recombination revealed that most primary rearrangements occur by inversions followed by secondary deletions (38). At the VH locus, the elimination of all 12-bp spaced signal sequences by any VDJ rearrangement would, in principle, hinder further recombination. Nevertheless, 70% of all VH genes harbor at their 3'-ends a sequence similar to the consensus heptamer. Such sequences have been reported to engage in secondary recombinations with the expressed VHDJH gene (39,40 and 41), but the extent of these events *in vivo* is unknown.

RAG PROTEINS

To isolate the genes involved in Ig recombination, Schatz and Baltimore relied on the fact that only immature lymphoid cell lines carried out V(D)J gene assembly (42); however, on transfection of human or mouse genomic DNA, 3T3 fibroblasts displayed V(D)J recombinase activity (42). The cloning and sequencing of the locus responsible for such an activity revealed the presence of two genes, with no apparent sequence similarity: RAG-1 and RAG-2 (43,44). These genes are separated in the mouse genome by only 8 kb and are aligned in opposite transcriptional orientation with respect to each other. The linked genomic organization, conserved in all vertebrates so far analyzed, and the lack of sequence resemblance between RAG-1 and RAG-2, prompted speculation that these genes arose as part of a fungal, or viral recombination system (44). Later studies, in fact, demonstrated a portion of the RAG-1 protein to be related to the homeodomain of the *Salmonella typhimurium* Hin invertase, which also recognizes a nonamer-like sequence in the genome (45).

V(D)J recombination also was linked to transposition based on the similarity between RSS sequences and the inverted repeats found at the end of transposons (46). This relationship was reinforced by mechanistic similarities shared by both reactions (47,48) and the similarities between the disintegration reaction carried out by the human immunodeficiency virus (HIV) integrase and the generation of hybrids and open-and-shut joints catalyzed by the RAG proteins (49,50). *In vitro* studies found RAG-mediated insertions of cleaved RSSs into new DNA by a mechanism akin to retroviral integration (51,52). Finally, the coordinate transcriptional regulation of RAG genes suggests that the original integration of the primordial transposon most probably occurred at a site near a transcriptional element that captured these proteins for the evolving immune system (53).

V(D)J RECOMBINATION

Much of what is known with regard to the molecular details of Ig gene recombination has been inferred from cell-free *in vitro* assays. The current model of V(D)J recombination derived from such studies describes first the recognition and cleavage of the heptamers signal sequences mediated by RAG1 and RAG2 proteins (43,44,54) (Fig. 13.5). The stereochemistry of this reaction shows that RAG proteins generate first a nick at the 5'-end of the heptamer. The 3'-hydroxyl group created by the nick then attacks the phosphodiester bond at the end of the 7-mer of the bottom strand (*transesterification*). This reaction creates a DNA hairpin at the coding end and a blunt end at the signal end (48). Although RAG proteins can cleave DNA alone *in vitro*, other factors, such as the high mobility group (HMG) proteins, have been shown to enhance the reaction (55,56).

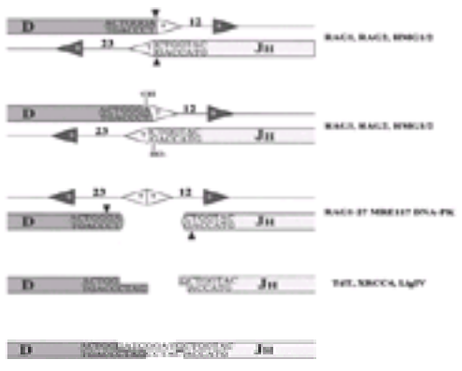

Figure 13.5. Proposed model for V(D)J recombination. **A**: Nicking. The RAG1 and RAG2 proteins recognize and bind to the recombination signal sequences (7, heptamer; 9, nonamer; 12 and 23, DNA spacers). RAG1 and 2 then cleave the recombination signal sequences (RSSs) at the 5'-end of the heptamer. **B**: Transesterification. The 3' nucleophilic OH group created by the nick (bases A and G) attacks the phosphodiester bond at the end of the 7-mer of the bottom strand (bases T and C), generating hairpins at the coding ends and blunt ends at the signal ends. **C**: Signal and coding end processing. DNA-PKcs, Ku70, and Ku80 form a protein complex around the DNA ends generated by the transesterification reaction. Signal ends are joined bluntly; the hairpins formed at the coding ends are opened by either RAG1 and RAG2 or the Mre11/RAD50/Nbs1 complex. **D** and **E**: N and P nucleotide additions. The terminal deoxynucleotide transferase (TdT) catalyzes the addition of nontemplate-dependent nucleotides (N nucleotides) at the opened hairpin ends. XRCC4, Ligase IV, and probably a polymerase seal the DNA gaps.

Hairpin intermediates were first characterized in severe combined immunodeficiency disease (SCID) mice, which accumulate such unprocessed ends due to a deficiency in the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) (57). This kinase, in association with two other factors, Ku70 and Ku80, form a protein complex not only required for V(D)J recombination (57,58 and 59) but also for repair of ionizing radiation (IR)-induced DNA damage (60). Ku70 and Ku80 bind as a heterodimer with high affinity to DNA ends (61). This binding has been suggested to protect the ends from nucleolytic degradation (62) and physically to juxtapose them for further processing, as revealed by scanning force microscopy (63,64 and 65). The Ku-DNA interaction is also believed to recruit the catalytic subunit DNA-PKcs to the DNA ends and enhance its kinase activity (66). From these observations, it can be concluded that the accumulation of coding ends in SCID mice would result from failure of DNA-PKcs to phosphorylate and activate the hairpin endonuclease. Hairpin opening was shown to be carried out by the RAG1 and RAG2 proteins (67,68), although another report evoked the concerted action of the nonhomologous end-joining factors Mre11, RAD50, and Nbs1 as possible candidates (69).

The opening of hairpins at asymmetric positions is believed to generate *p*alindromic repeats at some coding joints (P nucleotides) (70) (Fig. 13.5C). Another enzyme, terminal deoxynucleotide transferase (TdT), catalyzes the addition of nontemplate-dependent nucleotides (N nucleotides) (71) to the open DNA ends. N-nucleotide additions are commonly found at the heavy chain but are rarely seen in light-chain genes, perhaps as a results of transcriptional downregulation of the TdT gene by the µ-surrogate light-chain complex on the surface of preB cells (72). The addition of P and N nucleotides, as well as the deletions created by the imprecise joining reaction, generate junctional diversity.

The final step of V(D)J recombination involves the joining of the processed DNA ends. This step is mediated by two genes, the gene mutated in the radiographic complementation group 4 (XRCC4) (73), a ubiquitously expressed factor also essential to repair DNA double-stranded breaks caused by ionizing radiation, and ligase IV (74,75). *In vitro* studies demonstrated that the XRCC4 protein interacts with and is phosphorylated by DNA-PKcs (76). XRCC4 also binds and activates another member of the V(D)J recombination machinery, DNA ligase IV, which seals the last DNA gap (74,75). *In vivo* data supporting this hypothesis come from ligase IV-deficient mice, in which lymphopoesis is blocked at the V(D)J joining step (77).

Combinatorial Diversity

Shortly after V and J gene segments were discovered, Weigert's group performed a detailed analysis of Vl21 protein sequences and proposed that antibody variability could be explained in part by combinatorial diversity, that is, the random associations of V, D, and J DNA segments (78). Assuming no bias in the combination process, the maximal collection of antibody specificities possible would be equal to the product of the number of V, D, and J genes present in each of the Ig loci. Translating this assertion to the recently characterized mouse Vk locus (79) results in 100 (functional Vs) × 4 (functional Js) = 400 potential Vk genes generated.

Junctional Diversity

Because of the nature of V(D)J recombination, the point at which V and J segments are joined can vary over a range of several nucleotides. As a result, codon 96 of the light chain becomes the most variable position of the molecule, as comparative studies of light chain sequences reveal (14,25). Theoretically, in every V-J combination event, four different amino acids can be placed at position 96, which in turn would result in 1,600 potentially different Vk genes (400 × 4). For the heavy-chain locus, junctional diversity operates both at the level of V-D and D-J joints, increasing diversity by a factor of 16 (4 × 4). As noted, heavy-chain junctions also are diversified by the addition of N nucleotides by the TdT enzyme. Equally important, the random opening of hairpin intermediates generates palindromic (P) nucleotides in the process. Because of the extent of junctional diversity, antibodies show the greatest diversity at the CDR3 (Fig. 13.3), the portion of the Ig molecule that contributes the most to the antigen binding pocket.

Somatic Hypermutation

Burnet conceived the clonal selection theory to a great extent by extrapolating the darwinian concepts he acquired while working on bacterial genetics. He imagined lymphocyte populations undergoing constant physiologic and mutational changes. Much as antibiotics select resistant bacterial strains, antigenic determinants would induce lymphocytes with which they can interact to proliferate and secrete immunoglobulins. Above all, Burnet's theory suggested that subclones would constantly arise as a result of somatic mutation “in that region of the genome concerned with immunologically significant pattern” (17). The first direct evidence for somatic diversification of antibody variable regions came from a comparison of k1 protein sequences to the number of Vk1 genes (80). The study showed that of 19 k1 protein sequences analyzed, 12 were identical and seven differed in two to three amino acids. The interpretation was that there existed only one k1 gene in the genome and that the substitutions had arisen, as predicted by Burnet, by somatic mutational events. The idea became unambiguously validated by sequencing of the k1 genomic locus (81).

The mechanism of somatic hypermutation, which is confined to germinal center B cells, randomly introduces mutations downstream of the VH and VL promoters modifying the affinity or specificity of the antigen-binding sites of the Ig. Those B cells expressing higher affinity for antigen are selected for further expansion and differentiation. B cells that have lost the affinity for the antigen in question or those that react to self-antigens (change in specificity) are eliminated through apoptosis. A role for transcription, or at least for accessibility, has been demonstrated by several reports (82,83 and 84). Somatic mutation is not completely Ig gene specific because the Bcl6 oncogene has been found mutated in GC B cells (85,86).

IMMUNOGLOBULIN CONSTANT REGIONS AND CLASS SWITCHING

The existence of different isotypes was first hinted by the work of Bauer and Stavisky, who discovered that after immunization, antigen-specific antibodies appeared first with a sedimentation coefficient of 19S, followed within several days by the additional synthesis of a 7S form (87). The results demonstrated that distinct-molecular-weight antibodies were generated during an immune response. The favored interpretation at the time was that different plasma cells synthesized the 19S or the 7S immunoglobulins, and the idea that a single cell could “switch” from one isotype to another was proposed by Nossal in 1964 (88). Nossal isolated single antibody producing cells at different time points after immunization and characterized the antibodies they produced. In the first 5 days after antigen challenge, all plasma cells isolated produced the heavy 19S antibody form (IgM). At day 7 and later, only 7S (IgG)-containing cells could be found. At days 6 and 7, however, single cells were isolated, producing both IgM and IgG. That this switching was the result of another recombination event was first suggested by the demonstration that IgG1 cells shared the same variable domain with their IgM predecessors (89). The finding by Honjo and Kataoka that specific CH genes were deleted in mouse myeloma cells and that deletion accompanied class switching unequivocally validated the recombination theory (90).

Heavy-chain constant regions exist in five classes: mu (μ), delta (d), gamma (g), epsilon (e), and alpha (a). Accordingly, individual immunoglobulins are named based on their constant region isotype: IgM, IgD, IgG, IgE, and IgA. In the mouse, CH genes span a region of approximately 200 kb in chromosome 12 (91) and are organized from 5' to 3' in the order μ, d, g3, g1, g2b, g2a, e, and a. The human CH locus is located in chromosome 14q32 and is composed of two copies of the g-g-e-a unit (92). Contrary to the light-chain k and l constant regions, the CH proteins are functionally divergent as to their ability to dimerize, interact with different Fc receptors, activate the complement cascade, or cross the placenta (see later).

Isotype switching can be induced *in vivo* or in cell culture on activation with a variety of mitogens and cytokines. For example, culturing mouse B cells in the presence of lipopolysaccharide (LPS) and interleukin 4 (IL-4) induces switching from IgM to IgG1 and IgE. In the absence of IL4, however, LPS activates recombination to IgG3 and IgG2b. *In vivo* studies indicate that switching is not a random process. In the mouse, immunization with soluble antigens generates IgG1 responses, whereas carbohydrate antigens stimulate switching to IgG3. Viruses, on the other hand, induce IgG2a responses in mice and IgG3 and IgG1 production in humans. In summary, the environmental stimuli elicited by an immune response appear to target switching to particular isotypes. Switch factors include IL4, IL5, IL10, interferon g (INF-g), transforming growth factor-b (TGF-b), LPS, and CD40 ligand.

Class-switch recombination occurs between highly repetitive DNA sequences, known as *switch (S) regions* (93), located 5' of each constant region gene, with the sole exception of Cd, which is cotranscribed with Cμ, and requires mRNA differential splicing for its expression. Although all S regions are composed primarily of the nucleotides repeats GAGCT and TGGGG, the exact sequence composition and length vary among CH genes. Unlike V(D)J recombination, isotype switching occurs anywhere within or near these S regions, with no apparent regard to the sequence specificity of the site of recombination (94,95 and 96).

As explained, switching results in deletion of chromosomal DNA located between the two S regions engaged in the recombination process (97) (Fig. 13.6). The direct visualization of circular DNA products generated during switching and V(D)J recombination provided clear evidence that both these mechanisms occurred through the formation and excision of a DNA loop (98,99 and 100). Both of these lymphocyte-specific recombination events are associated with loci that are transcriptionally active before recombination (101,102). This observation led to the hypothesis that transcription may render the chromatin accessible to the DNA-binding proteins involved in the recombination. This hypothesis, known as the *accessibility model*, proposes that cytokines target switch recombination by inducing transcription of specific heavy-chain constant regions. Recent work suggested that proper splicing of switch transcripts and not transcription *per se* is required for recombination to occur (103,104).

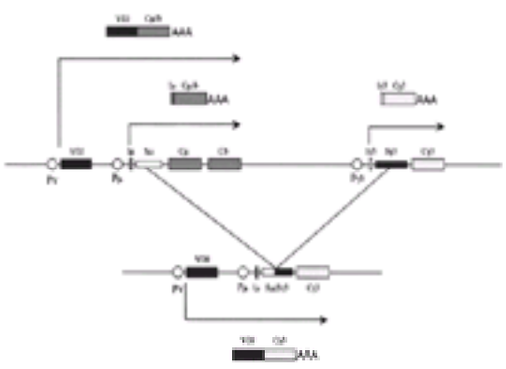

Figure 13.6. Class switching to g3. Before switch recombination, cytokines and B-cell activators specifically target the genes involved in the reaction (μ and g3) by initiating germline transcription from promoters (Pμ and Pg3) upstream of the respective CH genes. The I exons are spliced to the C exons, deleting the switch regions (S) from the germline transcripts. On switching, the intervening DNA is deleted from the chromosome, bringing the Cg3 into proximity to the VDJ gene so that the mature transcript initiated from the V promoter (PV) now contains the Cg3 domain.

Although the identity of the alleged class switch recombinase remains unknown, recent experiments indicated that some of the components known to participate in both V(D)J recombination and DNA double-stranded break repair (DNA DSB), such as DNA-PKcs, Ku70, and Ku80 are also directly involved in isotype switching (105,106

and 107).

BIOLOGY OF IMMUNOGLOBULIN ISOTYPES

Immunoglobulin M is the first antibody produced by plasma cells in primary immune responses and is expressed either in a membrane-bound form or as a secreted hexamer or more commonly as a pentamer. At the cell surface, IgM (as well as the rest of the isotypes) forms the B-cell receptor (BCR) composed of the Ig molecule noncovalently associated with two pairs of cytoplasmic subunits: Iga and Igb. These molecules have conserved amino acid sequences known as *immune receptor tyrosine activation motifs* (ITAMs), which interact with cytoplasmic tyrosine kinases transducing antigen–antibody signals downstream.

In its polymeric secreted form, IgM displays an increased avidity for antigen compared with the IgM monomer. Because primary immune responses proceed in the almost complete absence of affinity maturation, this increase in avidity would compensate for the overall lack of affinity for the antigen. Furthermore, IgM disposes of foreign particles by recruiting the classic complement pathway, leading to macrophage activation and phagocytosis of antibody–antigen complexes. This activation is initiated by the association of IgMs Cμ1 and Cμ3 domains with the complement components C3b and C1q (108).

Immunoglobulin D is expressed at high concentrations in the surface of mature B cells, but it is secreted only at extremely low levels and is present in serum at one order of magnitude lower than the other isotypes, with the exception of IgE. Although $IgD^{+}IgM^{-}$ germinal center and memory B cells exist in humans (109), IgD is most often seen coexpressed with IgM, and the function of the IgD only cells is not known. Coexpression is the direct result of the VDJ, Cμ, and Cd units being present in the same primary transcript. Alternative splicing ultimately dictates the isotype being translated. Despite the fact that the cytoplasmic levels of VDJ-Cμ mRNAs are consistently higher than those of VDJ-Cd, the mean IgD surface expression exceeds that of IgM by tenfold on mature B cells. Despite these differences between IgM and IgD, there is no known unique role for IgD in B cell development (110,111).

The four IgG subclasses constitute the most abundant Ig isotypes in serum and body fluids. Two of the most important functions of IgG molecules are activation of the classic complement cascade, which in turn leads to the destruction of antigens and pathogens. Likewise, IgGs exert their effector functions by interacting with Fcg receptors present in a variety of cell types. These interactions result in functional effects that range from modulation of the transducing signals generated by B-cell receptor cross linking, induction of phagocytosis in macrophages, or antibody-mediated cytotoxicity in mononuclear cells (112). Another fundamental role of IgGs is in protecting the fetus and newborn against infection. Before birth, IgGs are the only antibody isotypes capable of crossing the placenta. After birth, maternal IgGs found in milk are transported to the bloodstream of newborns via the neonatal Fc receptor (FcRn) expressed in the gut. It appears that FcRn binds IgG at the slightly acidic pH of milk in the proximal intestine and delivers it at the almost neutral pH of blood (113). The FcRN structurally resembles the major histocompatibility complex (MHC) class I molecules. Remarkably, the FcRn molecule, as their MHC counterparts, interacts with b_2 microglobulin and mice rendered deficient for this last molecule can not internalize maternal IgGs (114).

Immunoglobulin E antibodies are present in serum at the lowest concentration when compared with the other isotypes. This antibody class is unable to activate complement or opsonize antigen. Antigen recognition by IgE molecules bound to its high affinity receptor FceRI, expressed both in basophils and mast cells, leads to exocytosis of potent inflammatory mediators such as histamine and serotonin (115,116). This immune response, also known as *immediate hypersensitivity*, functions prevalently in the clearance of parasitic infections, although it often leads to anaphylaxis and allergic responses to innocuous molecules in predisposed persons.

Immunoglobulin A is the most abundant immunoglobulin in all body surfaces, with the exception of the skin, hence playing a key neutralizing role in mucosal immunity. In fact, IgA is synthesized at the amazing rate of 65 mg per kilogram daily, nearly twice as much as all other isotypes combined. In humans, IgA exists in two subclasses: IgA1, which is mostly found in serum as a monomer, and IgA2, secreted as a polymer by plasma cells found in the digestive, urogenital, respiratory, and mammary tissues. Once secreted, IgA is competent to cross the epithelial barriers by interacting with the poly-Ig receptor, also known as the *secretory component*. Oligosaccharides located at the a chain of IgA molecules prevent pathogens from adhering to mucosal epithelial cells, which would inevitably result in infection (117), although IgA-deficient mice do not appear more susceptible to mucosal viral infections than wild-type litter mates (118). Finally, IgA opsonizes antigens and pathogens, leading to their destruction via the FcaR found on polymorphonuclear leukocytes.

OTHER MEMBERS OF THE IMMUNOGLOBULIN SUPERFAMILY

The Ig domain first characterized in antibodies was later recognized in a variety of molecules with cell adhesion or molecular recognition functions. Remarkably, to date, more than 100 members of the “immunoglobulin superfamily” have been identified in species as diverse as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, zebrafish, and humans (119). Many of these members are grouped in multigene families such as VL, VH, MHCI, MHCII, TCRs, CAM, and FcRs.

Fc Receptors

Cells of the immune system “see” antigens through BCRs, T-cell receptors (TCRs), and Fc receptors. The last of these do so indirectly because they associate with the Fc portion of antibodies before eliciting effector functions. There are three groups of Fc receptors. The first group resembles TCRs and BCRs in that the ligand-binding component of the receptor is noncovalently associated with accessory proteins that signal through ITAMs (120). This group includes FceRI, FcaR, FcgRI, and FcgRIIIA. A second type of Fc receptor, typified by FcgRII, does not couple with ITAM-containing subunits but is instead a single-chain receptor possessing inhibitory motifs (ITIMs), which counteract the activity of the ITAM receptors (121). A third class of Fc receptors includes those that do not trigger or inhibit cell activation. As discussed, these receptors are implicated in transcytosis of antibodies through epithelia, such as the pIgR, specific for IgM and IgA, and the neonatal FcRn, which transports IgGs. Finally, Fc receptors exist as membrane-bound and soluble forms, the latter being the result of alternative RNA splicing or proteolysis of membrane forms (122). To date, the raison d'être of soluble Fc receptors is unknown.

The first FcR characterized was the high-affinity IgE receptor, which was identified shortly after the discovery of the IgE molecule (123). This receptor is expressed at relatively high levels in mast cells and basophils and at lower concentrations in human Langerhans cells, monocytes, eosinophils, and peripheral blood dendritic cells. The FceRI is composed of a ligand-binding a chain, which contains two Ig domains, a b chain, and two identical g chains, that are covalently linked by a disulfide bridge (Fig. 13.7). Both the b and g have ITAMs, which allow them to interact and activate downstream effectors such as the lyn and syk kinases (124). The g homodimer subunit was found not only to be structurally related to the TCR-associated molecule CD3z , but the former can be functionally substituted by the latter in transfected CV-I origin SV-40-7 (COS-7) cells (125). Most of the IgE released by plasma cells is thought to associate quickly and stably ($kd = 10^{-10}$ M) through its Ce2 and Ce3 domains to the FcaRI a-chain of basophils and mast cells (126). The binding of multivalent antigens to these IgE molecules induces aggregation of the FceRI molecules as well. Once activated, these granulocytes release histamine, serotonin, and other potent inflammatory mediators, such as prostaglandins, leukotrienes, and selected cytokines. A second IgE receptor, the FceRII or CD23, interacts with IgE molecules, but with lower affinity ($Ka = 10^{8}\ M^{-1}$) compared with FceRI ($10^{10}\ M^{-1}$) (127). This receptor, composed of a single transmembrane segment, lacks Ig domains and belongs instead to the calcium-dependent lectin family of receptors (128).

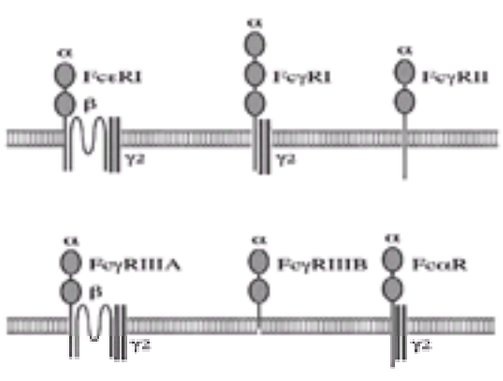

Figure 13.7. Fc receptors of the immunoglobulin (Ig) superfamily. The Ig domains of the a chains are depicted as *ovals* and associate with the Fc domains of antibodies in the extracellular milieu. b chains traverse the cytoplasmic membrane four times, and in conjunction with the g chains, they initiate intracellular signals through their immune receptor typrosine activation motifs.

FcgRI is expressed constitutively in macrophages and monocytes, and its expression can be induced in neutrophils upon IFN-g exposure (129). Activation through FcgRI is believed to stimulate phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) on these cells. FcgRI is able to bind all IgG subclasses, although it does so with much higher affinity to IgG1 and IgG3.

The inhibitory receptor FcgRII exists in at least six different spliced forms, some of which are secreted extracellularly. Its pattern of expression includes all hematopoietic cells, with the exception of natural killer (NK) cells and red blood cells. This single a-chain receptor functions as a negative regulator of ITAM-mediated signals and nullifies cell activation when coligated with stimulatory receptors (121). The inhibitory action of FcgRIIB (the only inhibitory version expressed in B cells) was first suggested by the observation that anti-µ F(ab') antibody fragments induced B cells to proliferate in culture, whereas whole IgG antibodies did not (130). Cross linking the BCR induces tyrosine phosphorylation of a variety of proteins and depletion of Ca^{2+} from intracellular stores and subsequently Ca^{2+} influx from the extracellular milieu. One of the early targets of the BCR, the lyn kinase, activates the FcgRIIB by phosphorylating Tyr-309 from its ITIM domain, leading to the recruitment and phosphorylation of the SH-2 domain phosphoinositol phosphatase SHIP (131,132). Once activated, SHIP partially abrogates BCR signaling by selectively blocking Ca^{2+} influx without interfering with intracellular Ca^{2+} mobilization (133). FcgRIIB-deficient mice exhibit three to ten times higher antibody titers than normal mice on T-cell-dependent and -independent immunizations (134), which shows that in B cells, FcgRIIB functions as a downmodulator of antibody production. Additionally, these mice are hyperresponsive to IgG- and IgE-mediated anaphylaxis as well as to collagen-induced arthritis and immune complex-induced alveolitis (134), suggesting that FcgRIIB normally sets thresholds to FcgRIII and FceRI (135,136).

The FcgRIII receptor is synthesized in two different forms: FcgRIIIA and FcgRIIIB. The former is expressed in monocytes, macrophages, and NK cells; the latter appears to be solely present in neutrophils. Analogous to the FceRI, the FcgRIIIA is a transmembrane glycoprotein that associates with the b and g2 signaling molecules. The FcgRIIIB, on the other hand, is anchored to the cytoplasmic membrane through a glycophosphoinositol linkage, hence lacking signaling activity (Fig. 13.7). Persons who have defective FcgRIIIB form suffer from a condition know as *paroxysmal nocturnal* hemoglobinuria, characterized by susceptibility to infections and delayed clearance of immune complexes (137,138). The physiologic role of FcgRIII receptors includes induction of superoxide synthesis, ADCC, and phagocytosis.

The most recently discovered and perhaps least characterized of the Fc receptors is FcaR (CD89). It is expressed in a variety of polymorphonuclear leukocytes, especially those found in mucosal areas, where IgA is the predominant isotype. Structurally, the Fca receptor, much like the FcgRs and FceRI, consists of an a chain associated with the FcRg-signaling homodimer (Fig. 13.7). Signals from this receptor result in phagocytosis and elimination of IgA-opsonized particles such as viruses and bacteria; thus, this receptor is thought to play a significant role in mucosal immunity (139).

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14 BIOLOGY OF B CELLS

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B cells can be defined as the population of lymphoid cells that, when antigenically stimulated, generates clones of antibody forming cells (AFC). It is through the production of antibodies that B cells function to protect the body against viruses, bacteria, parasites, and toxins. Adult mice have 2 to 4 x 10^8 B cells and adult humans have 10^{11}-10^{12} B cells distributed throughout the body. B cells are numerous not only in the spleen and bone marrow but are also present in lymph nodes, Peyer's patches, gut and bronchial associated lymphoid tissues, the peritoneal cavity, and throughout the lymphatic and circulatory systems. Within the spleen and lymph nodes, B cells distribute in and around the follicular areas. Structures within the lymphoid follicles known as germinal centers (GC) appear to be the major site of memory B cell generation and are highly enriched for B cells following immunization (see Chapter 2).

B cells express numerous cell surface antigens, some of which are known to function as adhesion molecules or as receptors for interleukins, hormones, mitogens, complement components, or immunglobulins while other cell surface antigens have been identified as essential for interactions with T cells (see Chapter 8 and Chapter 9). The cell surface antigens that are most characteristic of B cells are class II major histocompatibility (MHC) antigens, which function in the presentation of antigens to helper T cells (T) thus facilitating T cell–B cell collaborations (see Chapter 3 and Chapter 6), and immunoglobulin (sIg) which serves as the B cell's specific antigen receptor. By virtue of the efficiency of sIg in binding and concentrating antigens and the ability of B cells to process and present peptides of internalized antigens in a complex with class II MHC molecules, B cells subserve a second major function in the activation or inactivation of T cells.

CLONAL SELECTION AND B-CELL RESPONSES

The hallmarks of the humoral immune response are the enormous diversity of antibody specificities, the absence of reactivity to self-antigens (tolerance), the ability to selectively induce the production of antibodies that specifically recognize an immunizing antigen, and the capacity to mount an even more vigorous response to that antigen upon subsequent contact (immunologic memory). In order to account for these phenomenon, Burnet proposed in his “Clonal Selection Theory” that the specificity of humoral immune responses was a consequence of “the existence of multiple clones of immunoglobulin-producing cells each responsible for one genetically determined type of antibody” (1). He further posited that antigen would stimulate cells by interacting with an antibody representative of that cell's potential secreted antibody product expressed “on the surface of the cell which produced it.” This would ensure that a vast repertoire of antibody specificities would be clonally distributed among the population of B cells enabling antigen to either selectively stimulate or selectively inactivate specificities individually.

During subsequent years, most of Burnet's postulates have been validated and the underlying molecular mechanisms have been defined. By the early 1970s experiments had been conducted demonstrating that B cells bore cell surface immunoglobulins (2,3), that these immunoglobulins reflected both the specificity (4,5) and variable region antigenic determinants (idiotypes) of the cell's potential secreted antibody product (6), and that B cells and their clonal progeny were committed to the production of antibodies with a single variable region (clonotype) (7,8). This was consistent with earlier findings that neoplastically transformed B cells (myelomas) produced homogeneous immunoglobulins (9,10) and later successful efforts to obtain homogeneous antibodies by the fusion of B cells to myeloma cells (hybridomas) (11). The extensive heterogeneity of serum antibodies, therefore, reflected the participation in immune responses of numerous antibody forming cell (AFC) clones each producing a homogeneous antibody product (7). Since the early 1970s the molecular mechanisms responsible for generation of a vast array of heavy and light chain variable regions (12,13,14 and 15) and the expression of one antibody clonotype per cell (allelic exclusion) (16,17,18 and 19) have been defined (see Chapter 13).

Because an adult mouse has more than 2 x 10^8 B cells, each expressing a single clonotype, and any clonotype would infrequently be expressed by more than one B cell (20), the B-cell repertoire of a mouse exceeds 10^8 clonotypes (5,20,21,22 and 23). Thus, during the course of an immune response, antigen should have access to B cells expressing a diverse array of clonotypes. Most microorganisms present a spectrum of antigens, each of which is likely to display several constellations of amino acids or carbohydrates that could be recognized by B cell sIg receptors as distinct antigenic determinants. Furthermore, most individual antigenic determinants interact with sufficient affinity with sIg receptors of B cells of numerous clonotypes to enable stimulation to AFC formation. Therefore, foreign agents confronted by the immune system generally stimulate hundreds to thousands of B cells with distinct antibody clonotypes (5,21,22 and 23). The diverse array of antibody molecules produced by these B cells would comprise the humoral immune response to that particular immunogen and these antibodies would have in common the fact that they all can bind that immunogen.

The antigen combining sites of most antibodies are capable of accommodating a spectrum of antigenic determinants, including those that mimic the structure of the immunizing determinant as well as some that may be dissimilar but bind fortuitously to an alternate orientation of the combining site (24). Therefore, among antibodies within the response to a given antigen, some bind other antigenic determinants (cross react). However, because the entire population of antibodies binds with high affinity to the immunizing antigen, and only a few cross react with any other antigen, antibody responses as a whole are highly specific for the immunizing antigen and almost unlimited, at the population level, in their capacity to discriminate among antigens.

B-CELL DEVELOPMENT AND REPERTOIRE SELECTION

In mice functional B cells are first observed in the liver toward the end of the second trimester of fetal development (25). The liver remains a source of B cells throughout fetal development, but during the third trimester the fetal spleen becomes a source of B cells and remains so for several days after birth. After birth B-cell development is initiated in the bone marrow; and after the first week of neonatal life and throughout adulthood the marrow of large bones remains the major source of B cells in mice and humans (26). In adult mice approximately 5 x 10^7 B cells are generated from the marrow per day, which is sufficient to replace 15 to 25 percent of total mature B cells on a daily basis (26). However, the half-lives of B cells vary considerably, such that most newly generated B cells live for only a few days, whereas others may live for several weeks or months (27,28). Figure 14.1 depicts a scheme that correlates the molecular events that accompany B cell development in adult bone marrow with clonal differentiation and expansion. Multipotential stem cells, as defined by their capacity to generate all myeloid, erythroid, and lymphoid elements, have been isolated as fewer than 0.1 percent of bone marrow cells of mice and humans (29). Such cells can give rise to B cells, but it is not yet certain whether B cells routinely generate from such multipotential stem cells or alternatively from a more differentiated but still self-renewing stem cell population. The earliest detectable event in the commitment of bone marrow cells to the B cell lineage is the expression of various B lineage specific markers, including cell surface B220, the pre-B cell specific proteins V pre-B and l5, which together appear to constitute a light chainlike structure (surrogate light chain) (30,31 and 32), and Iga and Igb, which serve as part of the receptor complex on pre-B cells and B cells (see Chapter 13) (33,34 and 35). Additionally, enzymes such as terminal deoxynucleotide transferase (TdT), RAG1, and RAG2, which participate in V gene segment rearrangement, are expressed (36,37).

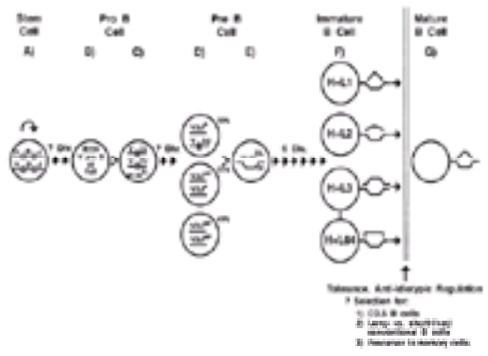

Figure 14.1. Precursors of B cells pass through several definable states during their development in the adult bone marrow: **A**: Multipotential stem cells self-renew (↷) and have the capacity to reconstitute all myeloid, erythroid, and lymphoid cells. **B**: Pro-B cells begin to express B lineage specific antigens, the enzymes necessary for Ig gene segment recombination, and V preB and l5 which together can comprise a surrogate L chain (SL) and co-receptors Iga and Igb. **C**: Pro-B cells initiate Ig gene segment recombination by recombining a D gene segment to a J gene segment on both chromosomes. It is possible that cells divide after this event. **D**: During the pre-B cell stage of differentiation, cells recombine a V gene segment to the rearranged DJ on one chromosome. If this rearrangement is productive (P) (~30%) and the resultant H chain is functional (assembles with SL) further V gene segment rearrangements are precluded (allelic exclusion). If the first V-D-J rearrangement cannot encode an H chain (non-productive = NP) a V gene segment of the second chromosome rearranges. Since ~30% of the second rearrangements will be productive, approximately half of the cells should have a productive rearrangement and synthesize an H chain. **E**: A proportion of pre-B cells that successfully express H chains will be selected, in part by H chain-SL chain interaction, to clonally expand. These cells will undergo at least 5-6 divisions prior to expressing L chains. Cells at this stage express the enzymes necessary for gene segment recombination, SL, and accessory molecules (Iga and Igb) for Ig membrane insertion. They express on their surface B220 and perhaps SL-μ complexes. **F**: Immature B cells represent those members of an expanded clone of pre-B cells that have rearranged a V and J gene segment productively and whose L chain interacts with the H chain to yield sIg expression. Each cell of the clone would presumably express the same H chain but a different L chain. At this stage, cells express on their surface IgM, as well as well as μ-SL complexes, MHC class II, and B220. At the stage at which immature B cells first express their sIg receptors, they are subjected to receptor-specific selective processes including inactivation by self-antigens (tolerance) that includes the potential for "receptor editing," anti-idiotypic regulation, and possibly subpopulation and longevity determination. In the presence of T and antigen, these cells can be stimulated to AFC clone formation but otherwise they do not divide. **G**: Mature precursor cells are tolerance resistant and do not normally divide. These cells express on their surface all B cell specific markers including MHC class II, B220, sIgM and sIgD. Upon stimulation, mature primary B cells give rise to AFC clones whereas mature precursors of memory cells give rise to memory B-cell clones.

As described in Chapter 13, the first critical molecular event in V gene expression is H chain V gene segment recombination and the generation of a productively rearranged H chain V region (14,18). The first known selective event in repertoire expression appears to be at the level of selection among nascent H chains, by virtue of their capacity to interact with surrogate L chains (V pre B + l5 (19,20,38,39 and 40). Cells with an appropriate nascent H chain form a pre-B cell receptor comprised of H chain, surrogate L chain and Iga and Igb. Such cells undergo at least 5-6 divisions prior to L chain V gene segment rearrangement (Fig. 14.1) (20,41). This process favors both the expansion of cells whose nascent H chains can interact with L chains and the expression of clones of B cells with identical H chains but a variety of L chains (20,42). Because the heavy chain-surrogate light chain complex is expressed on the surface of developing B cells, environmental selection by virtue of recognition of such complexes may also be possible (31).

The brief stage of B cell development following light chain gene segment recombination, wherein sIg expression is initiated, is the most critical with regard to repertoire selection. At this stage, immature B cells are highly susceptible to sIg receptor-mediated inactivation by environmental antigens (43,44 and 45) or anti-idiotypic antibodies (46,47 and 48). Furthermore, it may be at this stage that other receptor mediated interactions determine whether emerging cells will be long- or short-lived and what functional subpopulations they will enter (49,50 and 51). In addition to clonal inactivation of cells whose sIg receptors have high affinity for self-antigens, newly emerging B cells are also susceptible to a process called receptor editing (52,53 and 54). By this process, cells whose sIg receptors interact with self-antigen down-regulate their receptor expression, up-regulate or maintain RAG expression, and initiate further L chain rearrangement. Cells that are successful in expressing a new L chain re-express this L chain with their original H chain, thus creating a new receptor. If the "new" receptor does not recognize self-antigen the B cell can continue its maturation.

B-cell repertoire development during the fetal-neonatal period differs substantially from that described above for adults. In fetuses and neonates B-cell development occurs primarily in the liver and spleen. Although the sequence of gene segment recombination events is similar, the expression of various markers differs between neonates and adults (55,56). Furthermore, tolerance susceptibility of immature adult bone marrow B cells markedly decreases within hours of sIg expression (43), whereas neonatal sIg^+ cells appear to remain tolerance-susceptible for several days (44). Finally, repertoire diversity is far more restricted in neonates than adults (57,58,59,60,61 and 62). Indeed, among neonates of an inbred murine strain, not only do individuals express a limited clonotype repertoire (10^5), but also much of the same limited repertoire is reproducibly expressed from individual to individual. One reason for the limited neonatal repertoire is that neonates selectively overutilize certain V_L and V_H gene segments (especially V_H gene segments that are D_H proximal). However, the major basis for restricted repertoire expression during the fetal and early neonatal periods is the lack of TdT in developing fetal B cells and consequently a lack of N additions at the V_H-D and D-J_H junctions of the V_H regions (63,64 and 65). More than 95 percent of adult bone marrow B-cell precursors have N additions: as a consequence, diversity of the third complementarity determining region of heavy chains (HCDR3) exceeds 10^5 sequences for any pair of rearranged V_H and J_H gene segments (20). Because there are 10^2 to 10^3 V gene segments and four J gene segments, total V_H region diversity exceeds 10^8 sequences (20). In contrast, more than 95 percent of early neonatal B cells have no N additions in their heavy chains (63,64 and 65). The absence of N additions not only limits the potential for sequence diversity but also favors V_H-D and D-J_H recombinations wherein small overlaps of identical sequences occur in the 5' and 3' ends of the recombining gene segments (63,64,65 and 66). Thus, a few D-J_H and V_H-D junctional sequences frequently recur, which greatly limits HCDR3 diversity (64,65). Because sequences with a paucity of N additions recur within and among individuals (predominant clonotypes) and are often found to be specific for bacterial antigens or immunoglobulin idiotypes (66,67), they may represent specificities that are evolutionarily conserved (68,69,70 and 71). This situation is unlikely to be the case for the repertoire of clonotypes comprised of heavy chains with N additions, as such clonotypes should rarely recur whether with or among individuals (20).

B-CELL SUBPOPULATIONS

B cells are expressed as three subpopulations that differ functionally, developmentally, and in their expression of cell surface antigens. Each of these subpopulations can be perceived as occupying a distinct functional niche. Because the progeny of all three subpopulations can isotype switch, the antibody products of all three subpopulations contribute to the biologic effector functions that are peculiar to each immunoglobulin isotype (see Chapter 13).

"Conventional" Primary B Cells (B-2 B Cells)

"Conventional" primary B cells comprise most of the B cells in nonimmune adult mice. Generation of these cells from adult bone marrow is described above. Although as many as 5×10^7 of these cells may be generated daily from the bone marrow, most appear to be short-lived, however, selected longer-lived cells ultimately represent most B cells in the spleen and other peripheral tissues (26,28,51,72 and 73). Repertoire diversity among cells of this subset is extensive, as more than 95 percent express heavy chains with N additions at their V-D and D-J junctions. This subpopulation is characterized by expression of intermediate to high levels of cell surface IgM, IgD, MHC class II antigens, B220, and the cell surface heat-stable antigen (HSA) recognized by the J11D monoclonal antibody (74,75,76,77 and 78). These cells respond vigorously to various mitogens and to all categories of antigens including carbohydrates, bacteria, viruses, proteins, and hapten-protein complexes. Upon T_H-dependent stimulation, these cells give rise to AFC clones expressing multiple immunoglobulin isotypes; the progeny of these cells do not accumulate somatic mutations, however, nor do they generate memory B cells (77,79,80). Because of their abundance, broad distribution throughout the lymphoid system, and extreme repertoire diversity, "conventional" primary B cells are the subpopulation best suited to provide an early response to a broad array of pathogens. They would be particularly relevant in responses to pathogens such as viruses wherein repertoire diversity is essential to ensure determinant recognition (71,81).

CD5 B Cells (B-1 B Cells)

Like "conventional" B cells, stimulation of CD5 B cells, which represent a small proportion of B cells except in neonates and the peritoneal cavity of adults (43,76,82,83), give rise to AFCs but not germinal centers or memory B cells (77,80). These B cells can be distinguished from "conventional" B cells by their low levels of surface CD23 and their expression of CD43. Additionally, many of these cells express CD5 (T-cell marker) and Mac1 (macrophage marker). Among B cells, these cells are unique in that they produce high levels of IL-10 and may require IL-10 for their growth (43). Although under appropriate conditions these cells can respond to the same spectrum of antigens as "conventional" B cells, they respond more vigorously to bacterial antigens (43,76,82,83). In addition, these cells spontaneously produce high levels of IgM

antibodies being largely responsible for such antibodies in nonimmune serum.

Because B cells of the CD5 subpoplation can be generated from adult bone marrow, their repertoire may include V_H genes containing N additions and, therefore, extensive diversity (63). However, this subpopulation is enriched for cells whose V regions lack N additions (43,83). This appears to be the result of both selection of cells with certain V genes as cells acquire sIg receptors in the bone marrow (50,52) and because cells of this subpopulation, generated during fetal and neonatal development, self-renew and thus persist into adulthood (43,83). Although the repertoire generated in the absence of N additions is relatively restricted, the resultant antibodies are often antibacterial, and the B cells bearing such clonotypes may be present at high frequency (68). Therefore, such antibodies and the CD5 B cells that produce them may play a critical role in antibacterial responses, particularly when high concentrations of antibody are needed.

Memory B Cells

Memory B cells are the subpopulation of precursors whose generation requires antigenic stimulation. Memory B cells, like primary "conventional" and CD5 B cells, respond to antigenic stimulation by the generation of AFC clones. Also, as with primary B cells, the clonal progeny of these cells readily isotype switch (84,85). Memory B cells can be distinguished from primary B cells in that they express low to intermediate levels of HSA (74) and no surface IgD (84,86). Although some memory B cells express sIgM (85), most express a surface immunoglobulin other than IgD or IgM (84,85 and 86). Memory B cells are more likely than primary B cells to recirculate from the lymph to the blood and populate lymph nodes (27).

The most marked differences between memory and primary B cells is that the variable region sequences of memory B cells are generally highly mutated (87,88,89,90,91 and 92). These mutations accumulate by virtue of the process of somatic hypermutation that accompanies the generation of memory B cells in GC after antigenic stimulation (see Chapter 2).

Memory B cells appear to originate mainly from a small subset of primary precursors generated from the bone marrow of adult mice (77,79,80). These precursors of memory B cells are characterized by having relatively low cell surface levels of HSA and high levels of sIgD (77). Unlike primary CD5 and "conventional" B cells, upon stimulation these cells do not give rise to AFCs, but rather, generate clones of secondary B cells (77,79,80). Also, unlike primary B cells these precursors efficiently originate germinal centers and their progeny somatically hypermutate (77,79,80).

The V gene repertoire of the memory B-cell subpopulation includes sequences with N additions and those without. Thus, responses of this subpopulation are likely to be inclusive of both conserved and highly diverse clonotypes. Although the spectrum of clonotypes available for primary immune responses is likely to be inclusive of V regions that recognize most relevant antigenic determinants, the quality (affinity spectrum) of responses to any given determinant may be suboptimal. The special function of the precursor cell subpopulation that generates memory B cells may be perceived as providing the potential to refine and improve the clonotypes that recognize invading organisms by the combined process of somatic hypermutation and antigen selection. Because memory may be retained for months to years, this subpopulation also appears specialized to act as a repository for high affinity responses against microorganisms proved by previous contact to be a threat to the individual.

B-CELL TRIGGERING

Although B cells can be stimulated nonspecifically, the specificity of immune responses derives from the ability of any given antigen to selectively stimulate, from among a vast array of B cells, those whose sIg receptors interact with that antigen with sufficient affinity. This pivotal role in immunologic specificity of the sIg receptor is reflected in its role in B-cell triggering.

The sIg receptors contain the V region clonotype of the cell's productive V_H and V_L rearrangement and the membrane form of IgM and IgD, which are created by appropriate splicing of the immunoglobulin RNA transcript (see Chapter 13). Memory B cells can express sIg receptors of immunoglobulin isotypes other than IgM or IgD, which also represent an alternate splicing of RNA that provides a membrane insertion region. Regardless of the surface isotype, B cells express approximately 10^5 sIg receptor molecules. The sIg receptor functions as both a triggering receptor for B cells and a device for concentrating antigen on the B cell surface for either mitogenic stimulation or internalization, processing, and presentation to T cells.

Role of sIg as a Triggering Receptor

The triggering of B cells via the sIg receptor requires receptor cross-linking and appears maximized by the stable interlinkage of several receptor molecules in a complex with antigen (5,44,93,94). The aggregation of several such complexes can be visualized on the B cell surface as patches of receptor-antigen complexes (*patching*) (2). Owing to cell motility and cell surface fluidity such patches can aggregate to one pole of the cell (*capping*) (2). Although receptor interlinkage is a requisite for sIg-mediated B-cell triggering, neither patching nor capping appear to be essential for this process.

Receptor cross-linking by antigen is maximized by multivalent presentation of antigenic determinants and by high affinity receptor-determinant interactions. Multivalent antigen presentation is readily accomplished by bacteria, viruses, mammalian cells, certain polymeric macromolecules, and hapten-protein complexes, all of which can present multiple repeats of a relevant antigenic determinant. Smaller molecules, such as monomeric proteins, generally express only a single copy of each determinant but can be polymerized if bound by antibodies or on cell surfaces, which would enable them to be immunogenic.

For sIg receptor-mediated B-cell triggering to proceed, the affinity of the receptor-ligand interaction must exceed a minimum threshold affinity (5,44,62,93,94). Thus, low-affinity interactions are not stimulatory even when multiple sIg receptors participate, presumably because the stability of engagement of each receptor within the complex is not sufficient.

Although both sIgM and sIgD exist as complexes with isoforms of the intracytoplasmic accessory molecule Iga and Igb (33,34 and 35), sIgM appears to be the principle triggering receptor of primary B cells. The cascade of intracellular events initiated by sIg receptor engagement are described in Chapter 8. The stable interlinkage of sIg receptors rapidly induces protein tyrosine phosphoylation, the breakdown of phosphatidylinisitol biphosphate, and the resultant activation of protein kinase C and increased cytoplasmic Ca^{2+} (95,96). However, B-cell stimulation to proliferation and AFC generation generally requires engagement of other cell surface receptors such as those that mediate T_H-B cell interactions or mitogen receptors (95) (see Chapter 9). The functional consequences of interlinking a B cell's sIg receptors is most apparent in the process of B-cell inactivation by antigen (*tolerance induction*). This process is generally mediated by sIg receptor engagement alone. As is discussed below, relatively immature B cells or newly generated adult primary or memory B cells, appear to be most susceptible to sIg receptor-mediated inactivation (44,45,93,97). Although most of the subcellular events triggered by sIg receptor engagement appear to be similar for mature and immature B cells, immature cells show a much higher propensity for "receptor editing" (53), exhibit altered phosphatidylinisitol hydrolysis and may fail to activate the egr growth competence gene which appears necessary for activation of mature B cells (98,99). Whether these differences correlate with the increased sIg receptor mediated tolerance susceptibility of immature B cells is not yet known; however, the inactivation process of any of these immature cells can be reversed by concomitant T_H-mediated stimulation.

sIg Receptor-mediated Antigen Presentation

B cells are generally stimulated through collaborative interactions with T_H (see Chapter 9). This process requires that antigen be internalized by B cells for processing and presentation of the resultant peptides in association with class II MHC molecules (see Chapter 3). Although in the presence of high antigen concentrations internalization can ensue via pinocytosis, the usual means of concentrating and internalizing antigens is via their binding by the B cell's sIg receptor (100). This process ensures that B cells, whose receptors are specific for a given antigen, present peptides of that antigen in their MHC molecules (see Chapter 6). By this process B cells with high affinity receptors for an antigen are stimulated selectively, especially at low antigen concentrations (100). Furthermore, antigens that present determinants multivalently and thus bind to sIg receptors multivalently stimulate B cells more efficiently because of the increased affinity of such interactions. However, unlike triggering via the sIg receptor per se, the mode of B cell triggering that utilizes the sIg receptor as an antigen concentration and internalization device requires neither that the receptor-ligand interaction exceed an affinity threshold nor that antigenic determinants be presented multivalently. Thus, although high affinity and multivalent interactions are advantageous, this triggering mode can be accomplished by high concentrations of low affinity monovalent interactions.

Numerous macromolecules contain determinants that can interact with one of the myriad non-sIg receptors on the surface of a B cell, including receptors for mitogens (see below) and for immunoglobulin constant regions (Fc receptors). An additional mode by which B cell sIg receptors function in B cell triggering is by concentrating such macromolecules on the cell surface, thereby enhancing their interaction with other B cell surface triggering receptors (101). In some instances, such as with mitogens, these interactions facilitate stimulation of B cells whose receptors interact with determinants on the macromolecule; they thereby serve as an important component in the generation of specific immune responses (101). In other instances, such as with focusing immunoglobulins on the cell surface for interactions via Fc receptor binding, the interaction may foster either B cell activation or, more often, inactivation (102). Because the V regions of sIg may bind the same antigen as serum antibodies they can concentrate immune complexes on the B cell surface and in so doing would facilitate triggering via immunoglobulin-Fc receptor interactions (103).

T Cell-B Cell Collaboration

B cell stimulation is maximized by the collaborative interaction with antigen-specific T_H (see Chapter 6 and Chapter 9). At the core of these interactions is the dedication of T receptors to the recognition of class II MHC-peptide complexes and the efficiency with which B cells, by virtue of their sIg receptors, bind antigens for internalization processing and presentation.

Although T_H-B cell interactions maximize B cell stimulation, the effects of such interactions on the participating T cell depend on the state of activation of both B and T cells. On activation, B cells up-regulate cell surface co-receptors for T cell activation (e.g., B7) (see Chapter 2, Chapter 3, Chapter 4, Chapter 5, Chapter 6, Chapter 7 and Chapter 8). These up-regulated co-receptors appear to be essential for stimulation of collaborating T cells because in the absence of co-receptor participation resting T cells can be inactivated by interacting with antigen-presenting B cells (103).

The mutual stimulatory interaction of B and T cells mediated through T_H recognition of the B cell's class II MHC-peptide complex and co-receptors results not only in triggering of the B cell to initiate the cascade of intracellular events that lead to proliferation and differentiation but also the stimulation of T_H cells to release interleukins. The composition of the set of secreted interleukins is determined by the subset of participating T_H in that T_H cells release IL-2 and interferon-g, and T cells release IL-2 as well as IL-4 and IL-5. The importance of the released interleukins is not only their role in propagating T_H and B cell proliferation but also their capacity to selectively enable the secretion of antibodies of various isotypes.

In the RNA of primary B cells the V region is juxtaposed to the first domains of the µ constant region. These cells express an RNA transcript that encodes the V region as well as the µ and d constant domains (see Chapter 1). Primary B cells splice this RNA such that the predominant form of mRNA encodes the V_H region in conjunction with the membrane from the µ and d constant regions. Upon T_H-dependent antigenic stimulation, RNA synthesis is increased and RNA splicing changes to greatly favor the expression of V_H in conjunction with the secreted form of the µ constant region. The physical interaction of T_H and B cells also appears to affect the DNA encoding some of the other heavy chain isotypes in that the accessibility of their switch regions and their capacity to generate sterile transcripts increases (104,105). This situation, in conjunction with the action of various interleukins, enables the V region to splice with switch regions of downstream isotypes; it also enables expression of immunoglobulins comprised of the B cells' V regions with either membrane or secreted forms of isotypes other than µ or d (104,105).

Nonspecific B-Cell Activation

B cells have surface receptors that, upon interaction with moieties on various microorganisms, induce proliferation. Such moieties are deemed mitogens, and often such mitogenic stimulation yields AFC formation (101). Neither engagement of the sIg receptor nor T_H recognition is required for this form of B-cell activation (see Chapter 1). Although not all B cells have receptors for all mitogens, when a high concentration of a given mitogen is present, as many as 30% to 50% of all B cells can be stimulated. In these cases a highly diverse array of antibodies can be produced (*polyclonal stimulation*). Physiologically, this form of stimulation is most relevant when low amounts of the mitogen are present. In this case, the sIg receptors of B cells that recognize other determinants on the microorganisms concentrate the mitogen to the surface of those B cells, facilitating mitogenic stimulation and the production of antibody against the pathogen. This form of stimulation plays an important role in responses where T cell help is not available presumably because of a paucity of peptides derived from the antigen that would be appropriate for class II MHC presentation. Because of an absence of T_H-B cell interaction with this form of stimulation, isotype switching and memory B-cell generation are minimal.

B-Cell Differentiation and Regulation

On binding antigen to a B cell's sIg receptors, activation can ensue by several pathways. If antigenic determinants are presented multivalently and are bound with high affinity, the stable interlinkage of sIg receptors can initiate the cascade of intracellular events described above. Although this process does not necessarily lead to B-cell proliferation, it can enhance this process and alone can initiate the inactivation of immature cells.

The binding of antigen by sIg, whether monovalent or multivalent, can also facilitate the engagement of the major pathways of B-cell stimulation, antigen processing for T_H collaboration, and mitogenic stimulation. Once stimulated, B cells initiate mitosis, dividing every 8 to 16 hours for several days. These cells begin to synthesize and secrete IgM antibodies. At the DNA level, cells stimulated via interaction with T_H begin to express their V regions in conjunction with downstream constant regions (see Chapter 13). This is generally accomplished via the process of switch recombination, wherein a switch region down-stream of the rearranged V region is spliced with an accessible switch region upstream of a g, a, or e constant region with the excision of intervening constant regions. This process can be repeated in single cells by further rearrangement to more downstream isotypes. The expressed downstream isotype appears, at least in part, to be dictated by the set of interleukins produced by the collaborating T cell or surrounding tissues.

Clonal analyses of B cells stimulated individually have demonstrated that essentially all primary B cells can generate progeny expressing the same V region in conjunction with any constant region isotype (106,107). Although individual AFCs may retain mRNA for more than a single isotype, eventually they are committed to the production of only one isotype. As AFC clonal differentiation proceeds, sIg decreases, the concentration of polysomes increases, the cell's nucleus becomes smaller, and the cell becomes a plasma cell. Ultimately, these cells no longer divide, although they can survive and continue to secrete antibodies for several days to several weeks.

GENERATION OF MEMORY B CELLS

A hallmark of the immune system is the capacity to mount more rapid and vigorous humoral immune responses upon second contact with an antigen. This enhanced responsiveness is the result of numerous alterations in the immune system resulting from the first (primary) antigen contact. Subsequent to primary antigen contact, the frequency and reactivity of T_H that recognize peptides of that antigen in the context of class II MHC molecules is greatly enhanced. Additionally, antibodies generated as a result of primary B-cell stimulation have had the opportunity to adhere to the surfaces of macrophages and dendritic cells, arming such cells to better bind and process antigen. Most importantly, substantive changes in the B-cell population eventuate from primary contact with antigen.

Because of their stimulation by initial antigen contact, primary B cells generate AFC and terminally differentiate, so their frequency is generally decreased after primary stimulation. Furthermore, presumably in response to the high levels of primary antibodies present after this initial stimulation, anti-idiotypic reactivity is generated and suppresses subsequent responses by primary B cells (108,109). However, as a consequence of primary antigenic stimulation, memory B cells are generated, ultimately exceeding the frequency of the original primary B cells by a few-fold up to several hundred-fold (5,23).

The dramatically improved reactivity of the B-cell population subsequent to primary stimulation is not due solely to the increased frequency of B cells responsive to that antigen; it also reflects qualitative differences between primary and memory B cells. Functionally, memory B cells differ from primary B cells in that their receptors and potential secreted antibody products can be of isotypes other than IgM or IgD (84,85 and 86), they are more readily stimulated (5), their stimulation is resistant to anti-idiotypic down-regulation (108), and they often accumulate in the bone marrow wherein their plasma cell progeny can secrete antibody for several months (110). Most significant is that, as a consequence of a gradual increase in the affinity for antigen during the course of the primary response (immunologic learning), the affinity for antigen of the antibody products of memory B cells is significantly higher than that of primary B cells (111). This is the result of two ongoing processes that accompany the generation of memory B cells. First, presumably owing to progressively decreasing concentrations of the immunogen and increasing amounts of serum antibody that could compete for antigen with sIg receptors, there is a progressive selection for B cells whose sIg receptors are of higher affinity (5,111). Second, within a few days of their stimulation, precursors giving rise to memory B cells begin to somatically hypermutate their rearranged V region genes. Mutations accumulate at a rate approaching one mutation per 10^3 base pairs per division (88,89,90,91 and 92). Whereas most mutations do not directly affect antigen binding and some are deleterious, occasional mutations increase affinity for antigen. B cells expressing such antibodies would be favored by antigenic selection. Evidence of the significance of somatic mutations to antigenic selection of memory B cells comes from analyses of accumulated somatic mutations in high affinity memory B cells. Mutations yielding amino acid substitutions can be shown to cluster in complementary determining regions (CDRs), whereas silent mutations are favored in regions that would not affect antigen binding (88,92).

The alteration of antigen-combining sites by somatic mutation might be expected occasionally to create new anti-self specificities. As is discussed below, newly emerging memory B cells, like newly emerging primary B cells, appear to pass through a phase of high tolerance susceptibility wherein memory B cells expressing such anti-self specificities could readily be inactivated (97,112,113).

The generation of memory B cells is a complex process that requires participation of at least three cell types and occurs in special sites within lymphoid follicles called germinal centers (80,86,114,115). In addition to the stimulatory T_H-B cell collaborative interaction, the progression of memory B-cell generation appears to require a dendritic cell within the lymphoid follicles [follicular dendritic cells (FDCs)] that is specialized for antigen presentation via antibodies that coat small bodies (iccosomes) along their extended processes (114). In the milieu of T_H and FDCs, newly emerging memory B cells rapidly divide (every 6-12 hours) to form a cluster of B cells that

are the clonal progeny of one or a few original precursors (115,116 and 117). These clusters of cells include numerous dividing cells, many dead cells, and cells that appear to have ceased dividing. Cells being generated in germinal centers acquire several activation markers, including receptors for peanut agglutinin, which distinguish them from cells in surrounding tissues.

The cellular origins of memory B cells remains a controversial issue. Evidence indicates that, although it is possible that cells giving rise to primary AFCs can also give rise to memory B cells, most memory B cells are derived from a separate precursor cell subpopulation (77,79,80). By enriching for cells from the spleen of nonimmunized mice on the basis of their low expression of HSA and high expression of sIgD, it has been possible to identify a subpopulation comprising 10-20% of all precursor cells (77). These cells appear unable to give rise to AFCs after primary antigenic stimulation, but they do give rise to memory B cells. Thus, if these cells are antigenically stimulated *in vivo* after being transferred along with primed T_H to mice with severe combined immunodeficiency (SCID) that have no T or B cells (118), they give rise to germinal centers and memory B cells but not serum antibody (77,80). Such mice respond by the generation of AFCs, serum antibodies, and fusion partners for hybridomas only after a second antigenic stimulation. Similarly, if stimulated *in vitro*, these HSA^{lo} precursor cells generate clones of memory B cells but no primary AFC (77). Additionally, the progeny of these cells accumulate somatic mutations both *in vitro* and *in vivo* (77,79). Conversely, under the same experimental conditions, “conventional” primary B cells and $CD5^+$ B cells give rise to AFCs but not germinal centers or memory B cells (77,79,80).

Following primary immunization newly generated antigen-responsive secondary B cells can be detected with 4 days (77). Although these early memory B cells have not accumulated somatic mutations, their sIg receptors may reflect isotype switching. Furthermore, because antigen is often retained for several days, these cells may be stimulated to AFC formation as early as the second week during the course of a primary immune response. Thus, depending on the availability of antigen and T_H, the immune response to a single dose of antigen is likely to progress from domination by the AFC progeny of primary B cells during the first week or two to a response dominated by the AFC progeny of newly generated memory B cells at later stages.

After a secondary B-cell response subsides, animals retain memory for the immunizing antigen; a third or fourth contact with antigen elicits tertiary or quaternary responses. These responses are often more vigorous and of higher affinity than the secondary response. Indeed, even somatic mutations appear to contine to be induced after several courses of immunization (79,88,89,117). One component of the immune system that appears essential for the maintenance and propagation of memory is the retention of antigen on FDCs (114). However, the responsible cellular mechanism may reflect in part the capacity of the subpopulation of cells that give rise to memory B cells to also generate new precursors of memory B cells. These “immune” precursors of memory B cells, which can be isolated from the spleen of immunized mice, differ from “nonimmune” precursors of memory B cells found in naive mice in that they are generally isotype switched and bear somatically mutated receptors. Thus, after immunization two precursor cell types dominate the repertoire, both of which are somatically mutated and isotype switched (negative for sIgD): (a) secondary B cells, which can give rise to AFCs; and (b) precursors to tertiary B cells, which cannot give rise to AFCs but whose progeny can further somatically mutate and generate germinal centers and tertiary B cells. Cells of this precursor lineage would continue to propagate responses upon multiple antigen contact.

SELF-NONSELF DISCIMINATION BY B CELLS

A major challenge for immune recognition is the generation of a repertoire that is sufficiently diverse to ensure recognition of myriad foreign antigens but devoid of clonotypes that recognize the vast array of self-antigenic determinants. This challenge is compounded by the fact that most relevant determinants, both foreign and self, represent constellations of amino acid or carbohydrate residues, and the distinction between foreign and self may be subtle.

Numerous mechanisms are utilized to minimize the existence or stimulation of B cells reactive to self-antigens. Because B-cell stimulation by most antigens requires T_H participation, in some instances B cells that recognize self-antigens do not respond because the relevant T_H cells are tolerant or their reactivity is suppressed. However, when T_H reactivity to a self-antigen is absent, B cells that recognize that antigen have the potential for being activated by the presentation of determinants of that self-antigen on a mitogenic macromolecule or on a nonself carrier whose peptides can be recognized by T_H. Therefore, mechanisms are available that render inactive B cells that recognize self-antigens (*peripheral tolerance*) or preclude them from entering the repertoire (*central tolerance*).

Peripheral inactivation of mature B cells can occur in several ways. Stimulation of B cells can be prohibited by the action of either antibodies or T cells that recognize idiotypic determinants on the sIg receptors (*idiotypic* or *network suppression*) (108,109,119). Similarly, occupancy of B-cell receptors by molecules that cannot be internalized or processed and presented as peptides can also prohibit B-cell stimulation (*antigen blockade*) (120,121). Additionally, monovalent antigens that cannot interlink sIg receptors can block access of the cell's sIg receptors to immunogens (5). In some cases, a blockadelike effect can be obtained with antigen-antibody complexes (102). Finally, certain interactions of B-cell receptors and multivalent (and possible monovalent) antigens have the capacity to induce a temporary state of nonreactivity wherein the B cell's sIgM (but not sIgD) receptors may be down-regulated (122). This state, called *anergy*, may be reversed with time or by providing the B cells with excessive mitogenic or T_H stimulatory signals (123).

The principal means by which self-reactive clonotypes are purged from the B-cell repertoire is central tolerance, which appears to represent the permanent inactivation or elimination of B cells. This inactivation is induced by the stable cross-linking of sIg receptors by multivalent antigens in the absence of T-cell help (44,45,93,97). Although mature B cells may be inactivated by the continuous high concentration of certain cell-surface antigens (121,124), nonbiodegradable macromolecules (120), or antigens that can engage Fc receptors (antigen-antibody complexes) (102), under most circumstances central inactivation obtains only to newly developing B cells (44,45,123). This dichotomy in the susceptibility to tolerance induction of immature versus mature B cells was anticipated many years ago by Lederberg as a means to explain the difference between self and nonself antigens (125). By these precepts, self is defined as antigens that are present in the milieu of newly developing B cells, whereas substances (even determinants of sequestered self-moieties) that are not in this milieu would first encounter B cells after they had matured and would be recognized as foreign. Thus, the discrimination of self versus nonself would be determined by the maturity of encountered B cells and not by any structural features of the antigen. The attractiveness of this mechanism is that, although self-macromolecules vary enormously in structure and concentration, if present in the environment of developing B cells they all could eliminate emerging B cells that recognize their antigenic determinants.

The general rules for the induction of tolerance appear equally applicable to newly developing neonatal, bone marrow, or memory B cells. These rules include the requirements that determinants be presented multivalently (44,93,94,97), that the affinity of interaction exceeds a minimum threshold (62,93,97), and that tolerance can be circumvented by concomitant T cell help (44,97,122). It should be noted that, except in the case of newly generating memory B cells, antigen reactive T_H would rarely be present in the milieu of developing B cells. Newly generating adult bone marrow B cells are tolerance-susceptible for only the first several hours after sIg expression, whereas neonatal B cells appear tolerance susceptible for several days (44,45). Newly generating memory B cells may also have a prolonged period of tolerance susceptibility and appear to have a lower affinity threshold for tolerance than primary B cells, as they are susceptible to inactivation by cross-reactive determinants (97). Indeed, the affinity threshold for tolerance induction of newly generating primary B cells is sufficiently high that low affinity antigen-stimulatable cells are likely to escape the tolerance gauntlet (62,93,94). As mentioned above, another unique mechanism termed “receptor editing” has been shown to play a major role in eliminating self-reactivity among newly generated bone marrow B cells (53,54 and 55). By this mechanism, cells whose sIg receptors recognize self-antigens are induced to further rearrange L chain genes and ultimately express a receptor with altered specificity due to the complex of their H chain with a non-L chain. This mechanism appears to obtain to cells soon after sIg expression and cells remain susceptible through their later stages of bone marrow maturation wherein cells have been characterized as “transitional cells” (126). This transitional cell stage, which is characterized by high HSA and IgM expression and low to negative IgD expression, is also characteristic of 5%-15% of splenic B cells that have recently emigrated from the bone marrow. However, once in the spleen these cells no longer appear competent to undergo receptor editing. Since both inactivation and “receptor editing” may be applicable at the same B-cell maturational stages, and cells that attempt but fail to receptor edit may be inactivated in any case, the relative contribution of these mechanisms to the elimination of anti-self reactivity is not yet known.

Given the requisites for central inactivation of newly emerging primary B cells, it would be anticipated that potentially self-reactive cells could escape tolerance induction and mature. It would be especially true of cells with relatively low affinity for self-antigens and those reactive to monovalent self-determinants. Because of this it is neither surprising that reactivity to self-antigens can be found among mature B cells (127,128), nor that numerous peripheral mechanisms are available to ensure against the generation of anti-self antibodies.

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15 ADHESION MOLECULES

Michael L. Dustin, Ph.D.

Adhesion molecules play key roles in mediating and regulating cell-cell interactions required for initiation of immune responses and for effector functions and leukocyte migration (1,2 and 3). Beyond the immune response, adhesion molecules are also critical for development, tissue integrity, blood coagulation, and wound healing (4). Adhesion molecules are cell surface receptors that physically link cells and transduce signals across the cell membrane (5). The processes of adhesion and signaling are tightly linked since signaling regulates adhesion (inside-out signals) and adhesion receptors generate signals (outside-in signals). This chapter will focus on a discussion of four examples of leukocyte adhesion that are presented as current paradigms of adhesion in the immune response. These are leukocyte extravasation, leukocyte migration, cell-mediated killing, and immunological synapse formation. These biological examples will be followed by a discussion of the major adhesion molecule families that participate in these interactions. Finally, the mechanistic basis of adhesion regulation will be addressed. An effort will be made to integrate the structural and mechanistic information back to the functional paradigms. Particular attention will be focused on diseases effecting adhesion in the immune system and what these have taught us about molecular mechanisms.

It is common in biology to define interacting molecules as receptors and ligands, however, in cell-cell interactions it is often difficult to make this distinction since both interacting molecules on apposing cells may be of similar size and may both be capable of transducing signals. Thus, we will use the term receptor and counter-receptor to describe the pair of molecules in an interaction. The time scale for the cellular interactions spans the range from fractions of a second to hours or even days. However, the underlying molecule interactions are almost always transient with half-lives on the order of 0.1-30 seconds (6). These surprising molecular dynamics are correlated with the requirement that all the important cell-cell interactions are eventually reversible. Thus, the long duration of some cell-cell interactions in the immune response is not determined by the duration of the molecular interaction, but by how large numbers of transient molecular interactions are organized to create a stable interface. Figure 15.1 outlines a continuum of four immunologically important cellular interactions in terms of their duration and relates this to the duration and number of the dominant molecular interactions. As can be seen, duration of cellular interactions is better correlated with the number of interactions than with their individual duration. An understanding of molecular interaction in cell adhesion requires an appreciation of both the intrinsic molecular interactions and the geometry and forces in the interface between the interacting cells. The molecular interactions can be characterized in terms of solution affinity and kinetic constants that can be determined experimentally by surface plasmon resonance. This technique is well suited to the low affinities characteristic of adhesive interactions (7,8 and 9). An even more difficult problem is the analysis of the molecular interactions in contact areas. We will focus attention on these issues throughout the chapter. The following sections focus on these four types of cellular interactions: rolling adhesion, migration, killer cell conjugates, and immunological synapse formation.

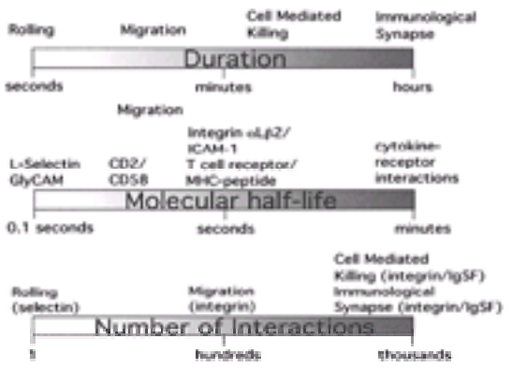

Figure 15.1. Cellular and molecular scale of adhesive interactions. IgSF = immunoglobulin superfamily.

FUNCTIONAL CONTEXTS OF CELL ADHESION IN THE IMMUNE RESPONSE

Leukocyte Extravasation

The process of leukocyte extravasation is critical for host defense against pathogens that enter the tissues. Leukocytes must enter tissue sites from the blood to isolate and clear these pathogens. Lymphocytes must enter lymph nodes and tissues to encounter antigen-presenting cells and cells infected with intracellular bacteria or viruses. Normal development of the hematopoietic system requires the migration of stem cells from the fetal liver to the bone marrow. In all of these processes, the challenge is to take cells that are flowing rapidly in the blood and have them arrest on the endothelial surface in a site-specific manner. The current paradigm for this process is based on multiple sequential adhesion steps with distinct molecular mechanisms (10,11). Figure 15.2 provides a schematic outline of the steps in the extravasation process. The first step is the tethering of the leukocyte to the vessel wall in a manner that allows the leukocyte to pause for a few seconds or to actually roll along propelled by the blood flow. The archetype molecular mechanism for this step is the interaction of receptors known as selectins with specific glycoprotein structures on the apposing surface (12). The time frame for this process is subseconds to a few seconds and one or a few molecular interactions are sufficient at any moment (13). The second step is the interaction of G-protein coupled receptors on the leukocyte with ligand on or near the surface of the endothelium. G protein coupled receptors, the same receptor type responsible for vision, are known for high speed signaling to the cytoplasm (14). In the third step, G-protein coupled receptors rapidly trigger the use of integrins, a class of adhesion molecules, with counter-receptors on the endothelium (15). The integrins on the activated leukocyte mediate the firm arrest within a few microns of the G-protein coupled receptor engagement. Hundreds or thousands of integrin to counter-receptor interactions mediate firm adhesion. Integrins also contribute to the movement of the leukocyte through intracellular junctions of the endothelium (16,17). This latter process is also facilitated by endothelial cells that respond to attached leukocytes through a contractile response that facilitates passage of the leukocytes (18). The individual steps of this multistep process are independently regulated and in combination can give rise to a large number of possible targeting or “area codes” for site specific leukocyte extravasation (19). Such combinatorial systems for guiding migration were previously proposed for the nervous system (20). Examples of area codes for site specific homing are included in Table 15.1.

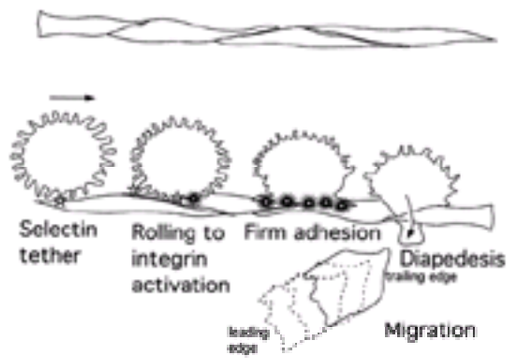

Figure 15.2. Leukocyte extravasation and migration. Five-pointed star = selectin interactions. Multipointed star = activating signals. Spread cells have many interactin interactions.

Target tissue	Tethering from flow	Activation	Arrest and diapedesis
Peripheral lymph nodes (naive and central memory T cells and naive B cells)	L-selectin/sulfated sialyl-Le^x or 6-sulfo sialyl-Le^x	CCR7-ligand(s) in lymphoid tissue chemokine (SLC, 6Ckine)	Integrin $\alpha_L\beta_2$ (LFA-1)/ICAM-1 and ICAM-2; integrin $\alpha_4\beta_1$ (VLA-4)/VCAM-1
Inflamed skin (effector memory T cells and monocytes)	PSGL-1 and/or sialofucosylated VLA-4	CCR4/TARC chemokine	Integrin $\alpha_4\beta_1$ (VLA-4)/ICAM-1 and integrin $\alpha_4\beta_7$ (LPAM-1)/ICAM-1 and ICAM-2
Mucosa (naive and subset of memory T cells that express β^7 integrin subunit)	CD44/hyaluronic acid and L-selectin/sulfotransferase 6 or 7/6-sulfo sialyl-Le^x	CCR9/TECK and CCR10/MEC	Integrin $\alpha_4\beta^7$ (LPAM-1)/MAdCAM-1, β^7 integrin $\alpha_E\beta_7$ (LPAM-1)/ICAM-1 and ICAM-2
Inflamed lung (subset of effector T cells [Th2] and eosinophils)	VLA-4/FIDAM-1	CC chemokine chemokine	VLA-4/FIDAM-1; β^7 integrin $\alpha_E\beta_7$ (LPAM-1)/ICAM-1 and ICAM-2

CCR = and C chemokine receptor; ICAM, intercellular adhesion molecule; LFA-1, leukocyte function antigen-1; MAdCAM-1, mucosal vascular addressin molecule 1; VLA, very late antigen.
These digits of [area code] are formed by combinatorial expression of receptors and counterreceptors on each step in extravasation. The format for each combination is expression/counterreceptor/FIMBO counterreceptor or ligand.

TABLE 15.1. Area Codes for Site-Specific Leukocyte Homing[a]

Leukocyte Migration

The migration of leukocytes is initiated by chemoattractants during extravasation and is sustained in the tissues by a hierarchy of chemoattractant and targeting signals (21,22). The environment in the tissue may include the surfaces of stromal and epithelial cells and an extracellular matrix. In contrast, the parenchyma of lymphoid tissues appears to be predominantly cellular, with no clearly defined extracellular matrix in contact with lymphocytes (23). Leukocyte migration requires adhesion molecule interactions and cellular polarity. The adhesion molecules that are utilized in migration include the integrins, CD44 and cell surface proteoglycans. Polarity is a cell biological concept that describes the difference behavior of one end of a cell from the other. In this context, one end of the cell is specialized for chemoreception and formation of adhesive interactions (the leading edge), while the other end is specialized to break adhesive interactions (the trailing edge) (Fig. 15.2) (24). The leading edge is rich in cytoskeletal proteins involved in actin polymerization, while the trailing edge is rich in myosin II, which may be involved in generating force that translocates the adhesive sites toward the trailing edge of the cells and for adhesion molecule detachment (25,26,27 and 28). Leukocytes can move at rates up to 10 µm/min in tissues (29). Since leukocytes are about 10 µm long this indicates that the longest duration of an adhesive interaction with another cell during migration is less than a minute. Migration involves a balance of directional cytoskeletal force and adhesion such that optimal migration may occur at relatively low levels of adhesion (30). When leukocytes reach a target cell or bacterium they may respond by stopping migration in response to high levels of cytoplasmic Ca^{2+} triggered by antigen receptors or other activating receptors (31).

Cell-mediated Killing

Cell-mediated killing is an important effector function carried out by leukocytes in tissues. Cell-mediated killing is directed at other nucluated cells and is triggered by T-cell antigen receptors or the immunoglobulin Fc receptors (32,33 and 34). The cells that carry out these functions are differentiated cytotoxic T cells, natural killer cells, neutrophils, and macrophages. Antigen receptors are very potent triggers for cell-mediated killing. It has been argued that a single T-cell receptor engaged by its counter-receptor, the MHC-peptide complex, can trigger the T cell to kill target cells (35). This great sensitivity is possible because adhesion molecules supply the preliminary interactions that are then augmented by antigen receptor signals (36,37 and 38). Adhesion molecules of the immunoglobulin superfamily such as CD2 and the integrin family such as LFA-1 play an important role in cell-mediated killing (Table 15.2). The killing mechanisms operate by triggering programmed cell death. The classical mechanism involves the use of perforin to form large channels in the target cell membrane through which specific proteases like granzyme B are introduced (39,40). The perforin and proteases are stored in granules in the cytoplasm of the killer cells and are discharged directly at the target in a process that relies on formation of a tight gasketlike adhesion zone into which the granules are exocytosed (Fig. 15.3) (41). The proteases do not digest the target per se, but trigger a protease cascade in the target that leads to DNA degredation and death by apoptosis within a few hours. A similar cascade leading to apoptosis can also be triggered by engagement of Fas on the surface of target cells by Fas ligand on activated T cells. The killer cell can trigger apoptosis in a few minutes and can engage and initiate death of many targets in the span of an hour (42,43 and 44). The peak junctional strength of the cytotoxic T cell–target cell interaction is so great that the cells are difficult to pull apart by mechanical means (45), but within a few more minutes the T cells can spontaneously separate to engage other targets. While the details of this signaling process are still poorly understood, the ability of adhesion mechanisms to display these rapid changes in avidity can be readily understood based on changes in killer cell polarity that accompany dissociation from one target to attack another.

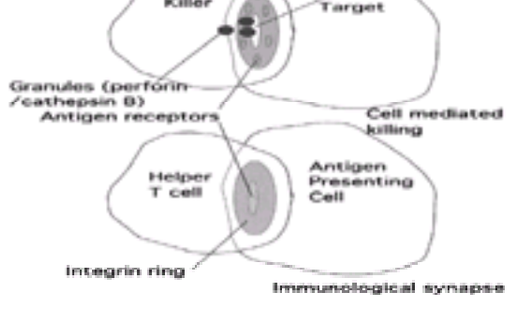

Figure 15.3. Cell-mediated killing and immunological synapse formation. Side-view showing the molecular pattern in the cell-cell interface at a small angle.

Receptor	Counterreceptor	Characteristics	Reference
CD2 (SRBC receptor)	CD58 (LFA-3) TIT3 on (mice) CD48 (rodents)	K_d 1 µM (k_{off} >5 s^{-1}); not regulated	[53a,179] [54]
3M	CD48 (human and rodents)	K_d 6 µM	[180]
Integrin $\alpha_L\beta_2$ (LFA-1, CD11a/CD18)	CD54 (ICAM-1), CD50 (ICAM-3), CD11a (ICAM-2)	K_d > 100 µM (k_{off} >1 s^{-1} to 0.03 s^{-1} [ligand induced]; Ac1: regulated	[57–59]
T-cell antigen receptor	MHC class I or II with cognate peptide	Vesicles: Ac1 0.1–40 µM (k_{off} < 0.1 s^{-1}	[1]
CD2a	gp120	K_d ~ 1 µM (k_{off} ~ 1 s^{-1}	[181]
ILAR and ILR	MHC class I allele; ILAR: cytotoxic killing and ILR: cytocidal killing	K_d ~ 1 µM (k_{off} ~ 1 s^{-1}); ILR has higher affinity than ILAC; ILR: form many interactions	[58,59,182]
CD8 (one or all forms)	MHC class I	Ac1↑ C_d↑ Ac1: regulated	[183,184]

ICAM, intercellular adhesion molecule; Ig, immunoglobulin; ILAR, killer cell activating receptor; K_d, dissociation constant; ILR, killer inhibitory receptor; LFA-1, leukocyte function antigen-1; MHC, major histocompatibility complex.

TABLE 15.2. Characteristics of Molecular Interactions in Cell-Mediated Cytotoxicity and Immunologic Synapse

Immunological Synapse Formation

The induction of T-cell help is perhaps the most critically regulated process in the adaptive immune response. The reason for this care is that the decision to mount an immune response and the nature of the response itself rests with these cells. Naive CD4 T cells are only activated by “professional” antigen presenting cells in a process that requires up to 20 hours of contact (46). Activated helper T cells are required for production of most antigen specific immunoglobulins by B cells, the ability of macrophages to kill intracellular bacteria and the generation of killer T cells (47). The long duration of the interaction appears to present a barrier to helper T-cell activation that may increase the fidelity of the process (48). The long duration interactions of helper T cells may also be important for the ability of the T cells to regulate the behavior of B cells, macrophages, and dendritic cells. Helper T cells direct secretion of cytokines at antigen bearing cells to deliver help for a variety of responses with high specificity (49,50). Bill Paul coined the term immunological synapse to describe this specialized cell-cell junction and its directed secretory function (47). The ability to sustain contact for prolonged periods is correlated with a specific molecular pattern in the interface between the T cell and antigen presenting cells. This pattern can be described in terms of the location of engaged integrin adhesion molecules in a ring around engaged antigen receptors in a pattern that evolves over a period of 0.5-10 minutes and is then stable for many hours (Fig. 15.3) (51,52). Other cell biological indications of immunological synapse formation include the transport of particles toward the contact area and the accumulation of glycolipid enriched membrane domains at the interface (53,54). This is the molecular pattern of a mature immunological synapse. Despite the stability of the immunological synapse, the molecular interactions are still highly dynamic. A possible exception to this is the interaction of T-cell antigen receptors with MHC-peptide complexes in the center of the synapse, which appear to be stabilized by an unknown process (52). Immunological synapse formation is determinative for full activation of helper T cells and is related to both the number of MHC-peptide complexes available and the kinetics of their interaction with a particular T-cell receptor. Besides integrins and antigen receptors other molecules can contribute to immunological synapse formation including CD28, ICAM-1, and CD2 (54,55 and 56). This emphasis on the pattern of molecules is important since overlapping sets of adhesion molecules are utilized in all of the examples of functional adhesion provided here, while the molecular interaction dynamics are similar, the cellular interaction dynamics are controlled by the organization of the contact areas.

ADHESION MOLECULES FAMILIES

The Selectin Family

The selectin family consists of three related gene products expressed on leukocytes or endothelial cells. The molecules are placed in cluster of differentiation (CD)62 with the three genes designated CD62L(for leukocyte) or L-selectin, CD62E (for endothelial) or E-selectin, and CD62P (for platelet) or P-selectin (57). L-selectin (schematic in Fig. 15.4) is constitutively expressed on many leukocyte populations, but can be lost from the surface on activation due to proteolytic cleavage (58,59 and 60). E-selectin is expressed on endothelial cells following endothelial cells activation by lipopolysaccharide or monokines like interleukin 1 or tumor necrosis factor (61). P-selectin is expressed on platelets and endothelial cells where it is stored in the a-granules or Weibel-Palade bodies, respectively, and can be moved to the cell surface by fusion of these granules with the plasma membrane within seconds of platelet or endothelial cell activation (62,63). Activated platelets can attach to the surface of leukocytes and can then impart P-selectin adhesive activity to leukocytes, which do not normally express this receptor (64).

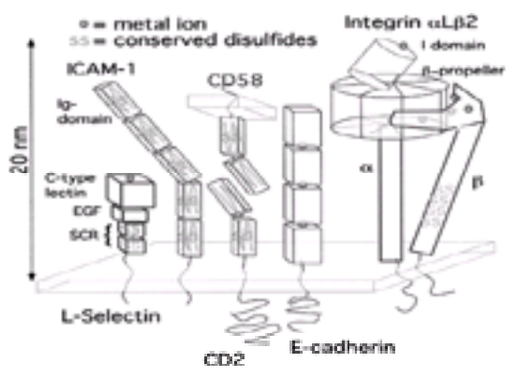

Figure 15.4. Adhesion molecule families. Schematics of molecules to scale. Integrin and ICAM-1 size based on electron microscopy images (185,186 and 187).

All selectins share a common structural plan. Selectins are type I transmembrane proteins with N-terminal lectin domains, a single epidermal growth factor (EGF) homology domain and 2-9 short consensus repeats similar to those in complement receptors (Fig. 15.4) (61,65,66). The lectin and EGF domains determine ligand binding specificity while the SCR domains are important for determining the distance of the ligand binding site from the membrane surface (67). The structure of the C-terminal lectin domain of E-selectin has been determined (68,69).

Selectin ligands are glycoproteins expressed on the surface of leukocytes and endothelial cells. The basic oligosaccharide motif is a fucosylated and sialated structure identical to or closely related to the sialyl-Lewis X blood group antigen (70). These oligosaccharides are generated at the termini of aspargine (N)-linked and serine/threonine (O)-linked oligosaccharides on a number of core polypeptides as they pass through the Golgi apparatus. The key regulatory enzymes that generate sialyl Lewis X are fucosyltransferases IV and VII (71). The interaction of selectins with sialyl Lewis X is Ca^{2+} dependent. The affinity of selectins for sialyl Lewis X is very low and is not sufficient for physiological interactions (72). Each selectin binds a family of ligands with modifications that enhance the affinity and bring it into a physiologically useful range. L-selectin prefers sulfated structures in which the sulfates are included in the vicinity of the sialyl Lewis X structure (73). Sulfation is regulated by sulfotransferases that are selectively expressed in specialized or activated endothelial cells (74). E-selectin prefers to interact with sialyl Lewis X structures on N-linked oligosaccharides (75). P-selectin prefers the core polypeptide P-selectin glycoprotein ligand-1 (PSGL-1) with sialyl Lewis X like structures on O-linked oligosaccharides in combination with sulfated tyrosines on an N-terminal domain (76,77 and 78). PSGL-1 is expressed on all leukocytes. However, the ability of PSGL-1 is dependent on the expression of the appropriate fucosyltransferases and tyrosyl sulfotransferases (79,80). For example, only a small subset of skin homing T lymphocytes express a functional form of PSGL-1 (81).

C-type lectin domains are utilized in a number of other important leukocyte interactions. The Ly49 family is another major group of immunologically relevant receptors that share the C-type lectin related domain with selectins (82,83). However, Ly49 family members are type II transmembrane proteins that form disulfide-linked dimers. The lectin domain is at the C-terminus and the N-terminus is in the cytoplasm. The C-type lectin domain retains the ability to interact with carbohydrates, but the known physiologically relevant ligands are actually MHC molecules that interact through protein surfaces (84,85 and 86). Negative regulatory forms of Ly49 and the functionally related immunoglobulin family members, the killer inhibitory receptors or KIRs, act by recruiting the inhibitory phosphatase SHP-1 to sites of cell-cell contact (87,88 and 89). The phosphatase is thought to extinguish signals transduced by activating receptors such as CD16 or activating NK cell receptors of the C-type lectin or immunoglobulin superfamilies. Another C-type lectin type II transmembrane protein has been described on dendritic cells. This molecule, termed DC-SIGN, interacts with the immunoglobulin superfamily member ICAM-3 to mediate initial interaction of denditic cells and naive T cells in lymph nodes (90).

CD44 binds hyaluronic acid and mediates initial leukocyte interactions with endothelium. CD44 is a type I membrane protein with an animo terminal “Link” domain (91). The Link domain is a homology unit also found in collagen binding extracellular matrix proteins. Determination of the Link domain structure revealed that it is structurally homologous to a C-type lectin domain, although they share little primary structure homology (92). Cell surface proteoglycans may also serve as counter-receptors for CD44 and as receptors for the heparin binding sites of fibronectin participate in signal transduction to regulate formation of focal adhesion and migratory behavior (93). These cell surface proteoglycans include the syndecans, which have multiple roles in regulation of cell migration (94,95). Syndecans are expressed in leukocytes, particularly antibody producing B cells (96).

A class of selectin ligands, particularly for L-selectin, are large mucins. These molecules are membrane or sectreted proteins that have serine and threonine rich ectodomains that are modified by O-linked oligosaccharides terminating in sialic acid. These molecules are very effective at presenting ligand for L-selectin because of the very high density of oligosaccharides. Members of this group include the transmembrane proteins CD43, CD34, and podocalyxin and the secreted protein GlyCAM (72). The selectin ligands P-selectin glycoprotein ligand 1 and MAdCAM also have mucin domains along with other regions (76,97). In addition to carrying selectin ligands on cells expressing the appropriate enzymes, mucins also are major charge carriers on the cell surface, and extend this charge a significant distance (>50 nm) from the plasma membrane surface. This charged surface results in repulsion between neighboring cells. In fact, it has been proposed the CD43 acts as a negative regulator of leukocyte interactions through this charge barrier mechanism (98,99). Charge repulsion is a barrier to cell-cell contact that is overcome by adhesion

molecules.

The Immunoglobulin Superfamily and Related b-Sheet Domains

The immunoglobulin superfamily is a very diverse group of interactive molecules that share a common folding motif (100). The basic immunoglobulin domain is formed from ~100 amino acids that form a sandwich of two anti-parallel b-sheets with a hydrophobic core. One or more disulfide bonds typically link the two b-sheets. The overall homology between Ig family member is frequently quite low (~20%) and inclusion in the family is based on shared patterns of hyrodphobic residues that define the hydrophobic core and structural determination (101). The domains form roughly rectangular globular structures of ~4 nm length and ~2 nm depth and ~3 nm width. Family members may have from one to 9 of these domains in a tandem array and many family members are part of dimeric or multisubunit complexes. The interacting surfaces can be the loops at the end of the domain as in classical immunoglobulins and the T-cell antigen receptor or through the flat faces of the domains as in CD2 interaction with CD58 (102,103). These interactions have a wide range of affinities for ligands with dissociation constants in the 10^{-12} to 10^{-5} M range. The high affinity interactions of immunoglobulins typically involve significant hydrophobic interactions, while low affinity interactions like CD2/CD58 involve electrostatic and hydrogen bond interactions that provide high specificity, but relatively low affinity due to fast off-rates (> 1 s^{-1}) (103). Immunoglobulin domains can also be combined with other structural motifs, such as the peptide binding domains of major histocompatibility molecules and the mucin domains (rich in O-linked oligosaccharides) of CD8 and MadCAM (97,104).

There are other families of interactive proteins that are closely related to Ig domains. These related folds include the fibronectin type III repeat and cadherin repeats. Fibronectin type III repeats are distinguished by a lack of disulfides and a different pattern of b-strands, but have similar overall geometry and functionality to Ig domains (105). Cadherin repeats are similar to immunoglobulin domains in the number of amino acids (~100) and the b-strand pattern, but are distinct in two important ways (106,107). The residues making up the hydrophobic core of cadherins are larger so the domains are more barrel-shaped than rectangular. In addition the domains are linked together by interdomain bound Ca^{2+} ions that make the arrays of domains very rigid. When Ca^{2+} ions are removed the molecules become flexible and lose their adhesive activity (108,109). Thus, the Ca^{2+} ion is a structural element of the cadherin. Cadherins are primarily expressed by epithelial cells and neural cells where they engage in interactions with other cadherins (110), but E-cadherin can be used as a ligand for integrin family member aEb7 on lymphocytes (111).

The Integrin Family

Integrins are a family of large heterodimeric glycoproteins expressed on the surface of all nucleated cells (5,112). The two subunits, identified as a and b are both large type I transmembrane glycoproteins. Integrins can be divided into three subfamilies based on subunits that each share multiple partners to form functionally distinct receptors: the b1, b2, and aV subfamilies (Fig. 15.5). Leukocytes express members of all three subfamilies. b subunits are rich in highly conserved cystein residues and have a highly conserved N-terminal regions that harbors many mutations that lead to leukocyte adhesion deficiency type I (see below) and thus appear to be involved in association with the b subunit. Integrin b subunits share a series of 7 repeats at the N-terminus. While there is no direct structural data on integrins it has been proposed that these 7 repeats form a b propeller structure, a motif also identified in heterotrimeric G protein b subunits and many extracellular proteins (113). Some integrin b subunits have an “inserted” or I-domain between the second and third repeat of the putative b propeller. The I-domain has sequence similarity to the A-domains of von Wilibrand factor that are implicated in interaction of von Wilibrand factor with collagen. The structures of the aL, aM, and a2 I domains have been determined, and they all have dinucleotide folds similar to low molecular weight G proteins (114,115 and 116). The I domains contain a novel divalent cation binding site (the Metal Ion Dependent Adhesion Site motif) at the distal end of the domain from its attachment site to the b-propeller (117). The I-domain can be incorporated into the b propeller model as indicated in Fig. 15.4. The configuration of the I-domain is similar to the configuration of the a and b subunits of heterotrimeric G proteins. Integrins that contain I domains use the I-domain as the major ligand binding site (118,119,120 and 121). I domains appear to exist in two conformations that may correspond to high and low affinity forms based on changes in the position of a C-terminal a-helix and the coordinate exposure of hydrophobic residues around the MIDAS motif (122,123). These changes may be regulated by the interaction of the I-domain with the b-propeller domain (124). In contrast, integrins that lack I domains form a ligand binding site from the b propeller domain of the b subunit and the highly conserved region of the b subunit (125).

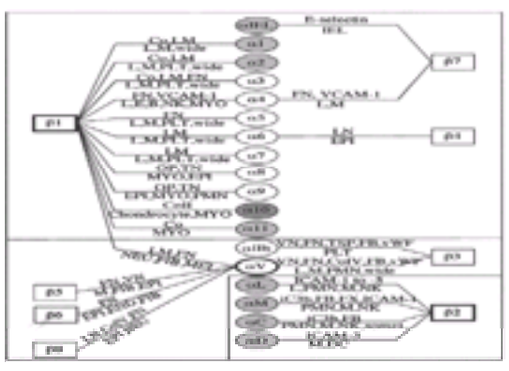

Figure 15.5. Integrin family. The association patterns among the known subunits is shown. In each case the ligands for a heterodimer are indicated above the line, and its tissue distribution is indicated below the line. Abbreviations: Co= collagen, LM= laminin, FN = fibronectin, VN = vitronectin, TSP = thrombospondin, FB = fibrinogen, vWf = von Willebrand factor, OP = osteopontin, FX = factor X, BSP1 = bone sialoprotein 1, L = lymphocytes, M = monocyte/macrophages, PMN = granulocytes, E = eosinophils, B = basophils, NK = natural killer cells, PLT = platelets, IEL = intraepithelial lymphocytes, PBL= peripheral blood lymphocytes, EPI = epithelial cells, ENDO = endothelial cells, MYO = muscle cells, NEU = neural tissues, MEL = melanoma, FIB = fibroblast.

Integrins on leukocytes undergo dramatic changes in activity (126,127). These changes regulate extravasation, migrations, and cell-cell interactions in tissues. Integrins on circulating leukocytes are inactive since they do not mediate adhesion to surfaces coated with ligands (127). Upon activation of leukocyte by chemoattractants there is a rapid increase in integrin activity that allows firm adhesion (15). Antigen receptor cross-linking also results in a rapid rise in integrin activity on leukocytes. While it was once proposed that simple affinity changes in the integrin could account for these changes in adhesiveness, this no longer seems likely. It is more likely that a number of events are required for integrin activation. Integrins exist in two affinity states with respect to ligand that can vary by as much as 10-1000 fold in apparent affinity (128,129,130 and 131). However, the most general mechanisms to generate the high affinity form is binding of the ligand itself, which favors a high affinity conformation of the integrin (132,133). This conformational change is detected by a number of monoclonal antibodies recognizing at least four different integrin heterodimers based on epitopes in the ectodomain and the cytoplasmic domain (133,134). There is only one report of a cytoplasmic ligand that will induce this conformational change in the inside to outside direction (135). In this case the N-terminal half of the cytoskeletal protein talin was shown to induce the binding of a ligand mimetic antibody to aIIbb3 (136). The current model for integrin activation involves coordination of cytoskeletal interactions with ligand engagement. A model for this process is discussed below.

The complex regulation process of intergrins appears to be amenable to inhibition by small molecules that have potential as therapeutic agents. This approach has been most broadly applied with respect to integrins that interact with ligands containing the RGD (arginine-glycine-aspartic acid) motif (137). Interestingly, recent progress has been made in finding small molecules that block function of non-RGD binding integrins such as aLb2 (138).

MOLECULAR MECHANISMS

Rolling Adhesion and Capture from Flow

The rolling adhesion of leukocytes observed in the first step of the extravasation process requires the formation of very few molecular interactions and has provided insights into the behavior of single molecular interactions under force (139). When selectin receptors are presented at very low densities, isolated tethering events can be observed between cells and the substrate *in vitro* that reveal the kinetics of the adhesive interaction. The important characteristics of the selectin/counter-receptor interaction were a relatively fast off-rate of ~1 s^{-1} and a small increase in the off-rate with increasing force. Thus, single selectin interactions can hold a cell against physiological blood flow for a fraction of a second, long enough for the next interactions to form to sustain rolling of the leukocyte. The conditions for rolling adhesion and efficient tethering from flow are relatively restrictive and not all adhesion mechanisms can perform these functions *in vivo*. In addition to selectins it has been demonstrated that CD44 interaction with hyaluronic acid (91) and a4b1 interaction with VCAM-1 can capture leukocytes from physiological flow (140). Furthermore, it has recently been reported that the interaction of the chemokine receptor CX3CR1 can mediate capture of leukocytes from flow through interaction with the membrane anchored chemokine called fractalkine (CX3C chemokine 1) (141,142 and 143). However, it is not clear how important this capability is *in vivo*.

Rolling adhesion can also be utilized for analysis of single molecule interaction kinetics in systems where the physiological interaction would not normally mediate rolling adhesion or tethering from flow. This approach has been utilized to study antibody interactions, interaction of CD2 and CD48 and the interaction of the integrin

aL domain with ICAM-1 (120,144).

Very small numbers of interactions also exist in at the moment when adhesion is initiated between two cells (145). The process of initial interactions under static conditions can be studied using micromanipulation to push two cells into contact for a controlled time followed by application of a separating force (146). This procedure is repeated many times under different conditions and the probability of forming an interaction can be used to determine the kinetic rate constants for initiation of a single or small number of interactions in an early contact area. This approach allows predictions to be made about how adhesion is initiated under different initial conditions.

Formation of contacts required for migration, cell-mediated killing, and immunologic synapse formation involve hundreds or thousands of molecular interactions in the interface between cells. The classic study relating large numbers of receptor interactions to the strength of adhesion employed a combination of fluorescence microscopy and micromanipulation (147). In this experiment a rat basophilic leukemia (RBL) cells expressing a high-affinity Fce receptor was coated with fluorescently labeled IgE (the bifunctional ligand for the Fce receptors). This IgE also would bind the small ligand dinitrophenol (DNP). When the RBL cells were brought into contact with beads coated with high densities of DNP, a time-dependent redistribution of the fluorescent IgE from the entire cell surface to the contact area was observed. This redistribution was not energy dependent, but was well correlated with the development of strong adhesion. The accumulation of IgE in the interface was dependent on the random lateral mobility of the Fce receptor on the RBL cell surface and the affinity of the IgE for DNP. The mobility and affinity led to random entry of the IgE/Fce receptor complex into the contact area between the cells and bead and trapping of the IgE/Fce receptor complex in the interface. Lateral mobility is a fundamental property of proteins in biological membranes that can be regulated by a variety of molecular interactions of the surface receptors (148,149). The fractional mobility and diffusion coefficient describes the average motion of molecules on the cell surface. The diffusion coefficient (D) is related to the time (t) required to diffuse a distance (r) is $t = r^2 \div 4D$. Thus as distance increases the time required for the average molecule to diffuse increases with the square of the distance. Lateral mobility contributes to interactions in two important ways. At the submicron level diffusion is an important component of the formation of the encounter complex between molecules that is a key component of the on rate (150,151). Thus, regulation of diffusion changes the effective on-rate. In addition, diffusion can allow receptor recruitment over distances of several microns. This transport effect was highlighted by the study above, which demonstrated large-scale motions of Fce receptors on the cell surface.

An alternative approach to examine interactions is to fix cell-cell conjugates with aldehydes or organic solvents followed by detection of specific surface receptors and cytoplasmic regulatory molecules with fluorescently tagged monoclonal antibodies. An advantage of this approach is that both surface and cytoplasmic proteins can be localized leading to an appreciation of interactions at both faces of the plasma membrane. For example, integrin interactions with counter-receptors are accompanied by accumulation of the cytoplasmic protein talin on the cytoplasmic side of the integrin containing membrane (51). This approach has yielded a number of insights and also has the advantage that many molecules can be examined based on available antibody reagents.

A model system based on supported planar bilayers allows a more quantitative approach to interactions in a contact area from which a two-dimensional dissociation constant can be determined. Supported planar bilayers are phospholipid membranes supported on a glass or quartz microscope slide (152). Counter-receptors can be tethered to the bilayers either through glycophosphatidylinositol moieties (151) or soluble proteins with C- or N-terminal 6 histidine extensions can be noncovalently attached to phospholipids with Ni-chelating head groups (153). In either case, the tethered ligand are laterally mobile in the plane of the bilayer. Fluorescent tagging of counter-receptors in the bilayer allows visualization of interactions between cell surface receptors and the counter-receptors (154). The two-dimensional Kd is obtained from a Golan-Zhu plot, which is based on a simple model for receptor movement on the cell surface that is applicable to highly mobile receptors (155). The key assumption is that the receptor on cells and the ligand in the bilayer can only interact in the contact area and that the free receptor is distributed uniformly on the cell surface. Therefore, the density of bound receptors is measured relative to the contact area, while the density of free receptors is measured relative to the entire cell surface area. The linearized plot is based on taking the mass action equation Kd = [complexes] ÷ ([free ligand]x[free receptor]) and substituting in the [free receptor] = (total receptor number – [complexes]x contact area) ÷ cell area. Algebraic rearrangement leads to a plot of [complexes] ÷ [free ligand] on the Y-axis, versus [complexes] x (cell contact area ÷ cell surface area) (Fig. 15.6). The negative reciprocal of the slope is the 2D Kd. Only a few adhesion mechanisms have sufficiently mobile and nonregulated receptors to apply this model, but these systems have provided important information. The interaction of the immunoglobulin super-family members CD2 and CD58 has a solution (3D) Kd of 2 (M and a 2D Kd of 1 molecules/(m^2. If it is assumed that the kinetics of the 3D and 2D interactions are similar, the geometry of interaction is the only difference between the 2D and 3D Kds. The 2D interaction has an entropic advantage over the 3D interaction because the membrane anchored adhesion molecules have fewer initial degrees of freedom compared to the resulting complex than for the corresponding soluble molecules. The ratio of the 2D Kd and the entropy corrected 3D Kd yields the confinement region (150,156) (Fig. 15.7). This is calculated to be 5 nm for the CD2/CD58 interaction. Thus, the action of hundreds or thousands of CD2/CD58 interactions results in alignment of apposed membranes with nanometer precision. This means that the CD2 and CD58 binding sites are concentrated in a attoliter (10^{-18}) volume in the interface between cells. In the future, it will be possible to determine 2D interaction parameters from interactions in cell-cell interfaces based on tagging proteins with intrinsically fluorescent proteins and developing new methods for calibration of the image data (89). The formation of thousands of transient interactions is an important process in T-cell mediated killing and immunological synapse formation.

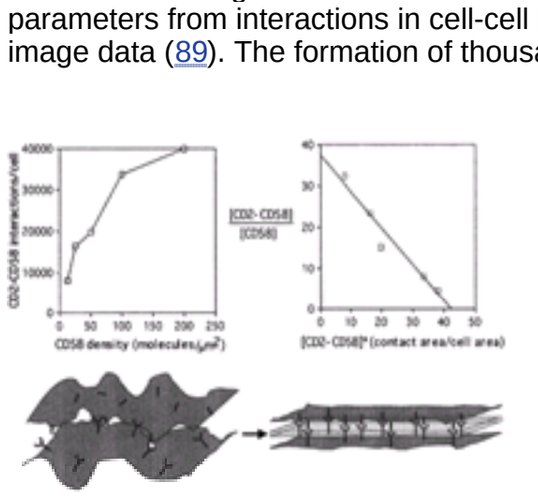

Figure 15.6. Measurement of two-dimensional affinity. The binding curves for CD2/CD58 interaction in contact areas between supported planar bilayers containing CD58 and T cells expressing CD2. The Golan-Zhu plot is based on the equations in the text. The data indicate that the initially dissordered contact areas become highly ordered through many independent interactions.

	3D	comparison	2D
Affinity	3D Kd Free energy	$\sigma = \frac{\text{2D Kd}}{\text{3D Kd}}$ Contact geometry	2D Kd Physiological affinity
Receptor number	Total number of receptors (N_t)	$f = \frac{\text{X-int*cell area}}{N_t}$ Lateral mobility	Maximum number of receptors in contact (X-int*cell area)

Figure 15.7. Interpretation of two-dimensional affinity. The parameters determined by two dimensional affinity and three dimensional affinity can be related to determine physical parameters important for adhesion.

Adhesion receptors of the integrin family undergo complex interactions with the cytoskeleton. Integrin activation is a multistep process that results in coordination of integrin engagement and cytoskeletal assembly that stabilizes the adhesive unit, a cluster of engaged integrins (157,158 and 159). Inactivity of integrins on resting leukocytes is due to lateral immobilization of a low affinity form of the integrin (131,160,161). The lateral immobilization of the integrin requires an intact actin cytoskeleton. There are three hypotheses for how lateral immobilization impairs integrin function. Lateral immobilization may prevent small-scale receptor rearrangements at the interface that are required to form complexes between the integrin and ligand (160). Lateral immobilization may also prevent the large-scale clustering of integrins that is correlated with higher activity (161,162). Finally, the interactions that lead to lateral immobilization may block interactions that are required to coordinate integrin engagement with cytoskeletal assembly that is a component of adhesion strengthening. The first step in integrin activation is mobilization (160). Mobilization is equivalent to an increase in random diffusion with a 10-fold increase in the diffusion coefficient. Mobilization increases both encounter complex formation and is also correlated with clustering. However, mobilization does not result in an increase in integrin affinity. The second step in integrin activation, at least in the context of lymphocytes, is the encounter of the low affinity form of the integrin with ligand. This low affinity interaction triggers a ligand-induced conformational change

that leads to two important effects: the half-life of the interaction (half-life = k/ln2) increases from a fraction of a second to ~30 seconds (for the aLb2 integrin) and new binding sites are exposed in the cytoplasmic domains. The third step is the interaction of poorly defined cytoskeletal factors with the ligand induced binding sites on the cytoplasmic domains. These factors may include the large actin binding protein talin, but may include other regulatory proteins that are not yet identified. These ligand induced cytoskeletal interactions trigger actin polymerization associated with cell spreading and integrin cluster formation. The cytoskeletal assembly process may act by increasing membrane alignment to the substrate, and thereby increasing two-dimensional affinity. Current evidence supports this activation sequence for (L(2 on lymphocytes.

Another important consequence of integrin interaction with the actin cytoskeleton is transport of engaged integrins over the cell surface. Directed transport of molecules on the cell surface is likely to involve that attachment of these molecules to actin-based structures that move due to myosin-based contraction of the actin. This type of transport is much more efficient over longer distances (a few µm) than diffusion and has the potential to transmit energy from the cytoplasm to the substrate for directed processes such as cell motility. The actin filaments that are moved by myosin-based contraction move at 20-40 µm/min. This movement can be transmitted to the engaged integrins to move leukocytes at up to 10 (m/min. Formation of the immunological synapse also involves striking directed transport of both engaged MHC-peptide complexes and adhesion molecules toward the center of the contact interface (Fig. 15.8). This results in an increase in integrin concentration toward the center of the contact, but this concentration drops off within a (m of the center. The central region is occupied by engaged antigen receptors that are transported from peripheral sites in the contact over a period of a few minutes.

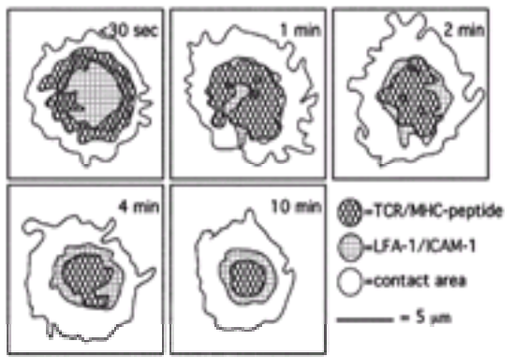

Figure 15.8. Molecular dynamics of immunologic synapse formation. The time course of adhesion molecule and antigen-receptor movements during functional T-cell activation.

LEUKOCYTE ADHESION DEFICIENCY DISEASES

Leukocyte Adhesion Deficiency Type I (LAD I)

LAD I is characterized by life threatening bacterial infections and skin and mucosal ulcers without puss formation (163). These deficiencies in innate immunity are caused by failure of leukocyte extravasation at skin and mucosal sites in response to bacterial products that would normally induce neutrophil infiltrates. LAD I is inherited as a recessive trait with no obvious phenotype in heterozygous individuals. The molecular defect in LAD I is absence or reduced expression of the (2 subunit common to all leukocyte integrins. In one family, the mutations resulted in expression of nonfunctional aLb2 and aMb2 (164). Without (2 integrins there is a complete absence of firm adhesion and extravasation of neutrophils to the blood vessel wall, whereas rolling adhesion is virtually normal (165). A number of LAD I mutations have been mapped and the defects include both large deletions resulting in no protein production, point mutations that block association with the (subunits, and point mutations that lead to nonfunctional complexes on the surface (163,164). Lymphocyte and monocyte migration is less severely affected in LAD I. In the mouse model of LAD I the compensatory mechanism appears to be the a4b1 integrin interaction with VCAM-1 (166). The relatively high level of T-cell function in LAD I patients is consistent with similar compensatory mechanisms in humans (167). Mice with deficiencies in either of the major (subunits of the leukocyte integrin subfamily, (L and (M, have much less severe defects in leukocyte extravasation (168,169). This is consistent with the observations that neutrophils use both (L(2 and (M(2 for interactions with activated endothelial cells. LAD I can be treated by non-MHC matched bone marrow transplantation (170). While the number of patients treated is small, the LFA-1 deficient recipients appears to become tolerant to non-MHC matched grafts with a higher success rate than expected for non-MHC matched transplants into immune sufficient recipients.

Leukocyte Adhesion Deficiency Type II (LAD II)

LAD II patients suffer life-threatening bacterial infections and skin and mucosal ulcers without puss formation, similar to LAD I patients (171). LAD II is also accompanied by severe mental retardation and growth defects. LAD II is inherited as a recessive trait. The molecular basis of the defect has not been fully determined, but it appears to be a global defect in fucose metabolism leading to an inability to generate sialyl Lewis X and related blood group antigens required for selectin function (172). The mental retardation and physical deformities in these patients suggests that fucosylated oligosaccharides have uncharacterized functions in the development of the nervous system and the musculoskeletal system. LAD II patients have defective lymphocyte rolling on endothelial cells that leads to greatly decreased extravasation (171). As with LAD I patients, the lymphocyte function is less profoundly affected than the neutrophil function. The ability of lymphocytes to home and migrate *in vivo* in the absence of selectin ligands may also be due to the ability of lymphocyte integrins like a4b1 and a4b7 to mediate rolling adhesion and the use of CD44 as a mechanism for interaction with endothelial cells. It has also been reported that delayed type hypersensitivity reactions are nearly normal in LAD II patients (173). The only defect noted was reduced redness and swelling at the delayed type hypersensitivity site. This result is consistent with expression of VCAM in delayed type hypersensitivity (174) and the potential role of (4 integrins in mediating tethering from flow.

Wiscott-Aldrich Syndrome (WAS)

WAS is an X-linked immunodeficiency caused by loss or mutation in the cytoplasmic protein WASp (175). WAS patients have deficiencies in T lymphocyte activation and platelet function. These defects are characterized by a loss of actin-based structures such as microvilli and abnormalities in cell shape. T cells from mice lacking WASp expression have defects in formation of T-cell receptor caps, which may be related to the process of immunological synapse formation. The WASp protein is expressed only in hematopoietic cells and platelets. A homolog N-WASP is expressed in a wide variety of nucleated cells and in lymphocytes. Both WASp homologs are multifunctional proteins with domains that interact with molecules involved in regulation of actin polymerization including CDC42, profilin, and VASP (176). The interaction of CDC42 with WASp can both recruit WASp to membrane sites and induce profound changes in WASp structure that enable interactions with the actin polymerization machinery (177). The link between T-cell antigen receptor engagement and WASp recruitment requires the nonreceptor tyrosine kinase ZAP-70 and the adapter proteins SLP-76 and NCK. Recruitment of NCK and WASp triggers rapid actin polymerization (178). The SLP-76 recruited Guanine nucleotide exchange factor vav may play an important role in this process through local activation of CDC42 [Fischer, 1998 #12]. Actin polymerization is likely to play a key role in regulation of adhesion and antigen receptor movements. Thus, WASp is a prototype for cytoplasmic proteins that regulate molecular interactions in the interface between T cells other cells.

PERSPECTIVE FOR FUTURE RESEARCH ON ADHESION MOLECULES

Important areas in research on adhesion molecules include the elucidation of the mechanism of integrin function and how integrin mediated adhesion is integrated with migration, primary activation, and effector functions of the immune response. While there is considerable data in this area and there are well-reasoned hypotheses regarding integrin function and the mechanism of adhesion in a variety of immunological contexts, there are still fundamental discoveries ahead. It will also be important to further evaluate the role of supramolecular organization of interfaces in cellular responses. These are areas where leukocyte systems are likely to provide key paradigms for mechanisms and processes of general importance in many areas of biology. These future endeavors will provide fertile ground for discovery of new therapeutic strategies to improve human health.

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16 CHEMOKINES AND CHEMOKINE RECEPTORS

Andrew D. Luster, M.D., Ph.D.

The recruitment of cells to sites of inflammation is an essential component of the host inflammatory response. Cell recruitment relies on the coordinated action of cell activation, cell adhesion, chemoattraction, and transmigration across the endothelial barrier. The chemokines (chemotactic cytokines) are a newly described superfamily of secreted proteins that play an important role in cellular chemotaxis. This chapter reviews chemokine structure, emphasizing how certain structural elements confer differential functional activity. Chemokine receptors, including their cellular expression and intracellular signaling mechanisms, also are included. The role of chemokines in cell trafficking is reviewed in terms of their role in homeostasis and development and in their participation in cell recruitment to sites of inflammation. Illustrative examples of chemokine action *in vivo* are drawn from specific human diseases and animal models.

CHEMOKINE STRUCTURE AND FUNCTION

The chemokines are a superfamily of small secreted proteins (molecular weight, 8–10 kd), that shares the ability to chemoattract leukocytes. To date, about 50 distinct chemokine molecules have been described (Fig. 16.1). The chemokines are structurally homologous and are subdivided into four families based on the position of conserved cysteine residues near the N termini of the molecules. CXC chemokines (also known as a-chemokines) are characterized by the presence of two cysteine residues near the N terminus that are separated by one amino acid (CXC motif). In contrast, CC chemokines (also known as b-chemokines) have two cysteine residues that are adjacent (CC motif). The CC and CXC chemokines represent the largest of the chemokine families and contain many members. The C-chemokine family contains only a single member, lymphotactin, which has one cysteine residue near the N terminus (C motif). Likewise, the CXXXC family has only a single member, fractalkine (also called *neurotactin*), whose N-terminal cysteines are separated by three amino acids (CXXXC motif). Fractalkine is unique in that it is expressed as a cell-surface glycoprotein with the “chemokine domain” setting on top of a mucinlike stalk and has both transmembrane and cytoplasmic domains.

Figure 16.1. Chemokines and their receptors. Chemokines are grouped by subfamily and listed individually according to the standard nomenclature: CCLn, CC chemokine ligand; CXCLn, CXC chemokine ligand where *n* refers to the corresponding human gene symbol number in the SCYAN nomenclature. The commonly used acronyms are listed alongside the new nomenclature with alternative names indicated in parentheses. Receptors for each chemokine are indicated where known.

The cysteines in the chemokine molecules form important intrachain disulfide bonds, which determine, in part, the secondary structure of these molecules. It was proposed that the chemokines be given numeric names, like the interleukins and the chemokine receptors. In the proposed nomenclature, CC chemokines are named CCLn, for CC chemokine ligand, where *n* is the number that corresponds to the gene symbol number given to each chemokine as they were mapped and called SCY “n” for a small secreted cytokine, followed by a number. Likewise, the CXC chemokines are named *CXCLn*, for CXC chemokine ligands. Only time will tell how widely accepted and used this nomenclature will be.

The CXC chemokines can be subdivided further based on the presence or absence of an ELR sequence (glutamatic acid-lysine-arginine) preceding the CXC motif, near the N terminus of the molecule (1). This structural difference is important because it delineates separate functional activities of chemokines in this family. In general, the CXC chemokines that contain an ELR sequence [e.g., interleukin-8 (IL-8) and neutrophil-activating peptide-2 (NAP-2)] are chemotactic for neutrophils, whereas non-ELR-containing chemokines [e.g., interferon-inducible protein 10 (IP-10) and B-cell attracting chemokine (BCA)] are active on lymphocytes.

In general, the CC chemokines are chemotactic for monocytes, eosinophils, basophils, and lymphocytes, with variable selectivity, but are inactive on neutrophils. Within the CC chemokines, the monocyte chemoattractant proteins (MCPs 1–5) and the eotaxin molecules (eotaxin 1–3) share approximately 65% identity and represent a subfamily within the b-chemokines (2). Like the CXC chemokines, the N-terminal amino acids preceding the CC residues of the CC chemokines are critical for their biologic activity and leukocyte selectivity. For example, the addition or deletion of a single amino acid residue at the amino-terminus of monocyte chemoattractant protein-1 reduces its biologic activity on monocytes by 100- to 1000-fold (3), and the deletion converts it from an activator of basophils to an eosinophil chemoattractant (4).

Several chemokines undergo N-terminal proteolytic processing after secretion that alters their activity, which can activate, inactivate, or even create natural inhibitors. For example, the inactive platelet granule chemokine, platelet basic protein, is N-terminally processed by monocyte proteases to generate NAP-2, which is a CXC chemokine that is active on neutrophils (5). In contrast, other chemokines, such as regulated on activation, normal T-cell expressed and secreted (RANTES), and stromal-derived factor-1 (SDF-1), are cleaved and inactivated by CD26, a leukocyte cell-surface dipeptidyl exopepdiase IV (6,7 and 8). CD26 cleaves the first two amino acids from peptides with penultimate proline or alanine residues, a sequence found at the N terminus of many chemokines. Still other chemokines, such as MCP-2, have posttranslation modification that removes the first five amino acids, resulting in a natural inhibitor of MCP-1, MCP-2, MCP-3, and RANTES (9). Thus, posttranslational modification may be a general mechanism whereby local factors can positively or negatively regulate chemokine activity.

CHEMOKINE RECEPTORS

Chemokines induce cell migration and activation by binding to specific G protein-coupled cell-surface receptors on target cells (10,11). Five human CXC chemokine receptors (CXCR1–5) and nine human CC chemokine receptors (CCR1–9) have been identified (Fig. 16.1). Most receptors recognize more than one chemokine, and several chemokines can bind to more than one receptor. There is receptor-ligand specificity within chemokine subfamilies, however, with CXC-chemokines binding exclusively to CXC receptors and CC chemokines binding to CC receptors. The molecular explanation for this ligand-receptor subfamily restriction may be related to structural differences between CC and CXC chemokines. Although they share similar primary, secondary, and tertiary structures, CC and CXC chemokines have different quaternary structures (12).

Chemokine receptors are expressed on multiple cell types, including all mature hematopoietic cells. Certain receptors have a restricted expression (e.g., CCR3 predominantly on eosinophils and basophils and CXCR1 and CXCR2 predominantly on neutrophils), whereas other receptors are more widely expressed (e.g., CXCR4 on most cells, including all leukocytes). Expression of a given receptor on a given cell type can vary with the state of cellular differentiation and activation. For example,

CCR1 and CCR2 are constitutively expressed on monocytes but are expressed on lymphocytes only after stimulation by IL-2 (13). Constitutive chemokine receptor expression can be downregulated following cellular activation. For example, CCR2 is downregulated by lipopolysaccharide, making the cells unresponsive to MCP-1. These cells remain responsive to chemokines, which signal through other receptors, such as macrophage inflammatory protein-1a (MIP-1a), which binds to CCR1 and CCR5 (14).

Certain chemokine receptors are expressed only on activated cells. For example, CXCR3 appears to be expressed only on IL-2 activated T lymphocytes (15). As such, transient upregulation of chemokine receptors on leukocytes allows for the amplification of immune responses. The expression of chemokine receptors has been characterized most extensively on hematopoietic cells; however, chemokine receptor expression is not restricted to cells of this lineage. For example, CXCR4 is expressed on endothelial cells, epithelial cells, and neurons (16,17 and 18), suggesting roles for the chemokine system in addition to leukocyte chemotaxis.

In addition to these receptors, the chemokines interact with two types of nonsignaling receptors. One is the promiscuous erythrocyte chemokine receptor, called Duffy antigen receptor for hemokines (DARC) (19). This receptor has been known since the 1950s as the Duffy blood group determinant and is expressed on erythrocytes and endothelial cells. Although DARC is structurally related to the chemokine receptors and both CXC and CC chemokines bind to it, chemokine binding does not induce a calcium flux. This receptor may function as a chemokine sink, clearing chemokines from the circulation.

The other type of “receptor” that the chemokines interact with is proteoglycans. The chemokines are basic proteins and bind avidly to negatively charged glycosaminoglycans (20,21,22 and 23). Proteoglycans serve to capture chemokines in the extracellular matrix and on the surface of endothelial cells, which may serve to establish a local concentration gradient from the point source of chemokine secretion (20).

Chemokine Receptor Signaling

The signal transduction events activated by ligand binding controlling leukocyte migration are not entirely defined and involve many different signaling pathways (Fig. 16.2) (24,25). A dramatic polarization of the cell occurs in response to a chemotactic gradient, which is the result of asymmetric signal transduction and the establishment of intracellular molecular gradients (26,27). Although all the molecular details remain to be elucidated, certain signaling molecules appear to play important roles in chemokine-induced cell movement. Chemokine receptors are coupled to the Gi subfamily of G proteins, and pertussis toxin (PTX), which adenosine diphosphate (ADP) ribosylates and irreversibly inactivates the Ga subunits of the ai class, inhibits most of the chemokine-induced effects on leukocytes, including chemotaxis, calcium flux, and integrin activation (28). Other signaling events activated by most chemokine receptors include the activation of phospholipase C (PLC), leading to generation of inositol triphosphates, intracellular calcium release, and protein kinase C (PKC) activation. Inhibition of chemokine-induced chemotaxis by wortmannin implicates phosphatidylinositol 3-kinase (PI3K) in chemokine receptor signal transduction. Mouse strains engineered with null a mutation of the p110 subunit of PI3Kg have defects in chemokine-induced neutrophil and macrophage migration, confirming the importance of this enzyme in directed leukocyte movement (29). Phorbol myristate acetate (PMA) activation of integrins implicates PKC as a potential mediator by which chemokines activate integrins. Chemokine signaling also leads to guanine nucleotide exchange on Rho, indicating the activation of Rho (30). Rac and Rho are small guanosine triphoshate (GTP)-binding proteins that are involved in controlling cell locomotion through regulation of actin cytoskeletal rearrangement leading to membrane ruffling and pseudopod formation.

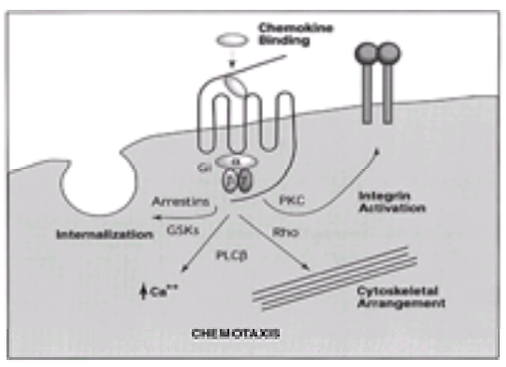

Figure 16.2. Chemokine receptor signal transduction. Chemokine receptors are a subfamily of G protein-coupled seven-transmembrane spanning cell-surface receptors. They are coupled to heterotrimeric G proteins of the Gi subclass, which are distinguished by their pertussis toxin sensitivity. Chemokine receptor activation leads to the stimulation of multiple signal transduction pathways, including the activation of phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC), leading to generation of inositol triphosphates, intracellular calcium release and protein kinase C (PKC) activation. Chemokine signaling also induces the upregulation of integrin affinity and the activation of Rho, leading to cytoskeletal reorganization. Agonist-stimulated receptors also activate G protein receptor kinases (GRKs), which leads to receptor phosphorylation, arrestin binding, G protein uncoupling (desensitization), and clathrin mediated receptor endocytosis (internalization).

Agonist-stimulated receptors also activate G protein receptor kinases (GRKs). Activated GRKs phosphorylate serine and threonine residues in the tail of G protein-coupled receptors (GPCRs) (31). Receptor phosphorylation is followed by the binding of arrestins, which bind specifically to phosphorylated receptor, uncouple the receptor from G proteins (*desensitization*), and function as adaptor molecules, leading to clathrin-mediated endocytosis (*internalization*). These internalized receptors are either dephosphorylated by phosphatses and recycled to the cell surface or targeted for degradation. Desensitization and recycling of chemokine receptors may be an important mechanism by which leukocytes maintain their ability to sense a chemoattractant gradient during an inflammatory response, whereas degradation of chemokine receptors may lead to termination of migration. Thus, chemokine receptors activate multiple intracellular signaling pathways that regulate the intracellular machinery necessary to propel the cell in its chosen direction.

ROLE IN THE MULTISTEP MODEL OF LEUKOCYTE RECRUITMENT

Leukocyte extravasation from the blood into the tissues is a regulated multistep process that involves a series of coordinated leukocyte–endothelial cell interactions (Fig. 16.3) (32,33). Several families of molecular regulators, such as the selectins, the integrins, and the chemokines, are thought to control different aspects of the process. In the multistep model, leukocyte extravasation begins with leukocyte rolling, a process that is dependent on selectins. Conversion of rolling to firm adherence depends on the activation of leukocyte integrins, a process that is dependent on cell signaling. Increasing evidence supports a role for chemokines as important activators of the leukocyte integrins.

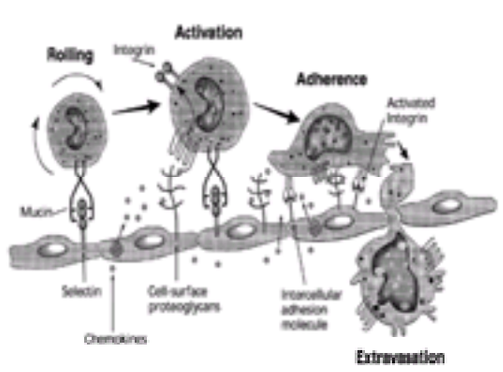

Figure 16.3. Chemokines activate integrins and convert leukocyte rolling to firm adhesion, which ultimately leads to diapedesis. Chemokines are produced by endothelial cells or transported across endothelial cells if produced by cells in tissue and presented bound to proteoglycans, which help maintain a local concentration gradient. Leuokocytes roll along endothelium in a process dependent on selectins and their mucin receptors sampling the endothelial surface. Ligand activation of leukocyte chemokine receptors induces a conformational change in surface integrins that favor binding to intercellular adhesion molecules on the surface of endothelial cells. This leads to firm adhesion and arrest of rolling leukocytes before extravasation through the endothelial layer.

The activation of integrins by chemokines has been shown for many leukocytes, including lymphocytes, monocytes, eosinophils, and neutrophils. The activation of leukocyte integrins by chemokines was demonstrated experimentally by examining the expression of integrin activation epitopes and the adhesion of leukocytes to

purified counterligands or endothelium under static and flow conditions. Using the more physiologically relevant flow-based adhesion assays, SDF-1, secondary lymphoid-tissue hemokine (SLC, also called 6Ckine), and MIP-3b (also called ELC, for Epstein-Barr virus-induced gene 1 chemokine ligand) induced the rapid adhesion of naive lymphocytes to vascular cell adhesion molecule (VCAM-1) (34) and mucosal cell adhesion molecule-1 (MAdCAM-1) (35). In contrast, firm adhesion of naive lymphocytes was not induced by other chemokines, including MIP-1a, MIP-1b, RANTES, growth-regulated oncogene-a (GRO-a), IL-8, interferon-induced protein 10 kd (GRO-a), or eotaxin, demonstrating the selective nature of this chemokine-induced adhesiveness. IP-10 and monokine-induced by g-interferon (Mig) induced firm adhesion of activated but not resting T cells to purified VCAM-1 and intercellular adhesion molecule (ICAM-1) (36). These chemokine-induced increases in integrin affinity and cellular adhesion were PTX sensitive, suggesting a dependence $G_i a$-linked chemokine receptor signaling.

Chemokine-induced adhesiveness to vascular endothelium has also been demonstrated for monocytes. Exposure of peripheral blood monocytes to MCP-1 or IL-8 led to rapid adhesion to vascular endothelium under flow conditions (37). Blocking studies with integrin-specific monoclonal antibodies suggested that this adhesion was mediated in part through b2 integrins.

An additional complexity of chemokine-induced integrin activation is beginning to be unraveled with the demonstration that individual chemokines can differentially activate specific integrin avidity on the same cell type. For example, MCP-1 differentially activates b1 and b2 integrins on lymphocytes (38), whereas RANTES and MCP-3 differentially activate b1 and b2 integrins on eosinophils (39), and IL-8 differentially activate b2, b3, and b7 integrins in a B lymphoblastoid line transfected with the IL-8 receptor (40). The exact signaling pathways downstream of PTX-sensitive G proteins, whereby chemokines induce activation of specific integrins, remains to be established and is an active area of investigation.

Taken together, these data support the concept that chemokines are important activating signals of leukocytes that convert the low-affinity selectin-mediated interactions into a higher affinity integrin-mediated adhesion. This ultimately leads to firm adherence of leukocytes to the vessel wall before the transmigration of the endothelial barrier. As such, at least certain members of the a and b chemokines can function not only as leukocyte chemoattractants but also as proadhesive agents in the recruitment of leukocytes to sites of inflammation.

ROLE OF CHEMOKINES IN DEVELOPMENT AND HOMEOSTASIS

Chemokines provide the directional cues for the movement of leukocytes in development and homeostasis.

Development

The importance of chemokines in development can be demonstrated by analyzing the effects of targeted deletions of chemokines and chemokine receptors in murine systems. Such experiments have elucidated unexpected roles for chemokines *in vivo*. For example, mice deficient in SDF-1 (41) or its receptor CXCR4 (42,43) have defects in B-cell lymphopoiesis and the recruitment of hematopoietic progenitors from the fetal liver into the bone marrow, suggesting an important role for this chemokine in these processes. These mice also demonstrated defects in cardiac, cerebellar, and vascular moprhogenesis. This dramatic phenotype underscores the possibility that, aside from directing the migration of leukocytes to sites of inflammation and infection, chemokines may play an important role in orchestrating the movement of cells during development.

This dramatic developmental defect appears to be the exception rather than the rule, however, for targeted deletions of chemokines or their receptors. For example, mice with a targeted deletion of CCR1 (44,45), CCR2 (46), CCR5 (47), MIP-1a MCP-1 (48), eotaxin (49), or IP-10 (unpublished observations) develop normally and do not manifest any significant pathologic lesions at baseline. In these circumstances, defects in immune function sometimes can be demonstrated after specific immunologic challenge. For example, deletion of MCP-1 and its receptor CCR2 resulted in mice that did not manifest any gross pathologic defect but manifested clear defects of macrophage and monocyte recruitment on stimulation (46,48).

Homeostasis

Chemokines are also believed to control the baseline trafficking of leukocytes through tissues. Lymphocytes continuously recirculate through the blood, tissues, and lymphatics in an organized manner, bringing naive lymphocytes into the lymph nodes, where they encounter antigen and memory lymphocytes into inflamed tissue to ensure immunity. T cells routinely patrol the body in search of foreign antigens and recirculate through the blood, tissue, and lymphatics, making more than 20 round trips each day. Several recently identified chemokines are believed to participate in guiding T cells in this process. One such chemokine that has been independently identified by several groups and given the various names of secondary lymphoid chemokine (SLC), exodus-2, 6Ckine, and thymus-derived chemotactic agent 4 (TCA-4), appears to play an important role in directing naive T cells into peripheral lymph nodes. A role for SLC in the trafficking of T cells into lymph nodes has been suggested from studies that have examined the mutant mouse strain DDD (50). This mutant mouse strain has a paucity of lymph node T cells (and hence also called *plt*) because of a defect in the lymph node stroma. Lymph nodes from the DDD mouse do not express the SLC chemokine (51) because of a mutation in one of two SLC genes that is normally expressed in lymphoid tissue (52).

Much the same way T cells recirculate, B cells also traffic through the body, and this process is also controlled by chemokines (53). The B-cell specific CXC chemokine independently identified as BCA-1 and BLC (B-lymphocyte chemoattractant) is an important participant in this process (54,55). BCA is a potent chemotactic factor for B cells and is expressed in the follicles of Peyer's patches, the spleen, and lymph nodes. BCA is a ligand for CXCR5, which is highly expressed on peripheral blood B cells. A role for BCA and CXCR5 in B-cell trafficking was revealed by the generation of a mouse strain deficient in CXCR5 (56). These mice have an impairment in the trafficking of peripheral blood B cells into lymph nodes.

Other leukocytes, such as macrophages, neutrophils, eosinophils, and mast cells, also traffic into tissue. Although these cells are produced in the bone marrow, they reside primarily in other tissues. The role of chemokines in regulating this process has begun to be elucidated from studies in mice deficient in chemokines. For example, the IL-8 receptor homolog in mice, murine CXCR2, plays an important role in drawing circulating neutrophils into tissues in unchallenged mice (57). Likewise, eotaxin plays a critical role in normal eosinophil recruitment into tissues, especially the gastrointestinal tract where eosinophils are thought to play an important role in host defense against helminthic pathogens (49).

ROLE IN ORCHESTRATING THE IMMUNE RESPONSE: LINKING INNATE AND ADAPTIVE IMMUNITY

Chemokines provide the directional cues necessary to bring together T cells, B cells, and dendritic cells to generate an immune response and to recruit leukocytes into sites of inflammation and infection (Fig. 16.4).

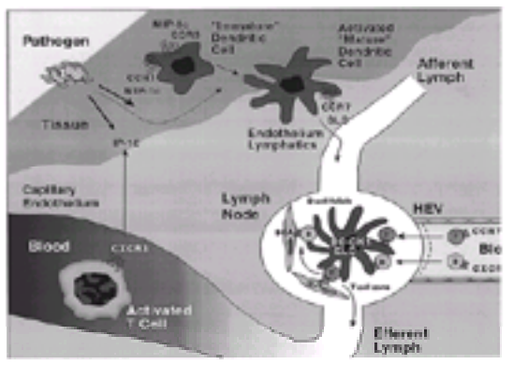

Figure 16.4. Chemokines orchestrate the trafficking of dendritic cells, T cells, and B cells necessary to generate an immune response. In the example depicted, LPS on the surface of bacterial pathogens stimulates the local release of chemokines, such as MIP-1a, MIP-3a, and IP-10. Immature dendritic cells are attracted to this site through the activation of chemokine receptors, such as CCR1 and CCR6, which they constitutively express. Immature dendritic cells are efficient at picking up antigen but need to mature and differentiate into cells capable of activating naive T cells. The local milieu into which the immature dendritic cell has been attracted contains factors, such as lipopolysaccharide (LPS) and tumor necrosis factor (TNF), that induce dendritic cell differentiation and maturation into potent antigen presenting cells. In this process, the dendritic cell downregulates CCR6 and upregulates CCR7, which leads to its migration into the afferent lymphatic. The CCR7 ligand SLC, which is expressed on the endothelium of afferent lymphatics, likely plays an important role in directing the migration of the antigen-loaded mature dendritic cells. Chemokines also play an important role in bringing naive T cells and B cells across high endothelial venules (HEV) into the lymph nodes and into contact with the activated dendritic cell. Although the molecular details remain to be elucidated, it is likely that chemokines, such as DC-CK1 and Epstein-Barr virus-induced gene 1-ligand chemokine (ELC), play an important role in juxtapostioning these cells in the lymph node. SLC and ELC are prodcued from stromal cells in the T-cell zone, whereas B-cell attracting chemokine (BCA) is expressed from stromal cells in the B-cell follicle, helping to guide cells to T- and B-cell areas, respectively. Some activated T cells downregulate CCR7 and upregulate CXCR5 and become directed toward the follicle, whereas other activated T cells upregulate CXCR3 and are attracted into inflamed

tissue. T cells activated in regional lymph nodes, following encounter with antigen-loaded dendritic cells, subsequently return to sites of inflammation by sensing chemokine gradients established at these local sites. Chemokines such as IP-10, a chemokine induced by LPS and IFNg, and a ligand for CXCR3, which is highly expressed on activated T cells, are believed to play an important role in this process.

Generation of Effector Lymphocytes

Dendritic cells are thought to play a pivotal role in generating an immune response by capturing and presenting antigen to lymphocytes in a process that leads to the activation of T and B cells. Dendritic cells in tissue capture and process antigen and transport it to local lymph nodes for presentation to lymphocytes. Several studies suggested that chemokines participate in this process (58,59). Immature dendritic cells reside in the tissue, where they are efficient at engulfing antigen but are not so efficient at activating lymphocytes. Immature CD34+ Langerhans-like dendritic cells respond to a number of chemokines, including the CC chemokine MIP-1a and MIP-3a, which has also been called liver and activation-regulated chemokine (LARC) or exodus. MIP-3a is a ligand for CCR6, which is highly expressed and functional on immature dendritic cells. MIP-3a is constitutively expressed by keratinocytes and venular endothelial cells in normal skin (60) and may be an important role in the trafficking of epidermal Langerhans cells to the skin. In addition, MIP-3a is expressed in tonsils by inflamed epithelium, a site known to be infiltrated by immature dendritic cells (61). Moreover, MIP-3a and MIP-1>a are induced by inflammatory stimuli, such as lipopolysaccharide (LPS) or tumor necrosis factor-a (TNFa). It has been hypothesized that MIP-3a attracts immature dendritic cells into the tissue, a process that is likely amplified by other “inflammatory” chemokines, such as MIP-1a. Once in the vicinity of the inflammatory stimulus, immature dendritic cells pick up antigen and then differentiate into cells more capable of activating lymphocytes. During this maturation process, dendritic cells downmodulate the expression of CCR6 and hence responsiveness to MIP-3a. At the same time, they upregulate their expression of CCR7, allowing them to respond to SLC and ELC. This switch in chemokine receptor expression and chemokine responsiveness results in the dendritic cells leaving the tissue and being drawn into the lymphatics and, ultimately, into the T-cell-rich regions of lymph nodes.

Although the molecular details remain to be fully elucidated, it is likely that the expression of chemokines by lymph node stroma and dendritic cells coordinate the juxtaposition of antigen-loaded dendritic cells with recirculating T and B cells. Chemokines that may play a role in this process are dendritic cell chemokine 1 (DC-CK1) [which was also identified as pulmonary and activation-regulated chemokine (PARC), alternative macrophage activation-associated GC chemokine-1 (AMAC-1), and MIP-4, monocyte-derived chemokine (MDC), thymus-expressed chemokine (TECK), and ELC (MIP-3b)]. DC-CK1 and ELC are expressed by activated mature dendritic cells and recruit naive T cells (62,63). In contrast, MDC is induced in Langerhans cells migrating from contact-sensitized skin during maturation into lymph node dendritic cells and chemoattracts antigen-specific T cells but not naive T cells (64). SLC and ELC are produced from stromal cells in the T-cell zone, whereas BCA is expressed from stromal cells in the B-cell follicle helping to guide cells to T- and B-cells areas, respectively. Some activated T cells may downregulate CCR7 and upregulate CXCR5 and become directed toward the follicle, whereas other activated T cells downregulate CCR7 and upregulate CXCR3 and are attracted into inflamed tissue.

T cells activated in regional lymph nodes, following an encounter with antigen-loaded dendritic cells, subsequently return to sites of inflammation by sensing chemokine gradients established at these local sites. Chemokines such as IP-10, a chemokine induced by LPS and interferon (IFN), and a ligand for CXCR3, which is highly expressed on activated T cells, plays an important role in this process (65) (Fig. 16.4).

Chemokines and Lymphocyte Effector Function: Localization of Cells to Tissue

Chemokines also participate in the effector phase of immune responses to coordinate the localization and specificity of the immune response. This is exemplified in T-cell-mediated immune responses. T-cell-mediated inflammatory responses can be segregated into distinct patterns based on the cytokine secretion pattern of CD4+ T-helper lymphocytes. T-helper (Th)-1 cells secrete mainly IL-2, IFN-g, and IL-12 and mediate immune responses characterized by delayed-type hypersensitivity with activated T cells and macrophages. In contrast, Th2 cells secrete IL-4, IL-5, IL-10, and IL-13, are involved in allergic inflammatory responses, and favor the development of humoral immunity [antigen-specific immunoglobulin E (IgE), and mast cell and eosinophil activation]. Studies suggested that, under certain conditions, Th1 and Th2 cells can express different chemokine receptors (66) (Fig. 16.5 and Fig. 16.6). In these studies, Th1 cells preferentially expressed CXCR3, CCR5, and CCR7 following activation, whereas activated Th2 cells expressed CCR3, CCR4, and CCR8. This differential expression of chemokine receptors may play an important role in the selective recruitment of polarized lymphocyte subsets in different disease states.

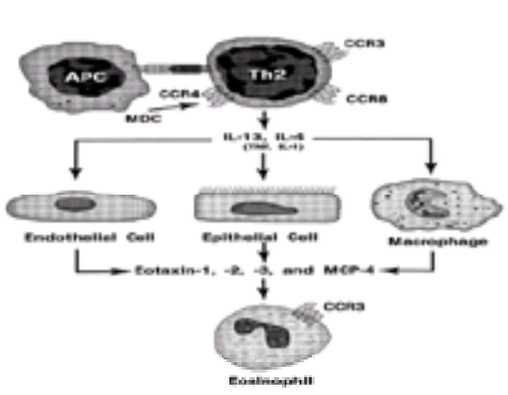

Figure 16.5. Chemokines link T-cell helper (Th2) lymphocyte activation and tissue eosinophilia. Antigen-activated CD4+ Th2 cells elaborate interleukin-4 and -13 (IL-4, IL-13), which synergize with proinflammatory cytokines, such as IL-1 and tumor necrosis factor (TNF), stimulating the production of eosinophil chemoattractants, such as eotaxin-1, eotaxin-3, and MCP-4, from epithelial and endothelial cells are tissue macrophages. These chemokines, in turn, attract activated eosinophils into the tissue, resulting in the hallmark of allergic diseases.

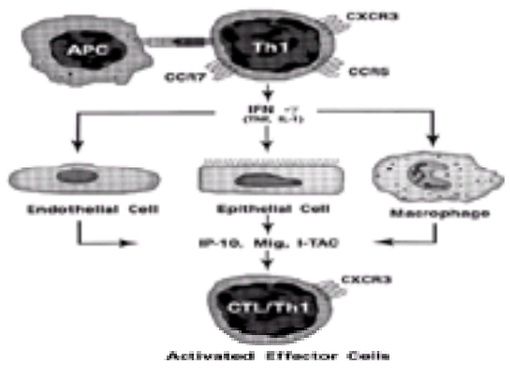

Figure 16.6. Chemokines amplify T-cell helper 1 (Th1) responses and recruit cytotoxic T-lymphocytes (CTLs) to sites of infection. Antigen-activated CD4+ Th1 cells elaborate IFNg, which synergizes with proinflammatory cytokines, such as IL-1 and TNF, stimulating the production of interferon-inducible protein 10 (IP-10), Mig, and interferon-induced T-cell µ chemoattractant (I-TAC) from epithelial and endothelial cells, which are tissue macrophages. These chemokines, in turn, attract activated Th1 cells and CTLs into the tissue.

Allergic pulmonary inflammation exemplifies a specific disease state in which a polarized inflammatory response (Th2 based) occurs. In certain persons, primary sensitization with an airborne allergen leads to the development of antigen-specific CD4+ T cells with a Th2 cytokine secretory phenotype. A secondary encounter with antigen leads to the elaboration of Th2 cytokines that promote the growth and maturation of eosinophils (IL-5) and mast cells (IL-3) and favor the class switch of Ig to the production of IgE (IL-4). These three developments are the hallmark of allergic inflammation. Th2 cells also secrete cytokines that promote the production of eosinophil-active chemokines, such as eotaxin-1, eotaxin-2, eotaxin-3, and MCP-4 (Fig. 16.5). The production of cytokines, such as IL-13, at the site of antigen deposition (e.g., respiratory tract) leads to the localized production of the eotaxins and MCP-4 from endothelial and epithelial cells, resulting in eosinophilic tissue inflammation (2).

Chemokines also play a role in the effector function of Th1 inflammtory processes, such as the host response to intracellular pathogens, and numerous chronic inflammatory diseases, such as multiple sclerosis, sarcoidosis, rheumatoid arthritis, and atherosclerosis. Activated Th1 cells express CXCR3 and CCR5 and respond to the CXCR3 ligands IP-10, Mig, and interferon-induced T-cell a chemoattractant (I-TAC), and the CCR5 ligands MIP-1a, MIP-1b, and RANTES (67,68 and 69). These *in vitro* studies were supported by studies that examined chemokine and chemokine receptor expression in diseased tissue. We reported that human atheroma express high levels of IP-10, Mig, and I-TAC and that virtually all the T cells found in atheromatous lesions were CXCR3 positive (70). Likewise, high levels of IP-10 were found in the bronchoalveolar fluid of patients with active pulmonary sarcoidosis, and all the BAL T cells were CXCR3 positive (71). Similarly, in rheumatoid arthritis patients, T cells recovered from synovial fluid expressed higher levels of CXCR3 and CCR5 than peripheral blood T cells (72). Lymphocytes infiltrating the liver of patients with chronic hepatitis C expressed CXCR3 and CCR5 (73). Whereas the CCR5 ligands MIP-1a and MIP-1b were confined to the vessels within the portal tract, the CXCR3 ligands IP-10 and Mig were selectively upregulated on sinusoidal endothelium. These findings suggest a specific role for CXCR3 ligands, as opposed to CCR5 ligands, in the recruitment of T cells into the hepatic parenchyma. Taken together, these studies implicate the IFNg-inducible CXC chemokines IP-10, Mig, and I-TAC, in regulating Th1 cell trafficking into tissue where there is ongoing Th1 inflammation (Fig. 16.6). In support of this hypothesis, we found that IP-10 plays a critical role in the recruitment of effector T cells into tissue following infection with the intracellular pathogen *Toxoplasma gondii* (65).

Chemokines also play a role in the effector function of CD8+ cytotoxic T-lymphocytes (CTLs). CD8+ lymphocytes play an important role in the host defense against viral infection, including human immunodeficiency virus-1 (HIV-1) (74). CD8+ T cells exert their effector functions through both cytolytic and noncytolytic mechanisms (75). Chemokines are important effector molecules in preventing viral entry into cells by HIV (76). CD8+ T cells are at least one important source of these chemokines. The release of chemokines from CD8+ T cells is dependent on the activation of these cells through their T-cell receptors for antigen (77) (Fig. 16.7). We found that these chemokines, including RANTES, MIP-1a, and MIP-1b, are stored preformed in cytolytic granules, colocalized with granzyme A and proteoglycans (78). Following CTL activation, the contents of the cytolytic granules, including chemokines complexed to proteoglycans, are coordinately released, where they participate in antiviral effector functions by inhibiting viral entry into uninfected cells. This finding demonstrates the coupling of cytolytic and noncytolytic antiviral functions of CD8+ T cells, which is regulated by antigen-specific T-cell activation.

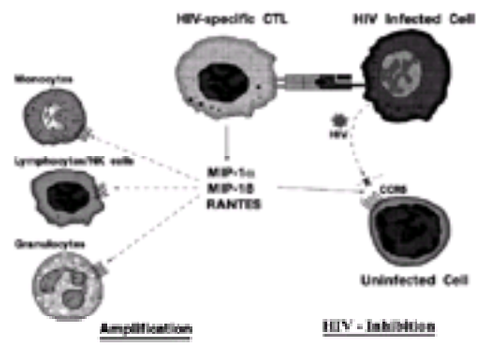

Figure 16.7. Chemokines released from cytotoxic T-lymphocytes (CTL) localize and amplify the immune response by recruiting leukocytes to the site of viral replication and inhibit human immunodeficiency virus-1 (HIV-1) entry into cells. Antigen activation of CD8+ cytotoxic T cells results in the release of MIP-1a, MIP-1b, and RANTES directly onto the target cell. The release of these chemokines at the site of infection serves as a beacon to call in additional leukocytes, such as macrophages, granulocytes, and natural killer (NK) cells, resulting in the amplification of the local immune response. In the case of HIV-1 infection, these CCR5 ligands have the additional property of inhibiting HIV entry into nearby cells.

The release of chemokines from CTLs following antigen specific activation is a general property of these cells and is not restricted to HIV-specific CTLs. CTLs specific for hepatitis B and C virus-infected cells also release large amounts of chemokines when activated by antigen (75). The release of chemokines directly onto the infected cell recruits other inflammatory cells, including professional phagocytes to the site of viral replication, amplifying the response to include the power of the innate immune system.

Role in Human Disease

It is likely that chemokines play a role in most disease processes that result in the accumulation and activation of leukocytes in tissues (79). Chemokines have been detected during inflammation in most organs, including the skin, brain, joints, meninges, lungs, blood vessels, kidneys, and gastrointestinal tract. In these organs, chemokines can be detected in many types of cells, suggesting that most, if not all, cells have the capacity to secrete them given the appropriate stimulus. Major stimuli for chemokine production are early proinflammatory cytokines, such as IL-1 and TNFa, bacterial products such as LPS, and viral infections. In addition, IFNg and IL-4/IL-13, products of Th1 and Th2 lymphocytes, respectively, can induce the production of chemokines and also synergize with IL-1 and TNFa to stimulate chemokine secretion. The capacity to control precisely the movement of inflammatory cells suggests that the various chemokines and their receptors might provide novel targets for therapeutic interventions to modify the courses of these diseases.

The type of inflammatory infiltrate that characterizes a specific disease is controlled, in part, by the subset of chemokines expressed in the disease. For example, many acute disease processes, such as bacterial pneumonia and the acute respiratory distress syndrome, are characterized by a massive influx of neutrophils into the tissue. The concentration of chemokines that are potent neutrophil chemoattractants, such as IL-8, are increased in the bronchoalveolar fluid of patients who have these diseases (80). Other acute diseases, particularly nonbacterial infectious diseases, such as viral meningitis, are characterized by the recruitment of monocytes and lymphocytes into the tissue. The cerebrospinal fluid concentrations of chemokines active on these cells, such as IP-10 and MCP-1, are increased in these patients, and the concentrations are correlated with the extent of mononuclear cell infiltration of the meninges (81).

Many chronic disease processes are characterized by tissue infiltration of lymphocytes and macrophages. The delayed-type hypersensitivity granulomatous lesions of tuberculoid leprosy, sarcoidosis, and multiple sclerosis is characterized by the accumulation of activated lymphocytes, and high concentrations of IP-10 have been detected in these lesions (71,82,83). In addition, levels of IP-10 in the bronchoalveolar fluid of patients with active sarcoidosis correlated with the number of T-lymphocytes in the fluid (71).

In atherosclerosis, macrophages and lymphocytes are the major inflammatory cells found in the diseased blood vessels. These cells are thought to be central to the pathogenesis of this disease, both as progenitors of lipid-laden foam cells and as a source of growth factors that mediate intimal hyperplasia. The CC chemokines [(MCP-1, MCP-4, RANTES), pulmonary and activation-regulated chemokine (PARC), and ELC)] and the CXC chemokines (IL-8, SDF-1, IP-10, Mig, and I-TAC) have all been detected in diseased vessels but not in normal arteries. A functional role for MCP-1 (84) and its receptor CCR2 (85) as well as for the mouse IL-8 receptor CXCR2 (86) in the recruitment of monocytes into atherosclerotic lesions was revealed when mice deficient in these genes were bred with mouse strains prone to develop atherosclerosis, such as the apolipoprotein-E-deficient and low-density lipoprotein (LDL) receptor deficient mouse strains. These “bigenic” (double transgenic) mice had a decrease in the number of lesional macrophages and a decrease in atherosclerotic lesion formation, establishing a role for chemokines in the pathogenesis of this disease.

In allergic diseases, such as asthma, rhinitis, and atopic dermatitis, the selective accumulation and activation of eosinophils and mast cells characterize the inflammatory reaction. Agents that induce the release of histamine from mast cells and basophils, so-called histamine releasing factors, are also strongly associated with the pathogenesis of allergic diseases. Chemokines, in particular eotaxin and the MCPs, are potent eosinophil chemoattractants and histamine-releasing factors, making them particularly important in the pathogenesis of allergic inflammation. In fact, the chemokines may be the major histamine-releasing factors in the absence of antigen and IgE antibody. Many chemokines have been detected in the airways of patients with asthma. In addition, several eosinophil-active chemokines are increased in the epithelial tissue of patients with atopic dermatitis, allergic rhinitis, and asthma after antigen challenge, making it likely that the chemokines are one of the molecular links between antigen specific immune activation and tissue recruitment of eosinophils. In animals with allergic pulmonary inflammation, expression of eotaxin, MIP-1a, and MCP-1, -3, and -5 precede the massive airway recruitment of mononuclear cells and eosinophils. From studies using antibodies that inhibit the action of these chemokines and studies of mice with a targeted disruption of the eotaxin gene, it is clear that all these chemokines are participating in the recruitment of eosinophils into the airways.

SUMMARY

Although initially identified as chemotactic factors profoundly induced by proinflammatory stimuli that mobilize the innate immune system and control the recruitment of leukocytes into inflammatory foci, the chemokines have emerged as important regulators of cellular trafficking in development and homeostasis. In addition, they are critical for the coordinated movement of dendritic cells and lymphocytes necessary to link the acquired immune response to the innate response and generate

long-lasting antigen specific immunity. Chemokines are highly expressed in human inflammatory diseases, and their specific roles in disease pathogenesis are being elucidated, revealing that these fascinating molecules offer a new class of targets for the treatment of a variety of human diseases.

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17 HEMATOPOIETIC CYTOKINES

E. Richard Stanley, Ph.D.

Blood contains red cells, monocytes, lymphocytes, megakaryocytes, and the various types of granulocyte. All mature blood cell types turn over rapidly. Investigators have estimated that humans produce 2×10^6 red blood cells every second. White blood cells, which represent at least 0.1% of red blood cell numbers, can be expected to be produced at a proportionally similar rate. In the adult, differentiated blood cells are all ultimately derived from a small bone marrow pool of undifferentiated, pluripotent hematopoietic stem cells. This process, involving extensive cell proliferation and differentiation, is known as *hematopoiesis.* Work carried out since the 1960s has indicated that hematopoiesis is regulated by a specific group of cytokines.

The studies of Till and McCulloch paved the way to our current understanding of the pluripotent hematopoietic stem cell (1), which gives rise not only to mature blood cells (erythroid and myeloid) but also to the lymphoid cells. Previous studies had shown that the intravenous injection of bone marrow cells into lethally irradiated mice could prevent their anemia and death. Till and McCulloch showed that if limiting numbers of normal mouse bone marrow cells were injected into the circulation of the irradiated mouse, colonies could be seen in the recipient animal's spleen 10 days after injection. With other researchers (2,3), they then showed that these spleen colonies were each derived from single cells, and that some contained not only several myeloid cell types but also cells that were capable of generating more splenic colonies after injection into a second lethally irradiated mouse. This finding supported the concept of a hematopoietic stem cell with the capacity to (a) self-replicate; (b) proliferate to produce many cell progeny; and (c) differentiate to generate all the mature blood cell types. Because of their low frequency and difficulty of isolation and maintenance *in vitro,* the existence and characteristics of hematopoietic stem cells have largely been inferred from an analysis of their progeny. In fact, only recently have conditions been established that allow proliferation of such stem cells *in vitro* without their differentiation to more mature cells (4).

It is now generally accepted that the mouse pluripotent hematopoietic stem cell must be capable of the long-term repopulation (longer than 6 months) of both the myeloid and lymphoid systems of lethally irradiated recipient mice. Studies in the mouse have shown that the pool of such stem cells represents only 10^{-5} of the total nucleated bone marrow cells (5). In the normal steady state, cells of this pool are either not cycling or are cycling slowly (6,7). When a stem cell undergoes a stem cell division, it gives rise to a daughter cell that is indistinguishable from the parent cell and a daughter cell that proliferates extensively but also differentiates. Because one exiting hematopoietic stem cell is capable of giving rise to millions of mature descendent cells, only a small proportion of the cells needs to exit the stem cell pool to proliferate and differentiate, despite the high turnover of blood cells. Under the control of several cytokines, the proliferating and differentiating stem cell gives rise to common myeloid (8) and common lymphoid (9) progenitor cells, which, as they proliferate and differentiate, progressively develop a more restricted capacity for differentiation and eventually give rise to cells that are capable of forming only one mature blood or lymphoid cell type (10) (Fig. 17.1).

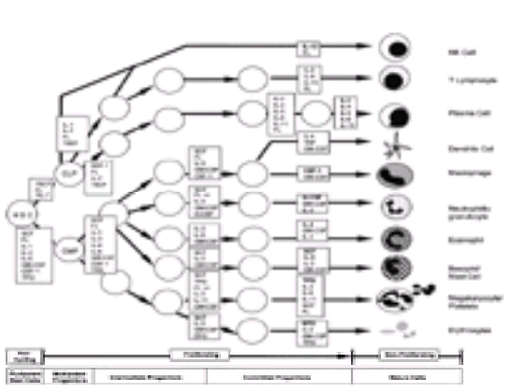

Figure 17.1. Hematopoietic cells, indicating the points of *in vivo* and *in vitro* regulation by hematopoietic cytokines. Note that some cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), may not regulate steady-state hematopoiesis but can be used to regulate hematopoiesis pharmacologically. Cytokines primarily regulating the proliferation and differentiation of committed progenitors of individual lineages are emboldened. CLP, common lymphoid stem cell; CMP, common myeloid stem cell; HSC, hematopoietic stem cell.

Our understanding of the hematopoietic cytokines arose from the description of semisolid culture methods for bone marrow in which progenitor cells proliferated and differentiated to form macroscopic colonies of mature granulocytes or macrophages (11,12). Colony formation required the presence of a source of a cytokine, or colony-stimulating factor (CSF), and it was subsequently shown that different cytokines caused the development of cells of different lineages. The number of colonies was linearly related to the number of cells cultured, and so these cultures could be used to assay specific progenitor cells in hematopoietic cell populations. In addition, because at a constant cell concentration, the number of colonies was proportional to the concentration of the cytokine, the cultures could also be used as cytokine

assay systems. These *in vitro* bioassays were used to monitor cytokine purification so protein sequence could be obtained and used to clone their genes (13,14 and 15). The analysis of mice with targeted inactivations of the genes encoding most of these cytokines and their receptors has greatly increased our understanding of their biology. Because of the dominant role of cytokines in the regulation of hematopoiesis and the importance of maintaining hematopoiesis in many clinical situations, the production of recombinant cytokines in large amounts has led to their rapid clinical application.

This chapter reviews our current knowledge of the nature and biology of the hematopoietic cytokines, including stem cell factor (SCF), fms-like tyrosine kinase 3 (flt3), ligand (FL), interleukin-3 (IL-3), IL-5, and IL-7, granulocyte-macrophage CSF (GM-CSF), CSF-1 (or macrophage CSF), granulocyte CSF (G-CSF), erythropoietin (EPO), and thrombopoietin (TPO). Also discussed are IL-1, IL-4, IL-6, IL-11, IL-15, leukemia inhibitory factor (LIF), chemokines, and other molecules that are also involved in the regulation of hematopoiesis. Table 17.1 lists the nature, chromosomal gene location, and sources of many of these cytokines, together with the primary hematopoietic phenotypes of the mice deficient in either the cytokine or the cytokine receptor (16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34 and 35). The hematopoietic lineages they regulate are indicated in Fig. 17.1.

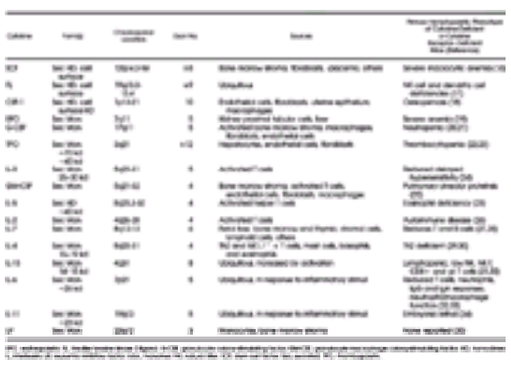

TABLE 17.1. Hematopoietic Cytokines

GENERAL ASPECTS OF CYTOKINES AND CYTOKINE REGULATION OF HEMATOPOIESIS

Sources and Regulation

The hematopoietic cytokines are glycoproteins, which are normally present in the circulation (e.g., CSF-1, SCF, FL, G-CSF, EPO, and TPO) or are induced to appear in response to infection or inflammation (e.g., GM-CSF, IL-3, IL-5, IL-6, and IL-11). They are found in picomolar concentrations, and their concentrations are individually increased in response to specific triggering conditions (e.g., EPO is increased in response to hypoxia) or to depletion of particular blood cell types in various cytopenic conditions (e.g., TPO is increased in response to platelet loss). Apart from EPO, which is synthesized by the kidney, these cytokines are often synthesized by multiple cell types of the bone marrow stroma and immune system. Many are also synthesized by fibroblasts, endothelial cells, and some by yet other cell types, including epithelial cells (Table 17.1).

Cytokine Structure

The carbohydrate moieties of these cytokines are not required for *in vitro* biologic activity, but they may be important for their half-life and localization *in vivo.* Even though there is little conservation of their primary structures, the crystal structures of many of hematopoietic cytokines, including CSF-1 (36), GM-CSF (37), G-CSF (38), IL-2 (39,40), IL-3 (41), and IL-6 (42), have been determined, and they have strikingly similar tertiary structures. In general, like growth hormone (43), they are composed of four a-helical bundles and an antiparallel b ribbon (Fig. 17.2). Some (SCF, CSF-1, FL, and IL-5) are homodimers, whereas the others are monomeric. Three of the cytokines, SCF, CSF-1, and FL, are expressed as membrane-spanning forms that are active at the cell surface and are involved in local regulation (Table 17.1).

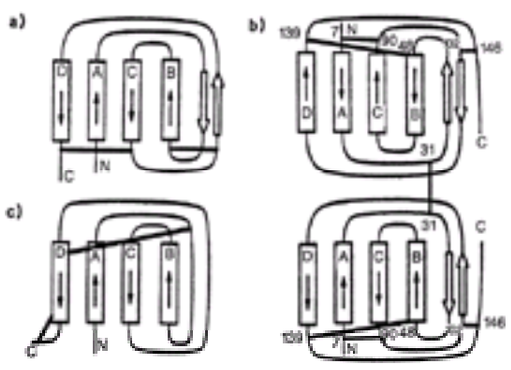

Figure 17.2. Similarity of the tertiary structures of the monomeric and dimeric hematopoietic cytokines with the structure of growth hormone. Topologic structures are evident, showing all the disulfide bonds. N and C, termini; A, B, C, D, a-helices; *unfilled arrows,* b strands. **A:** Granulocyte-macrophage colony-stimulating factor (GM-CSF). **B:** Colony-stimulating factor-1 (CSF-1) (amino acids 4 to 158 only). **C:** Growth hormone. (From Pandit J, Bohm A, Jancarik J, et al. Three-dimensional structure of dimeric human recombinant macrophage colony-stimulating factor. *Science* 1992;258:1358–1362, with permission. Copyright by the American Association for the Advancement of Science.)

Cytokine Receptor Signaling

Hematopoietic cytokine action on target cells is mediated by high-affinity cell-surface receptors (Fig. 17.3) that transduce the binding of their cognate ligands to signals for survival, proliferation, and differentiation. Binding of each of the cytokines to their cognate, high-affinity receptors or to ligand-binding subunits that lead to the assembly of high-affinity receptors results in extracellular cross-linking and dimerization of the signaling subunit and tyrosine phosphorylation of its intracellular domain. This occurs irrespective of whether the receptor contains an intrinsic intracellular tyrosine kinase domain, because the cytoplasmic domains of those that are not receptor tyrosine kinases are preassociated with tyrosine kinases. The end result is the phosphorylation of particular tyrosine residues of the cytoplasmic domains of the receptors that act as binding sites for signaling molecules possessing phosphotyrosine binding domains. Several aspects of the downstream, intracellular signaling pathways of hematopoietic cytokines are similar, because different activated receptor cytoplasmic domains often bind a common signaling molecule or family of signaling molecules.

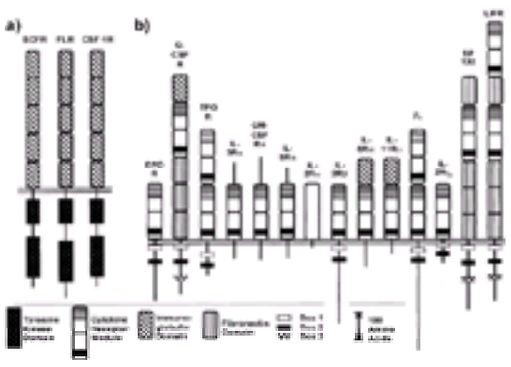

Figure 17.3. Some high-affinity hematopoietic cytokine receptors showing the similarity between those containing intrinsic tyrosine kinase domains **(A)** and the modular

nature of the “cytokine” receptor family **(B),** the signaling subunits of which are preassociated with a tyrosine kinases. The box 1, 2, and 3 motifs of the latter family are the only regions of homology in the cytoplasmic domains. Scale drawing.

Requirement of Multiple Cytokines for the Proliferation and Differentiation of Multipotent Cells

The *in vitro* proliferation and differentiation of primitive hematopoietic cells (pluripotent stem cells and multipotent progenitor cells) require a combination of cytokines (e.g., SCF, IL-1, IL-3, IL-6, GM-CSF, and CSF-1) (44,45). As expected from this multifactorial growth requirement, immature multipotent hematopoietic cells have been shown to coexpress different lineage-specific cytokine receptors at low levels (46). As these cells differentiate, they lose receptors for some cytokines (e.g., SCF or IL-3) while retaining the receptors for the late-acting cytokines (e.g., CSF-1 or EPO). Eventually, they reach the stage of committed progenitor cell, where their further proliferation and differentiation are along one particular lineage and are primarily regulated by one late-acting cytokine. In the case of CSF-1, this transition has been shown to be associated with an increase in the level of expression of the receptor for the late-acting cytokine (Fig. 17.1) that may be critical in conferring responsiveness of the cell to the single cytokine (47). Thus, in the absence of other cytokines, most late-acting cytokines (e.g., CSF-1, G-CSF, and EPO) regulate cells of primarily one lineage that selectively express their cognate receptors. The most primitive target cells within these lineages respond by both proliferating and differentiating (e.g., from committed macrophage progenitor into monoblast into promonocyte into monocyte into macrophage). However, these cytokines also regulate the survival of their target cells, often including the fully differentiated and nondividing end cells (48,49). In many instances, they also play an important role in regulating the function of the terminally differentiated cells. CSF-1 and GM-CSF, for example, prime mature cells for neutrophil and macrophage activation by other agents, such as bacterial lipopolysaccharide or immune complexes (50,51). Despite an apparent overlap in target cell specificity of several cytokines, their functions are largely nonredundant, as indicated by the distinct hematopoietic phenotypes of cytokine or cytokine receptor-deficient mice (Table 17.1).

Mechanism of Synergism between Cytokines

The phenomenon of synergism between late-acting, lineage-restricted cytokines, such as CSF-1, EPO, and G-CSF, with cytokines such as SCF, in stimulating the proliferation and differentiation of primitive multipotent cells, provides a mechanism for coupling the changes in levels of the late-acting cytokine, which are tightly regulated by the primary stimuli (e.g., hypoxia in the case of EPO), to the channeling of multipotent cells into a lineage to satisfy the demand for differentiated cells. The mechanisms underlying synergism among cytokines in the regulation of primitive hematopoietic cell proliferation could occur directly at the level of the receptors for the synergizing cytokines. Alternatively, synergism may occur at the level of the postreceptor signal transduction pathways. No strong evidence indicates that a cytokine directly regulates the increased expression of the receptor for the synergizing cytokine (47). However, in the one case studied in depth, the synergism between SCF and EPO in erythropoiesis, synergism is apparently mediated by the direct association of the SCF receptor (SCFR) with the EPO receptor (EPOR). The activation of the SCFR and its association with the EPOR apparently results in tyrosine phosphorylation of the EPOR at sites that differ from the those phosphorylated as a result of EPOR dimerization induced by EPO (52,53).

Permissive and Instructive Effects of Cytokines in Hematopoiesis

Because hematopoietic cytokines regulate survival and proliferation, one important question is whether their regulation of differentiation is “permissive” or “instructive” (Fig 17.4). Evidence for both mechanisms has been obtained in different systems. The permissive model is one in which the growth factor does not have a role in multipotent progenitor cell commitment but simply allows the survival and proliferation of committed cells. In support of this model, mice in which the thrombopoietin receptor gene was replaced with a gene encoding a chimeric receptor consisting of the extracellular and transmembrane domains of the TPO receptor (TPOR) and the cytoplasmic domain of the G-CSF receptor (G-CSFR) possessed a normal platelet count, a finding indicating that the cytoplasmic domain of the G-CSFR can functionally replace that of the TPOR to support normal megakaryocytopoiesis and platelet formation (54). This finding implies that the cytoplasmic domain of the TPOR does not have an instructive role, or that if it does, it must be shared with the instructive function of the G-CSFR cytoplasmic domain. In fact, the sharing of a common instructive signaling pathway is possible for these two similar receptors because their functions can be largely separated by their different patterns of expression on hematopoietic cells and in cells in which their expression overlaps, by the differential regulation of their cytokines, TPO being constitutively present in the circulation and G-CSF induced. In support of the instructive model, investigators showed that the common lymphoid progenitor cell (Fig. 17.1) can be redirected to the myeloid lineage by stimulation through exogenously expressed IL-2 and GM-CSF receptors, and that the granulocyte and monocyte differentiation signals are regulated by different cytoplasmic domains of the IL-2R (55). Thus, it is possible that both mechanisms are used, and that the one used depends on the receptors and commitment steps involved.

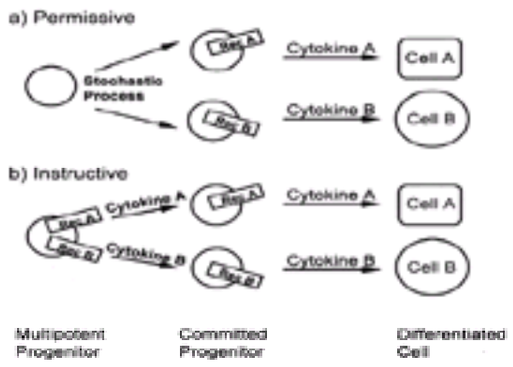

Figure 17.4. Permissive versus instructive models of hematopoietic cytokine action. In the permissive model, multipotent progenitors become committed to either lineage stochastically and consequently express either receptor A or receptor B. Cytokines A and B support the survival and proliferation of committed progenitors expressing their cognate receptor. In the instructive model, multipotent progenitors express receptors for both cytokines A and B. Cytokine A induces their commitment to committed progenitors of the A lineage, whereas cytokine B induces their commitment to the B lineage.

Role of Transcription Factors in the Commitment of Multipotent Progenitor Cells

Experiments with gene-targeted mice (56) and overexpression of transcription factors in multipotent cells clearly indicate that events regulated by nuclear transcription factors are critical for lineage commitment. Thus the transcription factor, GATA-1, reprograms avian myelomonocytic cells from a myeloblast fate into eosinophils, thromboblasts, and erythroblasts, and the transcription factor PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors (57,58). Furthermore, investigators have shown that pre-B cells from mice lacking the B-cell transcription factor, Pax 5, not only fail to differentiate to B cells, but also they acquire the capacity to differentiate to macrophages, osteoclasts, granulocytes, natural killer (NK), and T cells (59). These findings suggest that another mechanism underlying lineage commitment may involve the suppression of other lineage fates in multipotent cells by lineage specific transcription factors, such as Pax 5.

CYTOKINES SIGNALING THROUGH TYROSINE KINASE RECEPTORS

General Considerations

The three hematopoietic cytokines signaling through tyrosine kinase receptors, SCF, FL, and CSF-1, are members of a family of homodimeric cytokines that share some sequence similarity and are structurally similar to each other (60,61,62,63 and 64). Because of alternative splicing and differential proteolysis, all three cytokines are expressed as membrane-spanning, cell-surface, and secreted glycoproteins. All three growth factors are widely expressed in tissues. SCF and CSF-1 have been shown to have effects on nonhematopoietic as well as hematopoietic cells, whereas FL has not yet been characterized in this respect.

The SCF, CSF-1, and FL receptors for these cytokines are all members of the PDGFR family (63,65,66). Their mature forms all possess extracellular domains composed of five immunoglobulin (Ig)-like repeats that are heavily glycosylated with N-linked sugars, a transmembrane domain, and intracellular domains containing a juxtamembrane region, an *src*-related tyrosine kinase interrupted by a kinase insert domain, and a carboxy-terminal tail (Fig. 17.3A). The amino-terminal three Ig-like domains incorporate the ligand binding domains of the SCF and CSF-1 receptors. Binding of their cognate bivalent ligands by this class of receptors stabilizes their noncovalent dimerization at the cell surface and permits *trans*-tyrosine phosphorylation of one intracellular domain by the other (Fig. 17.5). Although tyrosine phosphorylation of cytoplasmic proteins in response to cytokine is not restricted to those proteins that stably associate with the receptor, the receptor phosphotyrosines act as “docking sites” for *src* homology region 2 (SH2) domains of signaling and adaptor proteins. These proteins may interact with plasma membrane-associated

proteins, such as the interaction of recruited Grb2/Sos with Ras, leading to their activation, or they may themselves become tyrosine phosphorylated. Many of the signaling pathways activated by these receptors, including the Ras/Raf/mitogen-activated protein kinase (MAPK) cascade, the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, Src family members, and phosphatidylinositol-3-kinase (PI3K), are shared (67). All three receptors are likely to exhibit ligand-induced, Cbl-mediated receptor downregulation (68). Two of the receptors, SCFR and CSF-1R, are encoded by the protooncogenes c-*kit* and c-*fms*, respectively. v-*kit* and v-*fms* oncogenes are present in retroviruses that cause sarcoma in cats. The viral oncogenes contain mutations in the cellular oncogenes (protooncogenes) that lead to constitutive activation of the receptor in the absence of cytokines (67,69).

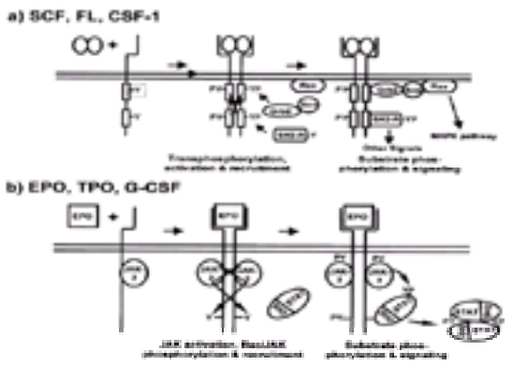

Figure 17.5. Model for the activation and signaling of hematopoietic growth factor receptors signaling through one receptor polypeptide chain. **A:** Receptors possessing intrinsic tyrosine kinase domains. **B:** Receptors without intrinsic tyrosine kinase domains.

Stem Cell Factor

STEM CELL FACTOR AND ITS RECEPTOR

For several decades before SCF was first characterized, the effects of its absence in the Steel (*Sl*) mouse mutants were carefully studied. There are several *Sl* alleles of varying severity, and affected mice variably exhibit defective development in the cell lineages involving pigmentation, hematopoiesis, and fertility (70). Precursor cells of all three lineages undergo migration during normal development for which the cell-surface form of the ligand is apparently important (62). SCF is widely expressed during embryogenesis and in a variety of adult tissues, including bone marrow stromal cells, fibroblasts, and endothelial cells, as well as yolk sac and placenta (71,72). It is synthesized in the endoplasmic reticulum as a 248-amino acid membrane-spanning precursor that dimerizes noncovalently and from which the mature secreted form is subsequently cleaved by metalloproteinases at the cell surface. Tissue-specific alternative splicing eliminates the exon encoding the extracellular proteolytic cleavage site in the membrane-spanning form. Both the secreted 30-kd SCF monomers and the extracellular domain of the cell-surface form are heavily glycosylated and biologically active. The secreted monomer consists of the first 164 residues, and evidence suggests that its tertiary structure is consistent with that described for CSF-1 (Fig. 17.2). An equilibrium exists between the monomers and dimers of both the secreted and membrane forms, but the monomer predominates under physiologic conditions. However, the noncovalent dimerization of the SCF is required for its biologic activity (72,63). The cell-surface form of SCF is more active than the secreted form as a result of more prolonged signaling (73,74).

As with SCF, the biologic effects of mutations in the SCFR gene in the mouse were extensively studied before its identification as the SCFR locus. The locus is known as the dominant white spotting (*W*) locus. Well before the relation between the products of the *Sl* and *W* loci was understood, the *Sl* product (SCF) was proposed to be the ligand for the *W* product (SCFR) on the basis of the complementary results of a series of elegant bone marrow transplantation experiments using mice with *Sl* and *W* mutations (70). Cells expressing the SCFR are frequently contiguous with cells expressing SCF, from the early presomite stage to the mature adult. SCFR is expressed in the germ cells, interstitial cells of Cajal in the gut, melanocytes, and early hematopoietic cells (63,71). SCFR expression in hematopoietic cells is low in primitive multipotent progenitors, is highest in committed progenitors, and decreases as cells mature (72). An alternatively spliced form in which four amino acids encoded by a short exon are inserted into the extracellular domain of the receptor is expressed in mast cells and in the midgestation placenta (75). The significance of this tissue-regulated alternative splicing is not understood, but the shorter isoform appears to have a low but constitutive level of activation in the absence of SCF. A soluble form of the SCFR, found in normal human sera, is able to bind SCF, although its physiologic significance is also unknown (63,71).

BIOLOGY

Apart from its effects on melanocytes and their precursors and on gonadal development, SCF has a broad spectrum of activity on hematopoietic cells. SCF alone cannot stimulate the proliferation of primitive SCFR-expressing progenitor cells. It acts synergistically with many of the hematopoietic cytokines, especially IL-6 and IL-3, to increase the numbers of precursors of most, if not all, lineages (76,77). However, it may not stimulate pluripotent stem cells that do not appear to express the SCFR (78) and that can survive *in vitro* in the absence of SCF (79). SCF-nullizygous *Sl/Sl* mice are embryonic lethals, but *Sl/Sl*^{d} compound heterozygotes bearing the partially functional *Sl*^{d} allele allow embryonic survival, yielding severely anemic mice (70). The severe anemia and mast cell deficiency in *Sl* mice indicate that the major effects of SCF are on erythropoiesis and mast cell development. In combination with EPO, SCF enhances the number of erythroid precursor cells and allows precursors to respond to levels of EPO that are too low to elicit a response in the absence of SCF. Differentiation of intermediate and later precursors requires the presence of SCF (80), after which they become responsive to EPO only. Mast cells and their progenitors require SCF throughout the differentiation of the lineage from early precursors to mature, primed tissue mast cells (72,81), and SCF regulates the migration, maturation, proliferation, and activation of mast cells *in vivo* (71). There is also strong evidence of biologic activity for SCF on the megakaryocyte, granulocyte-macrophage, and lymphoid lineages, yet the effects of the absence of SCF on their development is minimal, a finding implying some redundancy in cytokine action on these lineages. SCF acts on every stage of megakaryocyte development from progenitors to the mature megakaryocyte (72,82), yet circulating platelet levels are normal in *Sl/Sl*^{d}mice.

FL

FL AND FLT3 RECEPTOR

The discovery of the flt3, using cloning approaches to isolate novel receptor tyrosine kinases, preceded the discovery of its ligand, FL, which was identified by its ability to bind a soluble form of flt3 (83,84,85 and 86). FL regulates the proliferation of primitive hematopoietic cells. The predominant isoform of human FL is a biologically active transmembrane glycoprotein that is expressed on the cell surface. It may also be released by proteolysis. The predominant isoform in mice is an active cell-surface form that, although membrane associated, is not membrane spanning and arises from failure to splice an intron. The third isoform, present in both mouse and human tissues, arises from alternative splicing of exon 6 that introduces a stop codon near the end of the extracellular domain and generates a secreted biologically active protein that is relatively rare compared with the other isoforms. The structural similarity between FL and CSF-1, especially in the amino-terminal 150 amino acids essential for biologic activity, suggests that its tertiary structure is similar to that of CSF-1 (Fig. 17.2). Like SCF, FL exist as a noncovalently associated homodimer (63).

As far as the hematopoietic system is concerned, FL receptor (FLR) (flt3) expression is predominantly restricted to the progenitor–stem cell compartment (Fig. 17.1). Although bone marrow monocytes and a small fraction of lymphocytes also express FLR, the functional significance of this expression is not clear. One isoform of the mouse FLR, missing the fifth Ig-like motif in the extracellular domain, is still able to bind ligand and to become tyrosine phosphorylated. Its physiologic significance is not understood (63).

BIOLOGY

Like SCF, FL cannot alone stimulate the proliferation of its primitive hematopoietic target cells, but rather it synergizes with other hematopoietic cytokines. In contrast to SCF, FL has little or no effect on erythroid or megakaryocyte progenitor cells, and FLR knock-out mice have no defects on red cell, megakaryocyte, or platelet production. However, FLR- and FL-deficient mice have reduced numbers of pro-B cells, although they have normal numbers of mature B cells. (63,87,88). FL synergizes with IL-7 in stimulating the proliferation of primitive B lineage cells. FL also synergizes with IL-7 to stimulate proliferation of primitive thymic progenitor cells. FL synergizes with IL-15 to promote the expansion of NK cells from progenitor cells, and FL-deficient mice fail to develop splenic NK cell activity. In combination with GM-CSF, tumor necrosis factor (TNF), and IL-4, FL enhances the production of dendritic cells from BM progenitor cells. Treatment of mice with FL dramatically increases the number of functional dendritic cells in lymphohematopoietic and other tissues, and dendritic cell numbers are reduced in FL-deficient mice (63).

Colony-Stimulating Factor-1

COLONY-STIMULATING FACTOR-1 AND ITS RECEPTOR

CSF-1 regulates the survival, differentiation, and function of cells of the mononuclear phagocytic (monocyte-macrophage) lineage and the function of cells of the female reproductive tract (64,89,90 and 91). It is synthesized in the endoplasmic reticulum to yield a disulfide-linked, homodimeric, membrane-spanning precursor that contains 522 amino acids. In secretory vesicles, the mature forms of CSF-1—glycoprotein (approximately 100 kd) and proteoglycan (approximately 120 to 160 kd) (64)—are cleaved from this precursor and secreted. However, a smaller CSF-1 precursor (224 amino acids), encoded by an mRNA in which the proteolytic cleavage sites have been spliced out, is expressed on the cell surface when the secretory vesicle fuses with the plasma membrane. The amino-terminal 150 amino acids of CSF-1 are sufficient for *in vitro* biologic activity and possess the four a-helical bundle—antiparallel b-ribbon structure (36) (Fig. 17.2).

The approximately 58-kb CSF-1R gene maps to the long arm of chromosome 5, near the genes encoding GM-CSF and IL-3 (69). Extracellular ligand binding by the CSF-1R results in the formation or stabilization of a receptor dimer. Dimer formation is associated with receptor activation and the tyrosine phosphorylation of one intracellular domain of the receptor by the other. The activated, tyrosine phosphorylated CSF-1R mediates the tyrosine phosphorylation of many other, mainly cytoplasmic, proteins, some of which have been shown to have an important role in transduction of the signal for proliferation (92). After activation and signaling, the receptor-ligand complexes are internalized and are destroyed intralysosomally (68,93).

BIOLOGY

CSF-1 is synthesized by a variety of cell types, including fibroblasts, endothelial cells, bone marrow stromal cells, osteoblasts, keratinocytes, astrocytes, myoblasts, and, during pregnancy, uterine epithelial cells (64). Circulating CSF-1 is believed to be synthesized by endothelial cells lining the small blood vessels. Ninety-five percent of circulating CSF-1 is cleared by sinusoidally located macrophages, primarily Kupffer cells, by CSF-1R–mediated endocytosis followed by intralysosomal destruction. Thus, the number of sinusoidally located macrophages determines the concentration of the cytokine responsible for their production, a simple feedback control. The half-life of circulating CSF-1 at physiologic concentrations is about 10 minutes. The half-life of pharmacologic concentrations of CSF-1, which saturate the normal clearance mechanism, is approximately 1.6 hours (94). Circulating CSF-1 is elevated in response to bacterial endotoxin and bacterial, viral, and parasitic infections (64,89,90 and 91). Repeated injections of recombinant CSF-1 in mice elevate the circulating monocyte count about tenfold and increase macrophage numbers in certain areas of the periphery (95).

The role of CSF-1 in the development and regulation of mononuclear phagocytes has been studied in the osteopetrotic (*Csf-1^{op}/Csf-1^{op}*) mutant mouse. This mouse possesses an inactivating mutation in the CSF-1 gene and fails to produce CSF-1 (18,96). Compared with control mice, *Csf-1^{op}/Csf-1^{op}* mice exhibit impaired bone resorption associated with a paucity of osteoclasts and have no incisors, poor fertility, a lower body weight, a shorter average life span, an absence of evoked auditory and visual responses, a thinner than normal dermis, and deficiencies in blood monocytes and macrophages in certain tissues (97,98 and 99). Restoration of circulating CSF-1 in newborn *Csf-1^{op}/Csf-1^{op}* mice cured their osteopetrosis and monocytopenia and improved some but not all the tissue macrophage populations, consistent with local as well as humoral regulation by this cytokine (97,99). Both secreted forms are found in the circulation, and the cell-surface and secreted proteoglycan CSFs are believed to have important roles in local regulation, the latter through its sequestration by specific extracellular matrices (64,99). Studies in the *Csf-1^{op}/Csf-1^{op}* mouse clearly indicate that CSF-1 is the primary regulator of mononuclear phagocyte production. However, the pleiotropic phenotype of these mice and the restricted distribution of the CSF-1R to cells of the mononuclear phagocytic system and female reproductive tract suggest that the major role of CSF-1 is to generate and maintain macrophages that have trophic as well as scavenger (i.e., physiologic) functions and that are important for organogenesis and tissue turnover. The development of macrophages involved in inflammatory and immunologic (i.e., pathologic) functions apparently depends on other cytokines, which, in certain situations, may also regulate the activities of macrophages generated by CSF-1 (64,97,99). Thus, the development of lymph node and thymic macrophages is largely CSF-1-independent. However, CSF-1 does appear to play an important role in priming macrophages to respond to other stimuli, such as TNF-a, IL-1, and IL-6 (50).

The CSF-1R is expressed at low levels on primitive multipotent cells. There is a tenfold increase in its expression as these cells give rise to committed progenitors of macrophages and a further increase in expression in the more mature adherent mononuclear phagocytes. *In vitro* studies indicate that, in contrast to the committed progenitors, the primitive multipotent cells are unable to respond to CSF-1 alone. However, CSF-1 can synergize with other cytokines (e.g., IL-1, SCF, IL-3, IL-6) to stimulate multipotent cell proliferation and differentiation to committed macrophage progenitor cells (44,100,101). Apart from its role as a hematopoietic cytokine, CSF-1 is obviously also critically involved in bone metabolism through its regulation of osteoclast progenitor cell proliferation and differentiation. It is synthesized locally by osteoblasts and stimulates osteoclast differentiation by cooperating with osteoprotergerin ligand (OPGL). CSF-1 alone causes the development of small mononuclear cells, whereas OPGL stimulates the development of active osteoclasts in a CSF-1–dependent manner (102). CSF-1 regulation of macrophages plays an important role in both male and female reproduction (103), and the action of CSF-1 on trophoblast during pregnancy is critical in embryonic resistance to certain infections (104). Under the control of the female endocrine system, CSF-1 is synthesized by the oviduct and uterine epithelium during pregnancy and can influence several CSF-1R–expressing cell populations, including maternal macrophages and decidual cells, as well as early embryonic cells and various trophoblastic cell types (64,99). Circulating levels of CSF-1 are elevated in patients with myeloid and lymphoid malignant diseases and carcinomas of the ovary, endometrium, and breast. In ovarian cancer, elevated levels of CSF-1 in the circulation or ascitic fluid predict a poor prognosis. The etiologic role of CSF-1 in the pathogenesis of these tumors is not clear (64).

CYTOKINES SIGNALING BY DIMERIZATION OF ONE NON–TYROSINE KINASE RECEPTOR POLYPEPTIDE CHAIN

The receptors for EPO, G-CSF, and TPO are transmembrane glycoproteins that belong to a large family of receptors, defined by the presence of an extracellular cytokine receptor motif and referred to as the cytokine receptor family, that includes almost all of the receptor subunits shown in Fig. 17.3B. Within their cytokine receptor motifs, all have conserved short amino acid sequence elements, particularly two pairs of conserved cysteine residues and a Trp-Ser-X-Trp-Ser (WSXWS) motif near the transmembrane domain (105). The function of the WSXWS motif is not clear. EPO, G-CSF, and TPO appear to signal by causing the dimerization of receptors. Although it is clear that the receptor species identified for these cytokines dimerize, studies of all three receptors are still at an early stage, and perhaps other receptor subunits are involved in the high-affinity receptors for at least one of them. Although the receptor subunits for EPO, G-CSF, and TPO do not contain an intrinsic tyrosine kinase domain, activation of the receptor is associated with rapid tyrosine phosphorylation of several cellular proteins, including receptor itself. This is because of the prior association of the receptor with members of the JAK family (EPOR and TPOR with JAK2; G-CSFR with JAK1) that are activated by formation of the high-affinity ligand-receptor complex. Receptor activation on ligand binding leads to receptor tyrosine phosphorylation and recruitment of STATs, which are tyrosine phosphorylated and activated by JAKs, of additional JAKs and other tyrosine kinases, and of SH2 domain-containing protein tyrosine phosphatases (Fig. 17.5). The phosphatases can play positive and negative roles in signaling. The ras-MAPK and PI3K pathways are also activated in response to these cytokines through the recruitment of Grb2/SOS and PI3K. The receptors contain box 1 and box 2 sequences in their cytoplasmic domains that are essential for receptor function.

ERYTHROPOIETIN

Erythropoietin and Its Receptor

EPO, the primary regulator of erythropoiesis, was first of the hematopoietic cytokines to be discovered, its existence first suggested by the early studies of Carnot and Déflandre (106). It was purified from human urine, and the human gene was cloned in 1985 (107). Found at biologically active concentrations in the circulation, EPO is a glycosylated polypeptide of approximately 34 kd that is synthesized primarily by the proximal convoluted tubules of the kidney (108), but also by liver. Its synthesis is primarily regulated by tissue hypoxia sensed by a heme protein (109). The 3' flanking region of the EPO gene contains a hypoxia-inducible element for the assembly of a complex including the hypoxia-inducible factor-1 (HIF-1), hepatic nuclear factor-4 (HNF-4), and the transcriptional activator p300 (110). The 5' flanking region of the EPO gene also contains sites involved in induction by hypoxia and which also bind HNF-4, so HNF-4 may function to bridge the 5' promoter and 3' enhancer regions of the gene (111).

The human EPOR is a membrane-spanning, glycosylated protein of 64 to 78 kd. It appears that the homodimeric EPOR is necessary and sufficient for responsiveness to EPO (112,113), although, as discussed earlier, in response to SCF and EPO, it functionally and physically interacts with the SCFR. *In vitro,* like EPO and SCF, EPO and IL-3 synergize to stimulate the expansion of erythroid burst-forming units (BFU-E), and an interaction between the EPOR and the IL-3 receptor (IL-3R) b-chain has also been reported (114). The first cell within the erythroid lineage in which EPOR is expressed is the late BFU-E that express approximately 300 receptors per cell. Receptor density increases to approximately 1,100 per cell in erythroid colony-forming units (CFU-E) and erythroblasts and then decreases during the terminal stages of erythroid differentiation, to reticulocytes and erythrocytes, where they are virtually absent (115). EPORs are also found in megakaryocytic cells and in endothelial and neural cells.

BIOLOGY

EPO stimulates the proliferation and differentiation of late BFU-E through its synergism with SCF and stimulates expansion of all CFU-E. In addition, it stimulates the release of maturing normoblasts from the bone marrow and increases the amount of hemoglobin synthesized per erythrocyte. Whereas mouse embryos lacking EPO

die by day 13 because of severe anemia (116,117), their fetal livers contain both BFU-E and CFU-E that can be expanded by culturing in SCF and TPO (19), consistent with a primary role of EPO inducing the erthyroid cell survival (49).

Granulocyte Colony-Stimulating Factor

GRANULOCYTE COLONY-STIMULATING FACTOR AND ITS RECEPTOR

G-CSF was purified from the medium conditioned by cultured cells, and the gene was cloned in the mid-1980s (118,119 and 120). G-CSF is unusual in that it possesses only a single O-linked oligosaccharide chain, yielding a molecular weight of 19 kd. It shares significant sequence homology with IL-6. G-CSF is synthesized by a variety of cell types, including stromal cells, fibroblasts, and endothelial cells, but primarily in response to inflammatory stimuli such as lipopolysaccharide (LPS), TNF, and IL-1 (121,122). The G-CSF promoter region contains both kB and nuclear factor (NF)–IL-6 sites and cooperatively binds the p65 and C/EBPb transcription factors induced by IL-1 and TNF in fibroblasts (123,124).

The G-CSFR is 812 amino acids in length, with a large extracellular domain composed of an Ig domain, a cytokine receptor module and 3 fibronectin domains, and a cytoplasmic domain with box 1 and box 2 regions. It binds G-CSF with high affinity and with a 2:2 (2 molecules of ligand to 2 receptor molecules) stoichiometry (125,126 and 127). Hematopoietic control of G-CSFR expression is regulated by the transcription factors PU.1 and C/EBP (128,129).

BIOLOGY

G-CSF, the physiologic regulator of neutrophil production, stimulates the proliferation and differentiation of committed neutrophil progenitor cells without affecting the other granulocytic lineages. Circulating G-CSF concentrations are inversely related to neutrophil levels and are increased during infection (130,131). Administration of G-CSF results in neutrophilia (132). Whereas mice lacking G-CSF or its receptor are neutropenic, they possess some mature neutrophils and have only a modest reduction in the committed granulocyte-macrophage progenitor cells, a finding suggesting that G-CSF is not required for lineage commitment and that other cytokines can contribute to the proliferation and differentiation of the neutrophil progenitors (20,32). G-CSF synergizes with IL-3 or SCF to stimulate the proliferation and differentiation of primitive multipotent hematopoietic progenitor cells (133,134), thus providing a mechanism for expansion of the primitive cell compartment. G-CSF also enhances the survival of mature neutrophils, and it is believed that its local production primes their functional responses to agents, such as bacterial peptide f-Met-Leu-Phe, as well as IL-8 and macrophage inflammatory protein-1a (MIP-1a), that stimulate phagocytic activity, antibody-dependent cellular cytotoxicity, and intracellular killing (135,136 and 137).

Thrombopoietin

THROMBOPOIETIN AND ITS RECEPTOR

Although investigators suggested as early as 1958 that megakaryocytopoiesis was regulated by a circulating cytokine termed “thrombopoietin,” present in thrombocytopenic plasma (138), purification of TPO proved difficult, and it was the identification of the TPOR that led to the cloning of the TPO gene. The TPOR gene, *c-mpl*, was originally identified as the cellular counterpart of *v-mpl*, the oncogene carried by the murine myeloproliferative leukemia virus (139). *c-mpl* was shown to encode a membrane-spanning protein that was structurally and functionally related to members of the hematopoietic receptor superfamily (140,141) and restricted in its expression to megakaryocytes, platelets, and primitive hematopoietic cells (142). Furthermore, mice lacking *c-mpl* exhibited an 80% to 90% decrease in platelet counts with no alteration in the numbers of other differentiated blood cells (22). These results strongly implicated the *c-mpl* protein as the receptor of TPO, and the cloning of the TPO gene was reported by five independent groups in 1994 (142).

TPO is an approximately 70-kd heavily glycosylated 332-amino acid polypeptide that can be structurally and functionally divided into two domains. The amino-terminal (153 amino acids) shows 50% sequence similarity with EPO and is predicted to possess the 4 a-helix bundle characteristic of the cytokine family, whereas the carboxy-terminal domain is widely species divergent, has no homology with other known proteins, and is heavily glycosylated. Whereas the amino-terminal domain can induce the full spectrum of biologic responses, the carboxy-terminal domain is required for efficient biosynthesis and secretion and for stability (142,143). TPO is synthesized primarily by the liver and kidney, hepatocytes, endothelial cells, fibroblasts, and the proximal tubule cells of the kidney and is found in the normal circulation (142). High-affinity TPORs expressed on both platelets (approximately 30/platelet) and megakaryocytes (2 to 12 × 10^3/cell) actively internalize and degrade TPO (142,144).

BIOLOGY

TPO regulates all aspects of megakaryocyte and platelet development, including stimulation of megakaryocyte progenitor cell proliferation and differentiation, augmentation of endomitosis, induction of platelet specific proteins, stimulation of platelet release, and changes in platelet ultrastructure (145,146). As in the case of several of the circulating hematopoietic cytokines, it appears that TPO is removed from the circulation by megakaryocyte and platelet uptake and destruction through the TPOR (147). TPOR-nullizygous mice have high plasma TPO levels (22), as do patients with a low combined megakaryocyte and platelet numbers. In contrast, in thrombocytotic conditions, plasma TPO levels are low (148). Furthermore, it seems likely that TPO synthesis is constitutive and that regulation of plasma TPO occurs by its removal through TPOR-mediated endocytosis and destruction.

TPO- and TPOR-nullizygous mice possess an identical phenotype. However, surprisingly, despite their selective thrombocytopenia, both mutant mice also exhibit a 60% reduction in multipotent and committed myeloid progenitors (149), and they are deficient in stem cells capable of long-term repopulation (150). TPO has also been shown to be an important requirement for the *in vitro* proliferation of hematopoietic stem cells capable of long-term repopulation of both myeloid and lymphoid systems (4). Thus, apart from its regulation of megakaryocytopoiesis, TPO has a pleiotropic effect on hematopoiesis through its action on primitive cells.

CYTOKINES SIGNALING THROUGH RECEPTORS WITH A COMMON b SUBUNIT

The three cytokines signaling through a common b subunit, IL-3, GM-CSF, and IL-5, are members of a subfamily of cytokines that appear to share a common ancestry and structure despite the lack of significant amino acid sequence similarity among them. Their genes have a similar structure, and all map to the long arm of chromosome 5 in close proximity (151) (Table 17.1). The tertiary structures of all three are the four a-helical bundle and antiparallel b-ribbon structure shown for GM-CSF in Fig. 17.2, except IL-5 is a disulfide-linked dimer. All three cytokines signal through receptors composed of a cytokine-specific a chain, which alone exhibits low affinity for the cytokine and a larger, shared b chain, termed b common (bc), that can interact with any of the three low-affinity a chain–cytokine complexes to generate a specific high-affinity ligand-receptor complex (152) (Fig. 17.3B). The bc subunit does not bind cytokine by itself but significantly increases the affinity of the a subunit. Ligand-induced disulfide bonding between an a and b subunit and dimerization of the bc subunit are required for signaling (Fig. 17.6A). The high-affinity complex appears to be similar to the IL-6–IL-6R high-affinity complex, consisting of two receptor a chains, two bc chains, and two ligand molecules (153). Because of the shared b subunit, IL-3, GM-CSF, and IL-5 compete with each other for binding to their high-affinity receptors (Fig. 17.6A), although the biologic significance of this action is not well understood. Their a subunits, transmembrane glycoproteins of 70 to 90 kd, belong to the cytokine receptor family (Fig. 17.3B). The cytoplasmic domains of the these subunits are required for proper receptor function.

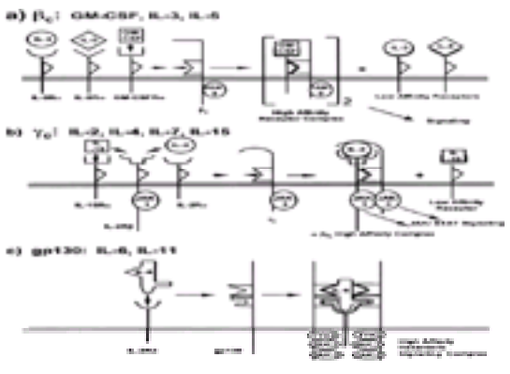

Figure 17.6. Model for the activation of hematopoietic growth factor receptors composed of multiple receptor polypeptide chains. **A:** Receptors sharing the common b subunit, b_c, illustrating the concept of cross-competition between cytokines for the b subunit that is necessary for the formation of the high-affinity signaling complex. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is shown to form the high-affinity complex preferentially, thus preventing high-affinity complex formation by interleukin-3 (IL-3), IL-5, and their a subunits. **B:** Receptors sharing the common g subunit, g_c, that forms a high-affinity receptor complex (abg_c) involving a second signaling subunit (b). **C:** Receptors sharing the common gp130 signaling subunit that forms a hexameric signaling complex with cytokine and subunits in a 2:2:2

stoichiometry.

The 120-kd b subunit also contains the conserved cytokine receptor motif found in the a subunit. It has a much longer cytoplasmic tail, which is required for proliferative signaling, and its box 1 and box 2 motifs contain the docking sites necessary for the recruitment of signaling molecules to the activated receptor. As discussed for EPOR, G-CSFR, and TPOR, receptor activation results not only in the tyrosine phosphorylation of the signaling subunit that creates docking sites for molecules with phosphotyrosine binding (PTB) or SH2 domains, such as the STATs, but also in tyrosine phosphorylation of the recruited signaling molecule and regulation of its function (Fig. 17.5B).

Interleukin-3

INTERLEUKIN-3 AND ITS RECEPTOR

Interleukin-3 is a secreted monomeric 25- to 30-kd glycoprotein with an intramolecular disulfide bridge that stabilizes the loop containing the receptor-binding site. It is essential for biological activity. The mature secreted protein consists of 133 amino acids and is extensively glycosylated (154). The IL-3R consists of a ligand-specific, low-affinity a subunit unique to the IL-3R (155) and the bc subunit. In the mouse, the situation is slightly more complex because a duplication of the bc gene has occurred. The second gene, bIL-3, has low affinity for IL-3 and interacts only with the IL-3 a chain (152,153).

BIOLOGY

IL-3 is synthesized almost exclusively by T cells in response to antigen stimulation (156). IL-3 is a pleiotropic hematopoietic cytokine supporting the proliferation and differentiation of both primitive multipotent progenitor cells and committed myeloid progenitors. IL-3 alone stimulates primitive hematopoietic cells to form multilineage colonies, composed of neutrophils, basophils, eosinophils, monocytes, and megakaryocytes. Moreover, with SCF, IL-1, and CSF-1, it stimulates the proliferation and differentiation of even more primitive precursors present in populations enriched for hematopoietic stem cells (101,157). In concert with other late-acting hematopoietic cytokines, IL-3 stimulates multipotent cells to become cells that are committed to the lineage regulated by the late-acting cytokine. For example, cells of the erythroid lineage do not require IL-3 for terminal differentiation, but in the presence of EPO, IL-3 apparently commits early multipotent progenitor cells to erythroid development and increased expression of the EPOR (154,158). Similarly, the combination of IL-3 and CSF-1 allows the proliferation of primitive cells that do not respond to CSF-1 alone to give rise to committed macrophage progenitors expressing high levels of the CSF-1R that do (100). These committed progenitor cells and their progeny no longer express the IL-3R and have lost the ability to respond to IL-3. Apart from these effects on primitive cells, IL-3 supports the terminal proliferation or differentiation of cells of the megakaryocytic, eosinophilic, and mast cell lineages (154,158). IL-3–nullizygous mice have diminished delayed-type hypersensitivity but no obvious steady-state hematopoietic phenotype (24). This finding, coupled with its highly restricted and regulated synthesis, suggests that this cytokine plays a role only during hematopoietic demand.

Granulocyte-Macrophage Colony-Stimulating Factor

GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR AND ITS RECEPTOR

GM-CSF is a 127-amino acid monomeric glycoprotein with a molecular weight of 18 to 32 kd, depending on the degree of glycosylation. The GM-CSF receptor (GM-CSFR), like the IL-3R, consists of a ligand-specific a subunit that confers low-affinity binding and the bc subunit. The GM-CSFR a subunit is a membrane-spanning glycoprotein of 80 kd containing the extracellular domain cytokine receptor motif (159). Three alternative transcripts are recognized for the a subunit: one for the main form described earlier, the second for a soluble form that has had the region encoding the transmembrane and cytoplasmic domains spliced out, and the third for an alternative membrane-spanning a subunit that has a slightly longer C-terminal end (160). All are functional, but their relative physiologic significance is not yet well understood.

BIOLOGY

GM-CSF is constitutively synthesized by macrophages, endothelial cells, and fibroblasts; it is inducibly expressed in a variety of cells, especially T cells, which can produce large quantities of GM-CSF after activation by antigens and other inducers (161). GM-CSF has been shown to have a broad range of biologic effects, acting on both progenitor cells and mature, terminally differentiated cells. The early progenitor cells of many lineages are stimulated to survive and to proliferate under the influence of GM-CSF (162). GM-CSF alone supports the survival, proliferation, and differentiation of all cells in the neutrophil, macrophage, and eosinophil lineages from multipotent progenitors to mature cells (163), and GM-CSF appears to be important in the activation and enhancement of function of mature cells of these three lineages (161). However, at most stages of development, the cells in these three lineages also require the presence of other, more specific hematopoietic cytokines: G-CSF, CSF-1, and IL-5. GM-CSF synergizes with EPO and TPO on primitive hematopoietic cells to generate erythroid and megakaryocytic progeny, respectively. *In vivo,* GM-CSF administration increases the number of circulating neutrophils, monocytes, and eosinophils and the number of tissue-fixed macrophages. GM-CSF-deficient mice have pulmonary alveolar proteinosis caused by a deficiency of the pulmonary alveolar macrophage (25). However, because they have normal granulocyte and macrophage production in both steady-state and stressed conditions, GM-CSF apparently does not play an important role in blood cell production.

Interleukin-5

INTERLEUKIN-5 AND ITS RECEPTOR

IL-5 is a secreted, disulfide-linked, 40- to 45-kd homodimer that is heavily glycosylated. Dimerization is essential for biologic activity, and its double 115-amino acid monomers are aligned in a head-to-tail configuration (164). The IL-5R is composed of the IL-5 specific a subunit and the bc subunit. The IL-5R a subunit is a 60-kd transmembrane glycoprotein that is predominantly extracellular, with a short, 54-amino acid cytoplasmic tail. An alternatively spliced form of the a subunit encodes a soluble form that is abundantly expressed (165).

BIOLOGY

Interleukin-5 was originally identified as a B-cell growth factor and was separately identified as a T-cell–derived eosinophil differentiation factor before the two activities were ascribed to the same molecule (166). It is produced predominantly by antigen-stimulated T lymphocytes, but also by NK cells, mast cells, B cells, eosinophils, and bone marrow endothelial cells (167). Eosinophils and basophils, their precursors, and some B cells express both subunits of the IL-5R, and IL-5 has relatively restricted biologic activity, acting principally on cells of the eosinophil and basophil lineages, but also affecting B-lymphocyte production and function. *In vitro* studies suggest that IL-5 is the primary late-acting cytokine for eosinophil proliferation and differentiation and that it also stimulates the survival, activation, and chemotaxis of mature eosinophils. In combination with GM-CSF, IL-3, and IL-4, IL-5 stimulates the survival, proliferation, and differentiation of the basophil-mast cell lineage (168). B-lymphocytes respond to IL-5 by proliferation and differentiation to Ig-secreting cells (169). *In vivo*, IL-5 is important throughout the development of the eosinophil lineage, and it plays a central role in the development of eosinophilia. Complete abrogation of the development of eosinophilia secondary to parasitic infections is observed in mice given anti–IL-5 antibodies or in IL-5–deficient mice (23,170). In contrast, although early eosinophil progenitors respond to IL-3 and GM-CSF, there is no alteration of eosinophil production in IL-3– and GM-CSF–deficient mice (24,25). Transcripts for the soluble IL-5R a subunit are normally more abundant in activated T cells than those of the membrane-spanning subunit. However, in patients with asthma, the expression of the membrane-spanning subunit is directly related to the severity of disease, whereas the expression of the soluble form is inversely related to disease, a finding suggesting that differential expression of surface and soluble receptor can affect eosinophil-mediated disease (171).

CYTOKINES SIGNALING THROUGH RECEPTORS WITH A COMMON g SUBUNIT

Four hematopoietic cytokines, IL-2, IL-4, IL-7, and IL-15, sharing a four a-helical bundle structure, signal through a high-affinity receptor composed of a cytokine-specific a chain and a common g chain (g_c), neither of which have catalytic domains (Fig. 17.3B). The g_c subunit to which these ligands fail to bind, or bind weakly, is also used in the receptor for IL-9 (IL-9R) (172,173,174,175 and 176). As for cytokines signaling through b_c, both a and g_c subunits are required for high-affinity binding, and the cytokine binds the a subunit with low affinity. In the case of IL-2 and IL-15, which map close together and probably have a common ancestry, a common b subunit (IL-2Rb), which binds both ligands, can replace the a chain, but it is usually included in a high-affinity abg_c complex (172,176). The minimum requirement for signaling is bg_c, which can respond to either IL-2 or IL-15. Mice lacking g_c suffer from a severe combined immune deficiency characterized by a tenfold reduction in the number of circulating mature T and B cells and complete loss of NK cells (172).

The known signaling pathways described for all these receptors are similar (Fig. 17.6B). These pathways involve the JAKs. On ligand binding and assembly of the high-affinity receptor-ligand complex, JAK1, constitutively associated with the a or b subunit, and JAK3, constitutively associated with the g_c subunit, become activated, probably by transphosphorylation. They then tyrosine phosphorylate the receptor chains, leading to the recruitment of phosphotyrosine-associating (SH2 domain–containing) signaling intermediates, including STATs 1,3, 5A, 5B, and 6, which, in turn, are tyrosine phosphorylated by the JAKs, thus allowing them to dimerize and move to the nucleus to direct transcription. The ras-raf-MAPK pathway is also activated by Shc interactions with the a or b subunit. The recruitment or involvement of the src-family kinases $p56^{lck}$, $p59^{fyn}$, and $p53/56^{lyn}$ has also been reported, and this, or the recruitment of insulin receptor substrates-1 and -2 to the receptor, leads to recruitment and activation of PI3K (172,173,174,175 and 176).

Interleukin-2

INTERLEUKIN-2 AND ITS RECEPTOR

The structural and functional aspects of IL-2 and the IL-2R have already been discussed. The IL-2Ra and IL-15a chains are closely related structurally and constitute a separate cytokine receptor family from the WSXWS sequence-containing cytokine receptor superfamily to which the IL-2Rb and g_c belong. The *de novo* synthesis and secretion of IL-2 and the expression of the IL-2R by mature, resting T cells are early consequences of their antigen or mitogen–induced activation (172).

BIOLOGY

The primary role of the 15.5-kd IL-2 glycoprotein is to promote rapid expansion of the effector T-cell population activated by antigen. The subsequent decline in expression of both the cytokine and its receptor leads to termination of the T-cell immune response. IL-2 also stimulates T-cells to produce other cytokines, including interferon-g (IFN-g) and IL-4. Targeted inactivation of the genes encoding IL-2 or the IL-2Ra subunits in the mouse indicate that IL-2 prevents autoimmunity and maintains homeostasis of the immune system. Despite normal *in vivo* T-cell responses, these mice die young and develop autoimmune disorders, including hemolytic anemia and inflammatory bowel disease (26,172).

Interleukin-7

INTERLEUKIN-7 AND ITS RECEPTOR

IL-7 is secreted constitutively by nonlymphoid stromal cells that support lymphopoiesis, including those of the fetal liver, bone marrow, and thyroid, as well as other nonlymphoid tissues in which gd T cells are relatively abundant, such as skin and intestine. It is also produced by secondary lymphoid tissues, a feature that reflects its role in the regulation of T-cell, NK cell, and monocyte-macrophage functions. The IL-7R is expressed on pre-pro-B, early pro-B, and late pro-B cells (175).

BIOLOGY

IL-7 is the major growth factor regulating precursor B cells. It is involved only in the antigen-independent stages of B-cell development (early and late pro-B cells) that take place in fetal liver and bone marrow. Although pre-pro-B cells express the IL-7R, their differentiation is independent of IL-7. Downregulation of IL-7 expression and of IL-7 responsiveness is correlated with rearrangement of k light chains and expression of surface IgM by immature B cells (174,175,177). Both IL-7Rg– and IL-7–nullizygous mice have markedly reduced numbers of late pre-B and mature B cells, although pro-B-cell numbers are also reduced in the IL-7Rg–nullizygous mice, probably because of the ability of at least one other cytokine, thymic stromal-derived lymphopoietin (TSLP), to bind the IL-7R (27,28,175). Ig heavy chain V-DJ recombination is also impaired in the latter mice, which, in addition, have reduced levels of the transcription factor, Pax5, that has been shown to regulate V-DJ rearrangements and to commit pro-B cells (59). IL-7 stimulates the *in vitro* proliferation of fetal and adult thymocytes, and antibodies to IL-7 block thymocyte proliferation. IL-7 synergizes with IL-2, SCF, FL, and TNF-a to promote thymocyte growth (175). Thymic cellularity and peripheral T-cell populations are markedly reduced in both IL-7Ra– and IL-7–nullizygous mice (27,28). Again, as in the case of B-cell development, thymocyte development was blocked at an early stage ($CD4^-CD8^-CD25^-$ cell) in the IL-7Ra–nullizygous mice compared with the IL-7–deficient mice, a finding probably reflecting the ability of the IL-7R to bind TSLP. IL-7 is preferentially required for the development of gd T cells because ab T cells develop, albeit with reduced survival, in both IL-7Ra– and IL-7–nullizygous mice, and it seems likely that IL-7 signaling through Stat 5 is essential for TCRg transcription after rearrangement of the TCRg gene complex. IL-7 can stimulate the proliferation of mature, peripheral T lymphocytes, and IL-7Ra–deficient mice have defects affecting peripheral T-cell activation, a finding suggesting a role for IL-7 in mature T-cell responses. NK cells develop and function normally in IL-7Ra– and IL-7–nullizygous mice. IL-7 may have effects on myeloid cells by synergizing with CSF-1, GM-CSF, or IL-3 or combinations of cytokines in stimulating the proliferation and differentiation of myeloid progenitors, as well as mature monocytes-macrophages, by stimulating their production of cytokines. It is also a regulator of T-cell growth in skin and intestine (175).

Interleukin-4

INTERLEUKIN-4 AND ITS RECEPTOR

IL-4 is a secreted glycoprotein of 15 to 19 kd. An alternatively spliced message yields a form lacking amino acids 22 to 37, termed IL-4d2, that is expressed more strongly in thymocytes and bronchoalveolar lavage cells and alone does not act as a costimulator for T-cell proliferation, but inhibits IL-4–induced T-cell proliferation. The high-affinity IL-4R is expressed on a wide range of cell types including T and B lymphocytes, monocytes, granulocytes, fibroblasts, and epithelial and endothelial cells and is upregulated by IL-4 itself. Two forms of the IL-4R are known. The classic form, consisting of the IL-4Rb and g_c subunits and requiring JAK3 for STAT6 activation, is predominantly expressed in hematopoietic cells. The alternative form, consisting of the IL-4Rb and IL-13Ra subunits and requiring JAK2 for STAT6 activation, is predominantly expressed in nonhematopoietic cells (178). A soluble form of the IL-4Ra binds IL-4 with high affinity (173).

BIOLOGY

IL-4 is expressed in TH_2 cells, which are generated from T-helper precursors through the action of IL-4, as well as $NK1.1^+$ T cells, basophils, mast cells, and eosinophils. IL-4 can act on many cell types and at various stages of maturation of a given cell type. Because it modulates cytokine production by many different cell types, some IL-4 effects may be indirect (173). *In vitro,* IL-4 stimulates B lymphocyte activation and enhances the antigen-presenting capacity of B cells toward T lymphocytes. It enhances antigen receptor and CD-40 triggering of B-cell proliferation and differentiation, but it antagonizes IL-2–induced costimulation, possibly because of its sequestering of the $g_c$chain. However, normal B lymphopoiesis is observed in IL-4–nullizygous mice, a finding indicating that the stimulatory and inhibitory effects that have been observed *in vitro* may be subserved by other cytokines *in vivo* (179). IL-4 stimulates the generation of Th2 cells and the proliferation and maturation of immature thymocytes, although it inhibits early T-cell development in fetal thymus organ culture. IL-4 inhibits CSF-1–induced macrophage colony formation and megakaryocyte colony formation, but it enhances G-CSF–induced granulocyte colony formation and, with IL-3, the generation of basophils, mast cells, and eosinophils. IL-4 regulates the production of mediators in a pattern consistent with its antiinflammatory role (173). IL-4–nullizygous mice have a Th2 cell deficiency and diminished IgG1 and IgE responses consistent with a primary role for IL-4 in the regulation of T-helper cell differentiation and Ig switching (29,180).

Interleukin-15

INTERLEUKIN-15 AND ITS RECEPTOR

IL-15 mRNA is expressed ubiquitously, and its expression can be enhanced by a variety of stimuli, including IFN-g, infectious agents, and bacterial lipopolysaccharide. However, translational and secretory control mechanisms play predominant roles in the production of IL-15 protein (174,176). The high-affinity IL-15R comprises IL-15Ra, IL-2Rb, and g_c subunits. Antibodies specific for either IL-2Rb or g_c block IL-15–induced responses, whereas antibodies specific for IL-2Ra have no inhibitory effect. Three alternatively spliced forms of IL-15Ra mRNA have been identified, and all three encode proteins that bind IL-15. One form is homologous to IL-15Ra, one has deleted an exon in the extracellular domain, and the third has an alternative cytoplasmic domain. As in the case of the IL-2–IL-2R system, IL-15Ra is required for high-affinity binding of IL-15, but not for signaling. In contrast to IL-2Ra, IL-15Ra alone binds ligand with high affinity and is more widely expressed. Because expression of the IL-2R subunits is more restricted than the expression of IL-15Ra, it is likely that IL-15Ra can function with other subunits in some tissues (174,176).

BIOLOGY

IL-15 can substitute for IL-2 in the induction of cytotoxic T lymphocytes and in the generation of lymphokine-activated killer cells. In addition, it augments antibody-dependent cellular cytotoxicity by human NK cells. IL-15 stimulates the proliferation of $CD56^{bright}$ human NK cells and cytokine production by NK and T cells. It also acts as a differentiation factor for these cells. It has other effects on several other cell types. The presence of IL-15 mRNA in many normal tissues and the

increased production of IL-15 in response to a variety of stimuli suggest that it plays a role in protective immune responses, allograft rejection, and the pathogenesis of autoimmune disorders (174,176). IL-15– and IL-15Ra–nullizygous mice are deficient in NK cells, NK T cells, and gd T cells (30,31,181).

CYTOKINES SIGNALING THROUGH A COMMON GP130 SUBUNIT

Three hematopoietic cytokines, IL-6, IL-11, and LIF, signal through a shared gp130 subunit, as do oncostatin M, ciliary neurotrophic factor, and cardiotrophin-1. The known components of the IL-6, IL-11, and LIF receptors include a cytokine-specific a subunit and the shared gp130 subunit (Fig. 17.3B). The binding of IL-6 to IL-6Ra induces the formation of a hexamer consisting of two molecules each of IL-6, IL-6Ra, and gp130 (Fig. 17.6C). One site on IL-6 binds IL-6Ra, and two additional sites on each IL-6 bind one gp130 and the other gp130, causing gp130 dimerization, which is required for signal transduction. The intracellular domain of IL-6Ra is not required for IL-6–mediated signal transduction. A similar situation is believed to exist for the IL-11R and LIFR, except IL-11 does not induce gp130 homodimerization, and thus another novel receptor subunit is implicated in the process. IL-6 signal transduction is mediated by the actions of JAK1, JAK2, and Tyk-2 tyrosine kinases that are constitutively associated with gp130 and STAT1, STAT3, and STAT5, which are activated, in turn. The ras-MAPK pathway is also activated through SHP-2 or Shc adapters. In addition, other nonreceptor tyrosine kinases are activated through the IL-6R. Two types of IL-6–responsive elements have been identified in the genes encoding acute-phase proteins. Type I IL-6RE is a binding site for NF-IL6/IL6DBP/LAP/C/EBPb. gp130-deficient mice die *in utero* because of severe deficiencies in hematopoietic and cardiac development.

Interleukin-6

INTERLEUKIN-6 AND ITS RECEPTOR

IL-6 is a multifunctional cytokine produced by both lymphoid and nonlymphoid cells. The IL-6R is also expressed on a wide range of cells (182).

BIOLOGY

Identified as one of the factors produced by T cells and macrophages that acts on B cells, IL-6 regulates immune responses, acute-phase reactions, and hematopoiesis. It acts on activated B cells, but not on resting B cells, to induce Ig production, and it is required for IL-4–dependent IgE synthesis, for IL-2–induced Ig production by B cells, and for antigen-specific antibody production by primary B cells (182). IL-6 also stimulates T-cell activation, growth, and differentiation and induces the biosynthesis of acute-phase proteins by hepatocytes. In hematopoiesis, IL-6 acts synergistically with SCF or IL-3 to induce the proliferation of primitive multipotential hematopoietic cells and to increase myelopoiesis and augment megakaryocyte production. IL-6–deficient mice showed reduced IgG and IgA responses and reduced numbers of cytotoxic T cells, and they are severely defective in the inflammatory acute-phase response after tissue damage or infection. They have decreased numbers of primitive multipotent progenitor cells, a reduction in megakaryocyte progenitors, and a predominance of monoblast, myeloblasts, and macrophages in granulocyte-macrophage progenitor cell colonies, as well as decreased neutrophil production and function. (32,182,183 and 184).

Interleukin-11

INTERLEUKIN-11 AND ITS RECEPTOR

IL-11 mRNA is expressed in a wide variety of cell types and tissues, but the protein is normally expressed at low levels and cannot be detected in serum. Depending on cell type, its expression can be induced by proinflammatory cytokines IL-1a and TNF-a, the antiinflammatory transforming growth factor-b (TGF-b), phorbol esters, retinoic acid, and histamine. The IL-11Ra mRNA is also widely expressed. Myeloid, erythroid, and megakaryocytic cells, as well as other cell lines, express IL-11Ra (185,186).

BIOLOGY

The effects of IL-11 on hematopoiesis are also largely synergistic and resemble those of IL-6, almost certainly because their receptors share the signaling gp130 subunit. IL-11 can enhance multilineage growth in combination with certain cytokines including SCF, KL, IL-3, GM-CSF, CSF-1, G-CSF, IL-4, IL-12, and TPO, in general paralleling the effects of IL-6. Moreover, like IL-6, IL-11 stimulates megakaryocytopoiesis *in vitro* through synergism with IL-3, KL, and TPO, by recruiting more progenitors to proliferate, by enhancing colony size, and by promoting megakaryocyte maturation and ploidy but without any effect on mature platelets. The synergistic effects of IL-11 on myeloid and erythroid progenitor cell proliferation and differentiation are less prominent, but it also enhances the generation of early B-cell progenitors. As predicted from *in vitro* studies, the predominant effects of *in vivo* administration of IL-11 are the stimulation of megakaryocyte and platelet production and progenitor stem cell expansion. Despite these effects of IL-11 on hematopoiesis, adult IL-11Ra–nullizygous mice are hematopoietically normal (187), and the residual megakaryocyte and platelet production in TPOR-deficient mice is not dependent on the action of IL-11 or any of the other cytokines known to signal through gp130 (188). Consistent with the broad expression of IL-11 and the IL-11R, like IL-6, IL-11 has many effects outside the hematopoietic system (185,186).

Leukemia Inhibitory Factor

LEUKEMIA INHIBITORY FACTOR AND ITS RECEPTOR

LIF is a monomeric secreted glycoprotein produced by monocytes and stromal cells in response to activating stimuli. The LIFRa is a membrane-spanning protein with a 238 amino acid cytoplasmic domain.

BIOLOGY

The hematopoietic effects of LIF are similar to those of IL-6 and IL-11 (189). LIF-nullizygous mice have no obvious hematopoietic phenotype. As mothers, they are unable to support blastocyst implantation (35).

OTHER CYTOKINES REGULATING HEMATOPOIESIS

Several additional cytokines not covered in detail appear to play important roles in hematopoiesis and are briefly discussed here.

Interleukin-1

The genes encoding three evolutionally related interleukin 1 polypeptides, IL-1a, IL-1b, and IL-1 antagonist (IL-1a), are clustered on human chromosome 2q, and their products mediate acute-phase and febrile responses to inflammatory stimuli. These three cytokines can interact with four receptor-like proteins, but signaling is only observed by ligand-receptor complexes involving either or IL-1a or IL-1b and the IL-1R1 and IL-1R AcP subunits, the latter increasing the binding affinity of ligand for IL-1R1. IL-1a–deficient mice remain able to mount acute-phase and febrile responses, whereas IL-1b–deficient mice fail to do so, a finding suggesting that IL-1b is biologically more important than IL-1a. IL-1R1 and IL-1R AcP are expressed on a wide variety of cell types (190,191,192 and 193). IL-1 stimulates synthesis of several hematopoietic cytokines, including G-CSF, GM-CSF, CSF-1, IL-6, LIF, and IFN-g, and it increases the expression of several cytokine receptors, adhesion molecules, and protooncoproteins (194). IL-1 also synergizes with IL-3, GM-CSF, and CSF-1 to allow multipotent cells to proliferate and differentiate *in vitro* (45,195).

Interferon-g

IFN-g is a noncovalent, homodimeric glycoprotein of 50 kd that is produced by activated or cytokine-stimulated T lymphocytes and NK cells. Its action is mediated by a receptor composed of two or three subunits: a ubiquitously expressed and ligand-binding IFN-g R-a subunit, an IFN-g R-b subunit that exhibits regulated expression, and a third subunit that is required for some responses (196). IFN-g–deficient mice have defects in the immune system that become apparent during infection. IFN-g augments immune responses by activating macrophages and enhances antigen recognition by increasing the expression of the major histocompatibility class I and II antigens. It is also a general inhibitor of cell proliferation. IFN-g inhibits hematopoietic cell proliferation by a variety of mechanisms (197).

Transforming Growth Factor-b

Of the various forms of TGF-b, hematopoietic tissues primarily produce TGF-b1. TGF receptors are expressed almost ubiquitously in the form of the type I and II membrane-spanning serine kinase receptor subunits (198). An important role of TGF-b1 is to inhibit cell proliferation by reducing the phosphorylation of Rb and by increasing the expression of the cell-cycle inhibitor, p27, and arresting cells in the G_1 phase of the cell cycle (199,200). In the case of TGF-b1, this antiproliferative

effect is clearly important for inflammatory cells because TGF-b1–deficient cells die perinatally with an intense inflammatory reaction (200). TGF-b1 directly inhibits the proliferation and differentiation of primitive multipotent cells, as witnessed by the enhancing effects of adding anti–TGF-b antibodies to cultured cells (201). Indirectly, TGF-b1 affects hematopoiesis by inhibiting the production of SCF and the expression of several hematopoietic cytokine receptors (202,203).

Tumor Necrosis Factor

Activated lymphocytes and macrophages are the major sources of TNF-a and TNF-b (also known as lymphotoxin-a). Receptors for these TNFs are essentially ubiquitous, and they serve important functions in sepsis, granulomatous disease, autoimmunity, and graft-versus-host disease, in particular as activators of neutrophils and macrophages. TNF-deficient mice have a disorganized splenic architecture and/or a peripheral lymph node deficiency, with a reduced ability to combat infection (204,205). The TNFs induce the production of the myeloid CSFs, IFN-g, IL-1, IL-6, and chemokines by various cell types, and they inhibit TGF-b production. Thus, although the TNFs directly inhibit the proliferation and differentiation of hematopoietic cells, they can also indirectly stimulate them, with varying results, depending on cell type and culture conditions (204,205).

Chemokines: Stromal Cell–Derived Factor-1a, Monocyte Chemoattractant Protein-1, Macrophage Inflammatory Protein-1a and 1b

Several chemokines have been shown to regulate hematopoietic cells. Many of them are synthesized by bone marrow stromal cells and can exhibit increased expression in response to inflammatory cytokines. Stromal cell–derived factor-1a (SDF-1a) stimulates the growth of pre-B lymphocytes, is a chemoattractant for primitive multipotent hematopoietic cells, and augments the action of TPO on megakaryocytopoiesis. SDF-1a–deficient mice die *in utero* or within 1 hour of birth. They exhibit severely reduced fetal liver B lymphocytopoiesis and bone marrow myeloid progenitor cell numbers (206,207). Monocyte chemoattractant protein-1 (MCP-1) induces the monocyte integrin expression necessary for chemotaxis and attracts and regulates several other lymphohematopoietic cell types. MIP-1a and MIP-1b attract and activate neutrophils and monocytes and attract $CD8^+$ (MIP-1a) or $CD4^+$ (MIP-1b) T cells (208). MCP-1 and MIP-1a and other chemokines inhibit multipotent hematopoietic cell proliferation and differentiation *in vitro* and also hematopoiesis *in vivo* (207).

Jagged and Notch

Notch is a membrane-spanning protein known to control fate decisions in *Drosophila melanogaster* and *Caenorhabditis elegans.* Evidence suggests that the mammalian paralogs of Notch and its ligands are involved in the regulation of hematopoiesis. Primitive hematopoietic cells have been shown to express Notch, and a Notch ligand, Jagged-1, is expressed by bone marrow stromal cells (209). Stromal cells expressing Jagged enhance primitive multipotent hematopoietic progenitor cell proliferation, and activation of Notch in a myeloid cell line inhibited granulocyte differentiation and increased cell proliferation (209,210). A gene broken at the t(7;9) breakpoint in several T-cell lymphomas is a mammalian *Notch* homolog, *Tan-1*(211).

PATHOLOGIC CONDITIONS INVOLVING HEMATOPOIETIC CYTOKINES

The bases of several hematologic conditions can be attributed to alterations in the expression of cytokines and their receptors or to mutations in the genes encoding them. Some of these conditions are summarized in Table 17.2. Analysis of the pathologic roles of cytokines and their receptors is in its infancy, and in many disease states not summarized in Table 17.2, a role for cytokines has been suggested. For example, several studies suggest that in the anemia of chronic disease, overproduction of IL-1 and TNF-a may lead to increases in other cytokines such as IFN-g and MCP-1 that can suppress erythropoiesis. In addition, in studies with mice, elevated levels of circulating CSF-1 before the development of frank leukemia have been shown to be associated with autocrine regulation of acute myeloid leukemic cells by CSF-1 (212). Table 17.2 also does not list conditions resulting from alterations in postreceptor signaling pathways (213,214,215,216,217,218,219,220,221,222,223,224 and 225).

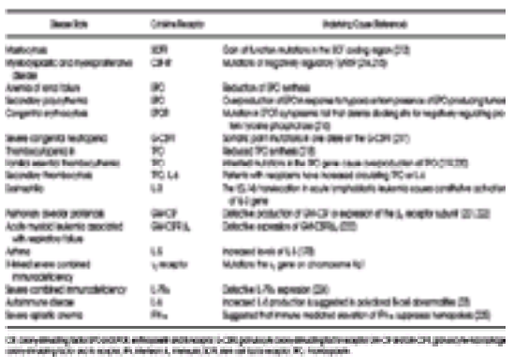

TABLE 17.2. Hematopoietic Cytokines and Receptors in Human Disease

CLINICAL APPLICATIONS AND POTENTIAL

Several hematopoietic cytokines, including EPO, TPO, IL-11, GM-CSF, and G-CSF are used clinically. EPO is used to correct the anemia of chronic renal disease. TPO and IL-11 are used to correct thrombocytopenic conditions. The most successful application of GM-CSF and G-CSF has been in reducing infections in patients with congenital and cyclic neutropenias, whereas their widespread application has been to accelerate white cell recovery and to reduce infections in patients receiving chemotherapy for solid tumors or in patients receiving hematopoietic stem cell transplants (226,227). In particular, the use of peripheral blood cells from donors previously injected with G-CSF to mobilize primitive hematopoietic cells has resulted in the increased use of peripheral blood rather than bone marrow for transplantation, in a decreased incidence of posttransplantation infection, and in a decreased requirement for platelet transfusions (228). Clearly, the hematopoietic cytokines will have extensive application in the future as we learn more about their regulation and mechanisms of action.

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18 T-LYMPHOCYTE EFFECTOR ACTIVITY

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The vertebrate immune system has evolved to counter infestation and invasion by foreign prokaryotic and eukaryotic microorganisms. The immune system is composed of two distinct but related components, the innate and the adaptive immune systems. The *innate immune system* consists of effector cells and soluble effector molecules present both in the blood and in tissues. These effectors are poised to respond rapidly to a variety of insults and use recognition mechanisms with broad specificity to distinguish self from nonself. The activity of these effector mechanisms typically is unaltered by prior exposure to an infectious agent and is therefore not adaptive. Cells of the innate immune system are both hematopoietic (e.g., monocytes and macrophages and neutrophils) and nonhematopoietic (e.g., fibroblasts and epithelial cells) in origin. The soluble mediators of the innate immune system range from the complex of interacting blood proteins comprising the complement and clotting systems to small, highly potent lipid mediators such as arachidonic acid metabolites. The cells and products of the innate immune system can be viewed more precisely as effectors of the vertebrate inflammatory system.

The *adaptive immune system*, by contrast, consists of two highly specialized and highly specific lymphocyte cell types, the B- and T-lymphocytes. These cells display receptors that have exquisite specificity for foreign antigen and that are clonally distributed among individual cells of the B- or T-lymphocyte lineage. Engagement of the antigen receptors on specific B- or T-lymphocytes leads to expression of the lymphocyte effector activities. Figure 18.1 illustrates the major adaptive immune effectors: antibodies produced by activated B-lymphocytes; lymphokines, the cytokines secreted by activated $CD4^+$ and $CD8^+$ T-lymphocytes; and cytolytic T-lymphocytes, activated T-lymphocytes capable of killing other cells by direct cell-to-cell contact. Unlike the cells of the innate immune/inflammatory system, which are constitutively active or rapidly induced, B- and T-lymphocytes exist before contact with foreign antigen as small “resting” cells that lack effector activity. Engagement of the antigen receptor on specific B- and T-lymphocytes by antigen results in the selective activation and differentiation of these resting cells into large blast-like cells that now exhibit effector activity. Adaptive immune cells exert their effector function directly as well as by orchestrating the effector mechanisms of the cells and products of the innate immune system. In the case of the antibody molecule, the Fc portion of the molecule interacts with the soluble proteins of the complement system and with Fc receptors on cells of the innate immune system (e.g., neutrophils and macrophages). In like manner, activated T-lymphocytes mobilize and orchestrate the response of the innate immune/inflammatory system by the release of soluble lymphokine and chemokine mediators. The usual outcome of this interplay between the adaptive and innate immune systems is the elimination of an invading microorganism and the establishment of an adaptive response (i.e., immunologic memory by the B- and T-lymphocytes responding to the organism). An abnormal response to self-molecules or an exaggerated (or inappropriate) response to foreign molecules by the adaptive immune system can lead to immunologic disease. This chapter deals with the effector activities of T-lymphocytes and the manifestations of these effector activities through the interplay of T-lymphocytes with the innate immune system.

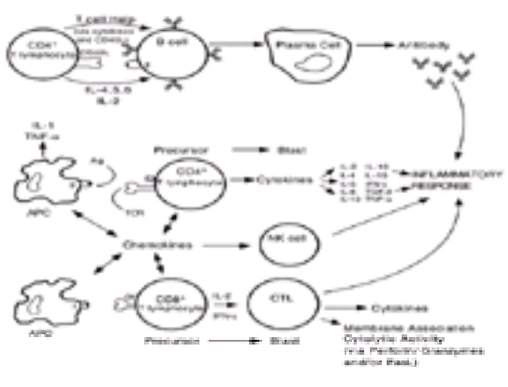

Figure 18.1. Cells of the adaptive immune system and their products. This diagram depicts the major cells types comprising the adaptive immune system, that is, T-lymphocytes and B-lymphocytes. The B-lymphocyte effector cell is the plasma cell. The primary effector product of the plasma cell is the antibody molecule. The T-lymphocyte effector cells are the $CD4^+$ and $CD8^+$ T-lymphoblasts. These blast cells produce cytokines in response to antigen. The $CD8^+$ T-lymphoblast also goes by the designation CTL (cytolytic T lymphocyte) because this cell exhibits cytolytic activity that depends on direct contact of the CTL with the antigen bearing cell. Both T- and B-lymphocytes start out as small resting cells that undergo activation and blast transformation after contact with antigen. See text for abbreviations.

EVENTS IN THE INDUCTION AND EXPRESSION OF T LYMPHOCYTE EFFECTOR ACTIVITY

The hallmark of the T-lymphocyte response to injury or invasion by foreign microorganisms is antigen specificity. The T-lymphocyte antigen receptor (TCR) must be engaged to activate the resting T-lymphocyte and to initiate cell proliferation and the differentiation program leading to the clonal expansion and the expression of lymphocyte effector activity by the progeny cells. This requirement for antigen recognition in the development of T-lymphocyte effector function is demonstrated both by primary (virgin) T-lymphocytes, which take days to transit through this activation and differentiation program, and by clonally expanded memory T-lymphocytes, which can rapidly express effector activity (within 12–24 hours of contact with antigen).

The antigen receptor on T-lymphocytes recognizes a complex between fragments of foreign antigens and major histocompatibility complex (MHC) molecules on cell surfaces. The TCRs must be engaged not only for primary and memory T-lymphocytes to activate but also for activated T-lymphocytes to express effector activity. Thus, the effector activity of T-lymphocytes is usually local, that is, focused on the antigen-bearing cell and its immediate environment. When antigen load is high or when activated T-lymphocytes encounter so-called superantigens, like the bacterial toxins producing toxic shock syndrome (1), however, systemic effects of T-lymphocyte effector activity are observed.

In response to TCR engagement, effector T-lymphocytes secrete soluble products, cytokines and chemokines, which function to recruit and activate cells of the innate and inflammatory systems. Major cellular targets of soluble effector molecules include leukocytes (i.e., granulocytes and monocytes or macrophages), vascular endothelial cells, tissue fibroblasts, and mast cells. Once activated, these cells can secrete cytokines and chemokines, which in turn amplify the local inflammatory response initiated by effector T-lymphocytes.

A second effector mechanism used by activated T-lymphocytes is the killing of antigen-bearing cells by direct cell-to-cell contact. This function is the most highly evolved and specific form of T-lymphocyte effector activity because it uses only the activated T-lymphocyte and requires both antigen receptor engagement and direct cell-to-cell contact.

T-LYMPHOCYTE RECOGNITION AND ACTIVATION

The primary functions of T-lymphocytes are to help B-lymphocytes produce antibodies, to interact with the inflammatory system and enhance the activity of inflammatory cells through the release of soluble mediators, and to act as antigen-specific cytotoxic effectors. The central event in this process is the recognition of antigen by the TCR (2). Like the antibody molecule, this receptor is a heterodimer and has characteristic N-terminal variable regions and C-terminal constant regions. The variable regions are generated by somatic rearrangement and diversification of a fixed number of germline genes (3). Unlike the antibody molecule, the TCR recognizes small peptide fragments of foreign and self-proteins expressed on cell surfaces in association with the highly polymorphic specialized cell-surface molecules, the MHC class I and class II molecules. Class I and II MHC molecules capture peptide fragments of proteins within the cells expressing the MHC molecule (see Chapter 6 for detailed discussion). MHC class I molecules primarily capture peptides during the synthesis of MHC class I molecules in the endoplasmic reticulum. These peptides are derived from a peptide pool generated by proteolysis of self- or foreign proteins in the cell cytoplasm. Cytosolic peptides are transported to the

endoplasmic reticulum, where peptides can bind to the nascent MHC class I molecule (4,5). In most instances, self- or foreign proteins must gain access to the cell cytoplasm to undergo the proteolytic fragmentation necessary for peptide capture by MHC class I molecules, although several alternative pathways for fragmentation and presentation of endocytosed extracellular proteins to class I molecules have been described (6,7 and 8). These alternative pathways of peptide presentation by MHC class I molecules may represent important mechanisms for the presentation of peptides from phagocytosed self- and foreign proteins produced by damaged and dead cells (9). MHC class I molecules are constitutively expressed at varying levels on most cells of the body, but the level of class I expression on cells can be upregulated through the action of certain cytokines, particularly the type I interferons (10,11).

The MHC class II molecules primarily capture peptides formed by proteolysis of endocytosed self- and foreign proteins in the endosome/lysosome compartment of MHC class II-expressing cells (12). In contrast to MHC class I molecules, the MHC class II molecules are constitutively expressed primarily on cells of reticuloendothelial origin that serve as antigen-presenting cells (APCs) (13). The expression of these molecules can be induced on other cell types (e.g., epithelial cells) by the action of cytokines, particularly type II interferon (IFN-g). MHC class I and II molecules must have a peptide bound to their peptide-binding cleft to form a stable molecule at the cell surface (4,5). Because MHC molecules are normally expressed at the surface of body cells before infection or injury, peptides derived from self-proteins make up most peptides complexed with MHC molecules on cell surfaces (14,15). These self-peptides are the presumed targets of the autoreactive T-lymphocytes responsible for tissue destruction in several autoimmune diseases.

One APC type, the dendritic cell, has been recognized as the critical cell for presenting peptide–MHC complexes to primary (naïve) T-lymphocytes (16). Dendritic cells not only constitutively express high levels of MHC class II and I molecules but also express ligands for coreceptors on T-lymphocytes that are necessary for the activation of primary (virgin) T-lymphocytes. Dendritic cells are strategically arrayed at epithelial surfaces and at sites of antigen accumulation in lymphoid tissues, that is, the lymph nodes and spleen, where they can capture foreign antigens for processing and presentation as peptides to T-lymphocytes (17).

The progenitors of effector T-lymphocytes are small, quiescent lymphocytes in the G_0 phase of the cell cycle present in the blood, lymphoid tissues, and epithelial surfaces. These resting small lymphocytes must activate, proliferate, and differentiate to give rise to activated effector cells (18). Engagement of T-lymphocyte antigen receptor by the appropriate peptide MHC complex displayed on an APC is necessary but not sufficient to complete this process. For $CD4^+$ T-lymphocytes, the cell-surface CD4 molecule serves as a coreceptor by binding to a nonpolymorphic (structurally conserved) region of the MHC class II molecule. This interaction serves to strengthen and stabilize the interaction of the antigen receptor with the peptide MHC complex and also concentrates at the site of antigen receptor engagement intracellular signaling molecules associated with the CD4 molecules, for example, the kinase *lck*, which is necessary for T-lymphocyte activation (19). The CD8 molecule is believed to play a corresponding role in $CD8^+$ T-lymphocyte activation by binding to conserved regions of the MHC class I molecule (20). This role of the CD4 and CD8 molecules as coreceptors for the TCR explains why $CD4^+$ T-lymphocytes preferentially recognize antigenic peptides bound to MHC class II molecules and $CD8^+$ T-lymphocytes primarily recognize peptide MHC class I complexes.

Although the CD4 and CD8 coreceptors must bind to their ligands (the MHC class II and I molecules, respectively) for activation of most quiescent T-lymphocytes, costimulatory receptors displayed on the surface of the resting T-lymphocyte also must be engaged for primary cells to differentiate into activated effector T-lymphocytes (21). The costimulatory molecules CD28, CD2, and lymphocyte function-associated antigen (LFA)-1 play a critical role in T-lymphocyte activation (22). Engagement of these receptors by their ligands [i.e., CD80/CD86, LFA-3, intercellular adhesion molecule (ICAM)-1/ICAM-2] on APCs leads to signal transduction events within the T-lymphocytes, which, along with antigen receptor–coreceptor engagement, initiate the cascade of gene activation events that are necessary for the antigen-dependent activation and differentiation of effector T-lymphocytes (23,24,25,26 and 27). As indicated, dendritic cells express high levels of these costimulating ligands and therefore serve as potent APCs for T-lymphocyte activation.

Engagement of the antigen receptor and costimulatory receptor on the resting T-lymphocyte leads to lymphocyte activation, that is, blast transformation and progression of the cell from G_0 to the G_1 state of the cell cycle. Early in the course of lymphocyte activation, a number genes will become transcriptionally active (28), including genes encoding transcription factors that control the expression of early cell-surface markers of lymphocyte activation, such as CD69, as well as the production of several lymphokines and the expression of lymphokine–cytokine receptors. Interleukin-2 (IL-2) is the principal lymphokine responsible for progression of the activated lymphoblast from the G_1 state to the S phase of the cell cycle and subsequent proliferation (29,30). This T-lymphocyte growth factor is produced by T-lymphocytes early (24–48 hours) after activation. Although produced by both $CD4^+$ and $CD8^+$ T-lymphocytes, $CD4^+$ T cells generally secrete higher levels of IL-2 than $CD8^+$ T cells and sustain the production of IL-2 during the subsequent proliferation and differentiation of the activated T-lymphocyte. IL-2 is both an autocrine and paracrine T-lymphocyte growth factor that functions both on the cell producing it and on neighboring activated $CD4^+$ and $CD8^+$ T-lymphocytes. These cells must express the high-affinity heterotrimeric IL-2 receptor to respond to IL-2. This receptor complex, which itself is upregulated on T-lymphocytes after activation and blast transformation, transduces the proliferative signal mediated by IL-2 (29). Other cytokines, such as IL-4 and IL-7, either alone or in combination, also can deliver an IL-2-independent proliferative stimulus to activated T-lymphocytes, but the physiologic significance of these growth factors for early T-lymphocyte activation is uncertain.

Once cell division commences, the activated T-lymphocyte and its daughter cells undergo a series of differentiation steps, culminating in the formation of activated effector T-lymphocytes that have the same antigen specificity (i.e., express an antigen receptor identical to that of their quiescent small lymphocyte progenitor). The differentiation program set in motion by antigen receptor engagement is not fixed. In the case of $CD4^+$ T-lymphocytes, the precursor lymphocyte can give rise to effector cells with one of several distinct patterns of cytokine production. The classic cell-mediated immune response (e.g., cutaneous delayed-type hypersensitivity) is an example of a type 1 or proinflammatory response and is orchestrated by effector $CD4^+$ T-lymphocytes of the T-helper cell-1 (Th1) subset. The response of these effectors is characterized by the preferential production of IFN-g and tumor necrosis factor-b (TNF-b) in response to antigen. Atopic dermatitis and allergic asthma exemplify type 2 responses mediated by effector $CD4^+$ T-lymphocytes of the Th2 subset. In response to antigen, these effector cells preferentially produce IL-4, IL-5, and IL-13.

During the differentiation of an activated $CD4^+$ T-lymphocyte and its daughter cells into effectors, the cells may activate the genes encoding type 1 or type 2 cytokines or both. Many factors appear to influence the commitment of activated $CD4^+$ T-lymphocytes to differentiation along the Th1 or Th2 pathway, including antigen dose and form (31,32 and 33), the APC type stimulating the resting $CD4^+$ precursor cell (34), and, most importantly, the cytokine milieu in which the lymphocyte activation and differentiation occur (35,36,37,38,39,40,41 and 42). As discussed elsewhere (Chapter 10 and Chapter 11), the commitment of effector $CD4^+$ T-lymphocytes to a type 1 or 2 cytokine response can affect profoundly the magnitude and character of the host response and immune-mediated injury. $CD8^+$ T lymphocytes can also differentiate into effector cells exhibiting a type 1 (Tc1) or type 2 (Tc2) cytokine profile (43,44). In most circumstances, however, $CD8^+$ T-cell differentiation is along the type 1 pathway, yielding Tc1 IFN-g-secreting effector $CD8^+$ T-lymphocytes.

Activated effector $CD4^+$ and $CD8^+$ T-lymphocytes have less stringent requirements for stimulation than their quiescent small lymphocyte progenitors. Although the TCR/coreceptor complex on effector cells must be engaged for the cells to secrete cytokines and kill antigen-bearing cells, engagement of costimulatory receptors (e.g., CD28) is not required. This is an important evolutionary adaptation by T-lymphocytes in dealing with invading microorganisms because most cells in the body that are susceptible to infection do not express the costimulatory ligands constitutively displayed by professional APCs. Effector T-lymphocytes, however, do display unique cell-surface adhesive molecules that are not present on small resting lymphocytes. These molecules facilitate the entry of effector cells into sites of inflammation and antigen deposition (as discussed subsequently).

An important outcome of the activation and differentiation of resting primary T-lymphocytes is the generation of an expanded population of memory T-lymphocytes directed to the stimulating antigen. Memory T-lymphocytes display characteristic cell-surface markers (e.g., the CD45RO molecule, a 180-kd isoform of the leukocyte common antigen (45,46,47 and 48)), which distinguish memory cells from primary (virgin) T-lymphocytes. Whereas the generation of effector cells from primary T-lymphocyte precursors requires 3 to 6 days, memory T-lymphocytes rapidly undergo blast transformation and cell division. Memory cells can express effector activity soon after exposure to the stimulating antigen, that is, within 24 to 48 hours (49). These cells and their progeny daughter cells are the predominant cell types involved in the expression of the cutaneous delayed-type hypersensitivity reaction.

TRAFFICKING OF LYMPHOCYTES INTO INFLAMMATORY SITES

The trafficking of lymphocytes to sites throughout the body is mediated by adhesion molecules on the cell surface of lymphocytes, which bind to their complementary ligands on the surface of endothelium (50,51). The expression level of various adhesion molecules on the cell surface of a lymphocyte depends on the activation state of the cell, and it is the differential expression of these various adhesion molecules that controls the migration of lymphocytes from the blood into peripheral tissues of the body (52). naïve T lymphocytes circulate through the blood and secondary lymphoid tissues, such as lymph nodes, Peyer patches, tonsils, and spleen. naïve lymphocytes display this restricted trafficking pattern because of their expression of high cell-surface levels of the carbohydrate binding lectin-like molecule, L-selectin (CD62L), the lymph node homing receptor for lymphocytes (53). CD62L expressed on the surface of a T-lymphocyte interacts with its carbohydrate ligand displayed by specialized vascular structures known as *high endothelial venules*, resulting in the extravasation of naïve, resting lymphocytes from the blood into lymphoid tissues (54). Constitutively high expression of CD62L on naïve T cells serves to keep naïve T cells circulating through the lymphoid tissues (55). Thus, the primary locations for the activation of naïve T-lymphocytes and subsequent differentiation into activated effector lymphocytes are the lymph node and the spleen, where there is an environment rich in professional APCs, such as dendritic cells.

In contrast to naïve T-lymphocytes, activated effector T-lymphocytes are able to exit the secondary lymphoid tissues through efferent lymphatics and to enter the

vascular compartment. Effector T-lymphocytes leave the bloodstream and enter peripheral tissues at sites of inflammation. This ability of effector lymphocytes to deviate from the normal recirculation pattern of naïve T-lymphocytes is the result of the differential expression of several distinct cell-surface adhesion molecules as a consequence of T-lymphocyte activation and differentiation (55,56 and 57). Lymphocyte activation, as discussed, induces a large number of genes to become transcriptionally active, including genes that encode various adhesion molecules. Activated effector T-lymphocytes express increased levels of the integrins LFA-1 and very late antigen-4 (VLA-4) as well as carbohydrate ligand for selectin. Thus, effector lymphocytes differ from naïve lymphocytes by expressing increased (e.g., CD44, LFA-1, and VLA-4) or decreased (e.g., CD62L) cell-surface levels of several adhesion molecules. The activation-dependent upregulation of several of these adhesion molecules (e.g., CD44) is often used as a marker for activated effector T-lymphocytes.

Besides their use as activation markers, several cell-surface adhesion molecules also contribute to the distinct homing preference for peripheral tissues exhibited by activated effector T-lymphocytes (55). The expression of CD44, LFA-1, and VLA-4, combined with the downregulation in the cell-surface expression of CD62L that occurs after lymphocyte activation, facilitates the migration of activated effector T-lymphocytes from lymph nodes via the blood to enter peripheral tissues such as the lung, gut mucosa, skin, and joints. For activated T-lymphocytes to enter tissues, they must bind to vascular endothelium. Only activated vascular endothelium expresses the appropriate combination of adhesion receptor ligands and the chemotactic proteins (termed *chemokines*) to induce the extravasation of activated effector T-lymphocytes into tissues (58,59). This requirement ensures that activated T-lymphocytes preferentially extravasate into tissues at sites of antigen deposition (i.e., inflammation).

The vascular endothelium is activated through the release of cytokines and chemokines by tissue phagocytes, particularly macrophages. Phagocytosis of foreign antigen by tissue macrophages leads to activation of the macrophages and release of TNF-a, a potent activator of vascular endothelium. Activation of vascular endothelial cells by cytokines such as TNF-a results in the upregulation of several different types of adhesion receptors (60,61). One of the earliest cell-surface molecules upregulated is the endothelial cell adhesion molecule E-selectin; this lectin-like carbohydrate-binding protein interacts with its carbohydrate ligand displayed on the surface of activated T cells (62,63). It is the T-lymphocyte's interaction with E-selectin that causes rolling along the endothelium. Firm adhesion of lymphocytes to endothelium is mediated by integrins. Two integrins important in lymphocyte/endothelial cell interactions are LFA-1, which binds to ICAM-1, and VLA-4, which binds to vascular cell adhesion molecule 1 (VCAM-1). These two integrin ligands, ICAM-1 and VCAM-1, are also induced on vascular endothelium activated by TNF-a. In addition, activated vascular endothelium is induced to express certain chemokines, such as IL-8, as well as to accumulate other chemokines produced by cells of the innate immune system via the binding of chemokines to the extracellular matrix of endothelial cells (64). Once the lymphocyte has become firmly adhered to the vascular endothelial cell, chemokines are required to induce the transition of integrins into a high-avidity state that is necessary to complete the extravasation of the activated lymphocyte into the inflamed tissue (Fig. 18.2).

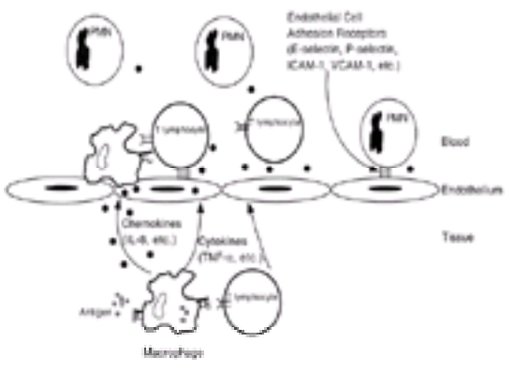

Figure 18.2. Recruitment of lymphocytes into areas of inflammation. Uptake of antigen in a tissue by resident macrophages leads to macrophage activation and the production of chemokines (e.g. IL-8) and proinflammatory cytokines [e.g., tumor necrosis factor-a (TNF-a)], which activate the vascular endothelium. Activation of the vascular endothelium by cytokines results in the upregulation of adhesion receptors such as E-selectin and P-selectin on the surface of endothelial cells. Additional macrophages, polymorphonuclear cells (PMN), and T-lymphocytes are recruited to the inflammatory site by following a chemokine gradient in the bloodstream. Interaction of these newly recruited cells with the adhesion molecules displayed on the surface of activated endothelium results in the extravasation of the cells into the inflammatory site.

Once lymphocytes reach these peripheral tissues, they probably interact with proteins and carbohydrates within the extracellular matrix, which, along with a chemokine gradient, regulates their migration through tissues. Activated effector lymphocytes express elevated levels of VLA-4, VLA-5, and VLA-6. These integrin receptors bind extracellular matrix proteins, such as fibronectin (a ligand for VLA-4 and VLA-5) and laminin (a ligand for VLA-6) (65). This interaction between effector T-lymphocyte integrins and the extracellular matrix likely aids in the movement of activated effector T-lymphocytes through tissues (66).

The events involved in the movement of effector T-lymphocytes from the vascular compartment at sites of inflammation to the sites of antigen deposition in tissues are beginning to be understood in molecular terms. Lymphocyte adhesion to the endothelium is a multistep process (52,67). Initially, lymphocytes are retarded in the blood flow by the transient binding of selectins to their carbohydrate ligands, a process termed *rolling*. The next step in the extravasation process, termed *activation*, is not well understood. It is thought that chemokines, which bind to seven transmembrane-spanning G-protein-coupled receptors expressed on target cells, mediate integrin activation and transition into a high-avidity state. Integrin activation leads to strong adhesion, or *tethering*, to endothelium, followed by transmigration into tissue. The entire spectrum of leukocyte cell types may enter a tissue during different stages of an inflammatory reaction, controlled by the action of various cytokines and chemokines that regulate expression of adhesion ligands (and receptors) on vascular endothelium.

Activated macrophages and endothelial cells secrete cytokines, and chemokines have chemotactic properties for various leukocytes. Chemokines represent a family of small basic chemotactic proteins that play a critical role in the movement of leukocytes during inflammation (68). Lymphocyte movement is directed toward an increasing concentration gradient of the chemokines. Chemokines usually are produced at the site of inflammation, where the concentration is the highest, and diffuse away from the site. A single chemokine can bind more than one receptor, and, conversely, a given chemokine receptor can bind several structurally distinct chemokines. A range of chemokines is known to induce lymphocyte chemotaxis, including IL-8, inhibitory protein (IP)-10, macrophage chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1a, and RANTES (regulated on activation, normal T-cell expressed and secreted). Chemokine receptors were shown to be differentially expressed on T-lymphocyte subsets. The chemokine receptors CCR3 and CCR4 are expressed on Th2 cells, whereas CCR5 and CXCR3 are expressed on Th1 cells, providing a possible mechanism by which Th subsets can be preferentially recruited into distinct inflammatory sites (69,70 and 71). Thus, lymphocytes follow a chemokine gradient toward the tissue involved in the inflammatory response.

The recruitment and extravasation of activated T-lymphocytes constitute a highly regulated series of interactions between the lymphocytes and vascular endothelial cells. Vascular endothelial cells express constitutive levels of MHC class I and II molecules, which can be upregulated by cytokines such as IFN-g. Therefore, vascular endothelial cells can present antigens to T-lymphocytes and further amplify T-lymphocyte responses at inflammatory sites. Cytokines such as IL-1, IL-4, IL-13, and TNF-a activate endothelial cells and upregulate the expression of adhesive receptors on the endothelial cell surface. TNF-a is produced by macrophages and activated T-lymphocytes (72). Thus, macrophages that have ingested antigen at a peripheral site can secrete TNF-a to initiate an inflammatory response, which later can be further amplified by TNF-a produced by activated effector T-lymphocytes that have migrated to the inflammatory site. The TNF-a produced by T-lymphocytes in turn further activates the vascular bed and recruits and activates monocytes and macrophages at the site of antigen deposition. The morphologic manifestations of a delayed-type hypersensitivity response reflect this interplay between the cells of the innate and adaptive immune systems.

T-LYMPHOCYTE EFFECTOR ACTIVITY MEDIATED BY LYMPHOKINES

As discussed, after initial stimulation with antigen, naïve lymphocytes secrete primarily IL-2. After additional exposures to antigen, IL-2-secreting T-lymphocytes will differentiate into effector cells capable of secreting multiple cytokines (73,74). Cytokines are small pleiotropic molecules that often have both autocrine and paracrine activity and mediate many diverse biologic effects (75). Most of the primary effector functions of activated T-lymphocytes are mediated by the cytokines that they secrete (39,76). Many of the later chapters in this text discussion detail the functionally distinct subsets (i.e., Th1 versus Th2) of T-lymphocytes that produce different types of cytokines. The type of cytokines released by effector T-lymphocytes exposed to foreign antigen can have important consequences for successful host defense.

Lymphokines have three important functions in the expression of T-lymphocyte effector activity: (a) They regulate lymphocyte activation, proliferation, and differentiation; (b) they activate inflammatory cells of the innate immune system (e.g., monocytes, neutrophils) and amplify the inflammatory response of cells of the innate immune system; and (c) they stimulate the production of inflammatory cells from precursors in the bone marrow and direct the recruitment of inflammatory cells from the bone marrow to sites of inflammation.

Four lymphokines—IL-2, IL-4, IL-15, and transforming growth factor-b (TGF-b)—directly regulate lymphocyte activation and growth. As discussed, IL-2 is produced by

newly activated T-lymphocytes and can serve as both an autocrine or paracrine growth factor to drive antigen-activated T-lymphocytes through the cell cycle (77). In addition, IL-2 also enhances the production of many other cytokines by activated effector T-lymphocytes. Thus, the primary role of IL-2 is to promote the proliferation and expansion of antigen-specific T lymphocytes. IL-2 also can stimulate the proliferation of natural killer (NK) cells and enhances their ability to secrete IFN-g. Finally, IL-2 serves as an important regulator of T-lymphocyte death by inducing the upregulation of several antiapoptotic genes such as bcl-2 (78). Therefore, IL-2 produced by effector T-lymphocytes can amplify the cellular immune response by supporting further effector T-lymphocyte proliferation and sustain the response through its antiapoptotic effects.

Although it is less well studied than IL-2, IL-15 shares a number of biologic activities with IL-2, including the stimulation and proliferation of antigen-activated T-lymphocytes. IL-15 also stimulates the proliferation of NK cells and, in concert with IL-12 made by macrophages, enhances the production IFN-g (79). In addition to these functions, IL-15 can enhance the cytotoxic activity of NK cells and also act as a strong chemoattractant for T-lymphocytes (80,81). In contrast to IL-2, however, the main source of IL-15 is macrophages, not T-lymphocytes.

Interleukin-4 is produced mainly by $CD4^+$ T-lymphocytes, although, as noted, some $CD8^+$ T-lymphocytes may make IL-4 under certain conditions. IL-4 serves as an autocrine growth factor for type 2 CD4 T-lymphocytes (82). IL-4 synergizes with stimuli provided by CD40 engagement on B cells to drive B-cell activation, proliferation, and differentiation, thus strongly enhancing antibody production by B cells, especially antibody of the immunoglobulin G4 (IgG4 isotype, IgG1 in mice). IL-4 also has a number of activities that promote allergic inflammation, including stimulating basophil recruitment and production, eosinophil chemotaxis, mast cell activation, and the production of IgE by B cells (83,84,85 and 86). Finally, IL-4 also plays an important role in the development of granulomatous inflammation by stimulating the morphologic transformation of macrophages into epitheloid cells and the formation of giant cells characteristic of granulomas.

The fourth regulatory cytokine, TGF-b is produced by many different cell types, including platelets, macrophages, and T and B cells; it has many diverse and sometimes opposing effects. TGF-b has several antiinflammatory activities, including the suppression of hematopoiesis, the reduction in the production of proinflammatory cytokines, and the inhibition of leukocyte adhesion to endothelium (87). A newly recognized subset of $CD4^+$ T-lymphocytes, termed *Th3 cells*, was proposed as $CD4^+$ T-lymphocytes that produce high amounts of TGF-b. Thus, through the production of the immunosuppressive cytokine TGF-b, these cells may play important roles in downregulating immune responses and may represent the suppressor T-cell population, the presence of which has long been suggested in the literature (87,88).

Interleukin-10 and -13 are two other effector cytokines made by Th2 cells. Both IL-4 and IL-13 induce human B cells to undergo Ig isotype switching to IgE, and both cytokines regulate inflammatory responses by suppressing TNF-a and IL-1 production by monocytes and macrophages (89). Unlike IL-4, however, IL-13 cannot induce T-cell proliferation. IL-10 is made by macrophages and T-lymphocytes, and it inhibits the production of proinflammatory cytokines and downregulates antigen presentation by macrophages (90,91). In addition, both IL-4 and IL-10 inhibit the synthesis of Th1 cytokines and the cell proliferation of Th1 cells (92).

Interferon-g is produced by type 1 $CD4^+$ and $CD8^+$ T-lymphocytes as well as NK cells and is an important mediator of inflammatory cell activation. IFN-g increases the generation of highly reactive oxygen species, such as superoxide anion and hydrogen peroxide in macrophages, increasing their phagocytic and microbicidal activity. IFN-g selectively inhibits the cytokine production and proliferation of Th2 cells as well as IgG1 and IgE production by B cells. IFN-g, however, is the major switch factor for antibodies of the IgG1 isotype (IgG2a in mice). In addition, IFN-g enhances the production of cytokines such as IL-1 and TNF-a by macrophages as well as the expression of MHC class I and II molecules on macrophages, B cells, and endothelial cells (93,94). These effects serve to amplify further an inflammatory response. Finally, IFN-g stimulates NK cell activity and plays a critical role in antiviral defense. This product of activated T-lymphocytes serves as a critical effector molecule involved in activating cells of the innate immune system.

The stimulation of bone marrow–derived leukocyte precursors is one last important effector function mediated by a group of cytokines. The cytokines IL-3, IL-5, macrophage colony-stimulating factor (M-CSF), and granulocyte macrophage–colony-stimulating factor (GM-CSF) stimulate immature hematopoietic precursor cells in the bone marrow (95). IL-3 is a growth factor for hematopoietic stem cells and mast cells. IL-5 stimulates the production of eosinophils from immature precursors in the bone marrow. In addition, IL-5 promotes the recruitment and activation of eosinophils. M-CSF and GM-CSF, secreted by T cells and macrophages, induce the growth, differentiation, and activation of precursor and mature granulocytes (GM-CSF) and macrophages (both M-CSF and GM-CSF). All these cytokines play a key role in signaling the bone marrow to increase the production of leukocytes to replenish those innate immune cells turning over at inflammatory sites. Thus, these cytokines act as a positive feedback mechanism to ensure that any leukocytes lost during an immune response can be quickly replaced. Because activated T-lymphocytes are an important source of these hematopoietic growth factors, the production of innate immune effector cells is regulated by TCR engagement and therefore by antigen availability.

Cells of the immune system must make several important decisions, including not only what is foreign to the host but also how best to respond to foreign antigen to mediate its removal from the host. The regulation of the type of T-cell response and other effector mechanisms chosen during an immune response can have important consequences for successful host defense. As described, many of the effector functions of T-lymphocytes are mediated by the cytokines they secrete. Effector T-lymphocytes that have differentiated into type 1 or type 2 cells (a process described in detail in Chapter 10 and Chapter 11) are capable of mediating quite different (and often directly opposing) immune responses.

The classic delayed-type hypersensitivity response is a manifestation of the response of $CD4^+$ T-lymphocytes of the Th1 type. The morphologic manifestations of the delayed-type hypersensitivity response are an intense infiltration of the site of antigen deposition with mononuclear cells, primarily monocytes/macrophages and lymphocytes. This response can be understood in terms of the cells and soluble factors that orchestrate it. Memory $CD4^+$ T-lymphocytes encounter antigen that has migrated to the regional draining lymph node either as free antigen or as processed antigen presented by the dendritic cells. Memory $CD4^+$ T-lymphocytes in the lymph node give rise to effector cells that migrate to the site of antigen deposition. Activated effector T-lymphocytes transit from the vascular compartment into tissue spaces through activated vascular endothelium at the site of antigen deposition. When these activated effector T-lymphocytes contact antigen, they release proinflammatory cytokines and chemokines that promote the recruitment and activation of mononuclear phagocytes into the site of antigen deposition.

The characteristic features of an allergic response include eosinophil accumulation in tissues, mast cell activation, and production of IgE antibodies. These features of the allergic response reflect the activity of T lymphocytes secreting a Th2 type cytokine profile. These Th2 type $CD4^+$ T lymphocyte effectors produce IL-3, IL-5, and GM-CSF, which promote the recruitment and activation of eosinophils at the site of inflammation. IL-4 produced by these T lymphocytes promotes B cell activation and the differentiation of B lymphocytes into IgE-secreting plasma cells. IgE binds to high-affinity receptors on eosinophils and mast cells. Cross linking of the IgE on these cells by antigen leads to eosinophil and mast cell degranulation. Various inflammatory mediators are released by the activated eosinophils, including leukotrienes, platelet-activating factor, and cationic proteins. The rapid degranulation of IgE-armed cells by antigen results in the classic immediate-type hypersensitivity response. The activation and recruitment of type 2 $CD4^+$ T-lymphocytes with the subsequent recruitment of eosinophils into sites of antigen deposition are characteristic of a more prolonged and sustained atopic response.

CELL-MEDIATED CYTOLYSIS BY CYTOLYTIC T LYMPHOCYTES

The second major strategy used by effector T-lymphocytes to eliminate antigens is to destroy, by direct contact, cells expressing the peptide MHC complex to which their antigen receptors are directed. This activity, called *cell-mediated cytolysis* or *cell-mediated cytotoxicity*, is carried out by cytolytic (or cytotoxic) T-lymphocytes (CTLs). Contact-dependent killing is a property of many cell types, ranging from unicellular protozoa to the cells of the vertebrate innate and adaptive immune system. Contact-dependent cell-mediated cytolysis by activated T-lymphocytes likely evolved as an effector mechanism to eliminate intracellular pathogens, in particular intracellular parasitism by viruses and certain bacteria. This effector activity of T-lymphocytes also may play a role in allograft rejection and tumor cell elimination.

Most CTLs express the cell-surface CD8 coreceptor and primarily recognize peptide MHC class I complexes displayed on cell surfaces (2). Because MHC class I molecules are ubiquitously displayed on most body cells, $CD8^+$ CTLs are particularly suited to survey the body for “abnormal” cells (i.e., allografts, tumors, infected cells) displaying peptide MHC complexes specific for the CTL antigen receptor. Although $CD8^+$ T cells and CTLs are frequently used synonymously, $CD4^+$ effector T-lymphocytes in certain instances (as discussed later) can exhibit CTL activity (96). Also, the antigen receptor engagement events resulting in the trigging of cytolytic activity by $CD8^+$ CTLs typically leads to the synthesis and secretion of cytokines by these activated effector cells. For this reason, $CD8^+$ Tc1 and Tc2 CTLs, both of which exhibit cell-mediated cytolytic activity, can be distinguished by the production of IFN-g by Tc1 CTLs and IL-4 and IL-5 by Tc2 CTLs in response to antigenic stimulation (43).

Cytolysis mediated by $CD8^+$ CTLs has been defined and characterized primarily through *in vitro* assays of target cell killing. CTLs use two distinct *in vitro* cytolytic pathways: the perforin-dependent granule exocytosis pathway and the FasL/Fas pathway (Fig. 18.3). As discussed subsequently, each pathway uses distinct effector molecules expressed by the CTLs to trigger killing, and each pathway requires the expression of distinct ligands by the antigen-bearing target cell to induce destruction of the target cell. Typically, both killing pathways are activated simultaneously by engagement of the antigen receptor on the CTLs. Under certain experimental conditions [e.g., suboptimal stimulation of the antigen receptor by the target antigen or by structural variants of the stimulatory antigenic peptide termed *altered peptide ligands* (97)], the perforin/granzyme or the FasL/Fas killing pathways can be selectively triggered (98,99). Engagement of costimulatory adhesive receptors normally displayed on the surface of activated CTLs, such as CD2 and the B-integrin LFA-1, leads to enhanced killing of target cells by CTLs. The ligands for these adhesive receptors need not be constitutively expressed on target cells for target-cell recognition and destruction by CTL. Many of these ligands, such as the LFA-1 ligand CD54

(ICAM-1), can be induced or upregulated on target cells through the action of proinflammatory cytokines produced by cells of the innate immune system. Consequently, at sites of inflammation or injury, cells displaying the appropriate peptide MHC class I complex can also express these adhesive ligands. This ensures the efficient recognition and selective destruction of these target cells by CTL.

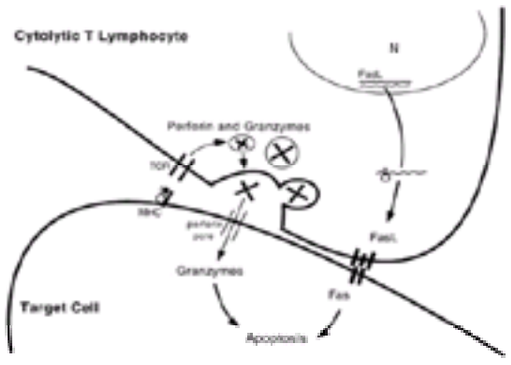

Figure 18.3. Mechanisms of T-lymphocyte-mediated cytolysis. Activated $CD8^+$ T lymphoblasts (CTLs) kill target cells by at least two distinct mechanisms: granule exocytosis and ligand receptor interaction. The T-cell antigen receptor (TCR) must be engaged for these killing mechanisms to be triggered. Engagement of the TCR on a mature CTL by peptide major histocompatability complex (MHC) on the target cell leads to the movement of granules containing perforin and granzymes to the site of TCR engagement and release of granule contents onto the surface of the target cell. Here the process of pore formation and apoptosis of the target cell is initiated. TCR engagement also leads to the transcriptional activation of the FasL gene and upregulation of FasL on the CTL surface. Engagement of Fas receptor on the target cell surface by FasL also triggers the cascade of events leading to apoptosis of the target cell.

Cell destruction mediated by CTLs occurs by triggering programmed cell death or apoptosis in the target cell. Apoptosis is a unique type of cell death distinguishable from other types of lethal cellular injury, for example, hypoxia, by characteristic morphologic and biochemical changes in the dying cell. Cells undergoing apoptotic cell death exhibit plasma membrane ruffling and vesicle blebing from the plasma membrane, mitochondrial swelling with alterations in mitochondrial membrane potential, and nuclear disintegration resulting in chromatin condensation and fragmentation of DNA into nucleosome sized pieces (100). The induction of apoptotic cell death is not unique to CTL. Apoptosis can be triggered by a variety of stimuli, such as ionizing radiation. Apoptosis is the mechanism used by most multicellular eukaryotic organisms to eliminate cells during tissue remodeling throughout development. The apoptotic process is mediated by a family of cytoplasmic cysteine proteases: the caspases. Members of this protease family exist as inactive proenzymes in all mammalian cells. They function in a cascade fashion with initiator procaspases (e.g., procaspases 8 and 10) undergoing autocatalytic proteolytic cleavage and activation in response to an apoptotic stimulus and terminal effector procaspases (e.g., procaspases 3 and 7) serving as substrates for the activated initiator enzymes. On activation, these caspases trigger the terminal events in the cell nucleus, mitochondria, and cytoplasm, resulting in the morphologic changes of apoptotic death. The cellular residue of this type of cell death, the apoptotic body, is susceptible to uptake by phagocytic cells, particularly macrophages. Phagocytosis of the apoptotic body is believed not to trigger macrophage activation, however (9).

PERFORIN/GRANZYME GRANULE EXOCYTOSIS KILLING PATHWAY

Quiescent naïve $CD8^+$ T-lymphocytes do not exhibit cell-mediated cytolytic activity, as already discussed. On engagement of the $CD8^+$ T-cell antigen receptor and costimulatory receptors by the appropriate peptide MHC class I complex and costimulatory ligands on an APC, the $CD8^+$ CTL precursors activate, proliferate, and differentiate into activated effector CTLs. During this differentiation process, the genes encoding products associated with CTL-mediated killing become transcriptionally active (101,102,103,104 and 105). The products include the pore-forming protein perforin, a family of serine proteases, the granzymes; and the lysosomal enzyme cathepsin C. These proteins accumulate in discrete secretory granules bound to a proteoglycan matrix in the cytoplasm of the differentiating $CD8^+$ lymphoblast. The presence of these lytic granules is a hallmark of the activated $CD8^+$ effector T-lymphocyte, the CTL. Exposure of the differentiating $CD8^+$ T cell to cytokines, particularly IL-2 and IL-7 (106,107), enhances the generation of effector CTL. Whereas these lytic granules are not present in naïve $CD8^+$ T-lymphocytes, they can be observed in circulating $CD8^+$ T cells expressing the $CD45RO^+$ memory phenotype (108). Granules that contain perforin/granzyme are also present in NK cells and in some T-lymphocytes of the g/d lineage.

Perforin/granzyme-dependent killing by CTL is through the process of granule exocytosis, described briefly later [for a more detailed description, see Henkart (109)]. In the mature effector CTLs, lytic granules are distributed throughout the cell cytoplasm. When the CTL encounters a cell displaying the appropriate peptide MHC class I complex, the CTL antigen receptor is engaged. The receptor-mediated signal transduction events result in rapid reorganization of the CTL cytoskeleton (within minutes) with polarized movement of granules (and other cellular organelles) to the site of CTL/target-cell contact resulting in release of the granules and their contents into the extracellular space at the contact interface between the two cells (101,102,104). The perforin molecule is liberated from the extruded granule into the extracellular space in a monomeric form. This 65- to 75kd glycoprotein monomer has several unique structural features, including a distinct domain with structural homology to the complement proteins constituting the pore-forming complement membrane attack complex (110), and a lipid-binding domain (111). In the presence of extracellular calcium, perforin monomers insert into the lipid bilayer of the target cell and polymerize to form structural and functional pores in the target cell membrane. Perforin pore formation can lead to target cell lysis, presumably because of the loss of intracellular macromolecules through these pores. Perforin pore formation alone does not induce the apoptotic death characteristic of CTL killing. Apoptotic death requires granzymes. The formation of perforin pores is believed to provide the granzymes access to the target cell cytoplasm.

At least eight members of the lymphocyte granzyme family of serine proteases (granzymes A–H) have been identified in the human (112,113). Two of these granzymes, A and B, have been directly implicated in CTL-mediated killing through the granule exocytosis pathway (114). Granzymes exist as proenzymes in the acid pH environment of the secretory granule. During degranulation, the proenzyme form of the granzymes is cleaved and activated by cathepsin C in the extracellular space at the site of CTL contact with the target cell. Although direct transfer of active granzymes into target cells has not been demonstrated, it is likely that the active enzymes enter cells through perforin pores. Granzymes are believed to trigger the DNA damage characteristic of the apoptotic process in the target cell by at least two mechanisms. Activated granzymes can bind directly to chromatin and cleave nuclear membrane constituents (115), thereby initiating nuclear damage and DNA fragmentation by a caspase-independent mechanism. Granzyme B also has been shown to cleave and activate both initiator and effector caspases (116). Therefore, granzymes may trigger apoptosis by activating the caspase-dependent cell death pathway. *In vitro* studies of CTL-mediated lysis with caspase inhibitors used to block killing or CTL effectors genetically deficient in specific granzymes also implicate caspase activation by granzymes as an important step in target cell killing by CTL through the granule exocytosis pathway (109).

Definitive information on the contribution of the perforin/granzyme killing pathway to $CD8^+$ T-lymphocyte effector activity *in vivo* has come from studies of mice rendered genetically deficient in perforin or specific granzymes by targeted gene disruption (reviewed in 109). In most instances, mice deficient in perforin or granzymes demonstrate normal resistance to viral and bacterial infections (117,118,119,120,121 and 122), reject allografts and tumors normally, and show no enhanced resistance or susceptibility to autoimmune injury (123), although exceptions have been noted under certain conditions (124). These findings are perhaps not surprising because $CD8^+$ T-lymphocytes also express their effector activity by cytokine and chemokine release and by killing through the Fas/FasL pathway.

THE FASL/FAS PATHWAY

The second major pathway used by CTL to kill cells is engagement of the 43-kd Fas (CD95) molecule on target cells by FasL (CD95L) displayed on activated T-lymphocytes (125). Fas is a member of the nerve growth factor (NGF) receptor superfamily. This receptor family also includes the TNF receptor types I and II (TNFRI and TNFRII) and the CD40 molecule. The Fas monomer is readily detected *in vitro* on cell lines of many different lineages and normally is expressed at the cell surface *in vivo* at high levels on lymphocytes and at varying levels on the surface of cells of other tissue types. The Fas molecule has in its cytoplasmic tail a novel “death” domain. Ligation of the extracellular domain of the Fas monomer by FasL results in the formation of a Fas trimer and activation of the cytosolic death domain. Trimerization of Fas triggers the activation of the caspase-dependent apoptotic pathway and death of the target cells (100). A structurally homologous death domain is also present in the cytoplasmic tail of other members of the NGF receptor family, including the TNFRI. The trimerization of the TNFRI receptor on cell surfaces by TNF also can lead to caspase activation and cell death. The presence of a cytoplasmic death domain on both Fas and TNFRI reflects the role of these molecules as inducers of cell death.

FasL is a member of the NGF superfamily and, like other members of this growth factor superfamily, it is a type II membrane glycoprotein. The molecule is present at low or undetectable levels on primary (resting) T-lymphocytes. The activation and differentiation of a resting T lymphocyte after TCR engagement also results in transcriptional activation of the Fas L gene and its expression on the surface of activated T-lymphocytes. FasL exists on the cell surface as a homotrimer. It is this property of the molecule that allows it to trimerize and activate Fas molecules when this ligand encounters its receptor on target cells. Unlike perforin and granzymes, which are exclusively expressed in cells of the lymphocyte lineage, FasL has been reported to be constitutively or inducibly expressed *in vivo* on nonlymphoid cells in

the eye, testes, and lungs (126).

The expression of FasL on activated lymphocytes is under tight control at both the transcriptional and the posttranslation levels. Expression of the FasL gene in effector lymphocytes requires TCR engagement by antigen, and sustained cell surface expression of the FasL trimers on effector T lymphocytes appears to require continuous antigenic stimulation. The FasL trimer on the surface of activated T-lymphocytes is also susceptible to cleavage by extracellular metaloproteases (127). The proteolytically cleared FasL trimer has been reported to be functional, that is, capable of engaging Fas and inducing apoptosis, but the soluble FasL more likely serves as a competitive inhibitor of cell-associated FasL, which blocks the interaction of membrane-bound FasL with the Fas receptor. The tight control of FasL expression on the surface of effector T cells ensures that FasL will preferentially engage Fas on cells displaying the appropriate peptide MHC complex.

The FasL/Fas killing pathway differs from the perforin/granzyme pathway in several respects. Although both killing pathways are normally simultaneously activated by engagement of the CTL antigen receptor, the intracellular signaling requirements for granule release and FasL expression may differ (98,99). Also, whereas perforin/granzyme-dependent killing requires extracellular Ca^{2+}, killing through FasL/Fas interaction is Ca^{2+} independent. In addition, FasL-dependent target cell lysis *in vitro* is slower than perforin-mediated killing, presumably reflecting the need for *de novo* expression of FasL. Finally, engagement of Fas by FasL does not always lead to apoptotic cell death. Different Fas-expressing cell types vary in their susceptibility to this killing mechanism. This represents the balance of proapoptotic and antiapoptotic mechanisms within the target cell regulating Fas-dependent cell death.

To date, little evidence suggests a primary role for the FasL/Fas killing pathway in T-lymphocyte effector activity against infectious agents or in graft rejection. The interaction between FasL and Fas on lymphocytes appears to be crucial for lymphocyte homeostasis. As discussed later, both humans and mice with genetic defects in the expression of functional Fas or FasL suffer from lymphoproliferative disorders and immune dysfunction, leading to autoimmune disease.

CELL-MEDIATED CYTOLYTIC ACTIVITY BY $CD4^+$ T-LYMPHOCYTES

Although the expression of contact-dependent killing is a characteristic of differentiated effector $CD8^+$ T-lymphocytes, $CD4^+$ T-lymphocytes also can express killing activity in certain circumstances. $CD4^+$ cytolytic effector T cells usually are detected when T-lymphocyte populations are maintained in long-term culture (128,129) or after exposure of resting T-lymphocytes to high levels of IL-2 during the stimulation of the $CD4^+$ T cells *in vitro*. During the normal process of $CD4^+$ T-cell activation and differentiation *in vivo*, the genes for perforin and granzymes are not transcriptionally active (130), suggesting that the expression of the granule-based killing pathway is not a typical property of $CD4^+$ effector T-lymphocytes. Under experimental conditions where $CD8^+$ T lymphocytes are eliminated, $CD4^+$ T-lymphocytes can differentiate *in vivo* into effector cells with lytic activity (131). The significance of $CD4^+$ CTL as effector cells is presently not clear. These cells have been proposed to play a role in regulating immune responses by eliminating antigen-bearing MHC class II positive APC and B-lymphocytes (128,132,133 and 134).

RESOLUTION OF THE EFFECTOR T-LYMPHOCYTE RESPONSE

Antigen drives both the induction and expression of T-lymphocyte effector activity. Once effector T-lymphocytes have responded and the stimulating antigen is eliminated, this expanded pool of antigen-specific effector T-lymphocytes is no longer needed. Most of these cells are eliminated from the lymphoid compartment. Without such a mechanism to remove effector T-lymphocytes from the lymphoid compartments (e.g., the lymph nodes and spleen as well as peripheral sites such as the gut, skin, and respiratory tract), these sites would rapidly fill with effector cells. In addition, new populations of effector cells entering these compartments in response to another unrelated pathogen ultimately would be crowded out by accumulated effector cells from prior infections (135,136).

During the resolution of the cellular immune response, activated effector T-lymphocytes in lymphoid tissues and sites of inflammation are eliminated by several related mechanisms, resulting in the apoptotic death of the T-lymphocyte effector cells. Engagement of the TCR on activated T-lymphocytes leads not only to the expression of lymphocyte effector activity but also to the transient expression by the T-lymphocytes of genes encoding antiapoptotic proteins (e.g., members of the bcl-2 gene family) as well as the transient synthesis and release of lymphocyte growth factors such as IL-2. Activated T-lymphocytes require the growth factor IL-2 to sustain their viability, and IL-2 interaction with its high affinity receptor on activated T-lymphocytes in turn also induces the expression of antiapoptotic proteins. After antigen is eliminated, the genes encoding growth factors and antiapoptotic proteins are no longer active and the effector T-lymphocytes undergo programmed cell death as a result of growth factor withdrawal. Activated effector T-lymphocytes also are eliminated by a mechanism operating at the level of TCR engagement through a process called *activation-induced cell death*. As mentioned, engagement of the TCR on activated T-lymphocytes results in the upregulation of FasL. Activated T lymphocytes are particularly sensitive to Fas-mediated apoptotic death. There is now compelling evidence that activated T-lymphocytes (particularly $CD4^+$ T-lymphocyte effectors) are in part eliminated during the resolution phase of the cellular immune response by FasL/Fas-mediated activation-induced cell death (137). Humans (and experimental mice) lacking functional FasL or Fas molecules suffer from lymphoproliferative syndromes that frequently are associated with autoimmune diseases. The T-lymphocytes that accumulate in the lymphoid tissues of these patients no longer express the CD4 (or CD8) coreceptor molecule (138). These “CD4/CD8 double-negative” lymphocytes are believed to be accumulated T-effector cells that were not eliminated during the resolution phase of past immune responses as a result of a defective FasL/Fas interaction. The TNF/TNFR pathway also has been suggested to play a role in activation-induced cell death, particularly in the elimination of activated $CD8^+$ T-lymphocyte effectors (139).

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19 NATURAL KILLER CELLS

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Natural killer (NK) cells were first identified as a population of leukocytes from humans or experimental animals without known or deliberate immunization that were able to kill certain tumor cell lines in *in vitro* cytotoxicity assays (reviewed in 1). The initial characterization of the cells mediating this activity indicated that they had a lower buoyant density than most B or T cells and were predominantly "large granular lymphocytes" (2). Most NK cells are slightly larger than the average lymphocyte, and they display prominent azurophilic granules in the cytoplasm. However, morphology alone is inadequate to distinguish these cells from cytotoxic T lymphocytes, which look quite similar. Moreover, a subset of NK cells is small and agranular, and thus morphologically indistinguishable from other lymphocytes (3). A precise classification of NK cells requires a detailed analysis of their expression of cell-surface leukocyte differentiation antigens.

Lymphocytes comprise three distinct lineages: B cells, T cells, and NK cells. B and T lymphocytes are defined by the rearrangement and expression of their immunoglobulin (Ig) and T-cell antigen receptor (TcR) genes, respectively. NK cells do not rearrange Ig or TcR genes and are identified by their characteristic cell-surface antigenic phenotype (4). In humans, all mature NK cells bear the phenotype $CD3^-$, $CD56^+$, and most of these cells also coexpress CD16 (a low affinity receptor for IgG), CD94, and CD161 [natural killer receptor (NKR)-P1], although subsets lacking these other antigens have been described (reviewed in 5). Classification of these lymphocytes as NK cells requires that they be CD3 negative because minor populations of $CD3^+$ T cells express CD16, CD56, CD94, and CD161. Although rodent NK cells do not express CD56, in mice they can be identified as $CD3^-$, $DX5^+$ (or $NK1.1^+$ in certain mouse strains) lymphocytes, and in rats, as $CD3^-$, $NKR\text{-}P1^+$ lymphocytes.

NK cells have been implicated not only in the lysis of certain tumors, but also in innate immunity against intracellular bacteria, viruses, and parasites and in the regulation of hematopoiesis and adaptive immune responses (6,7). In many cases, these effector functions are mediated by their ability to secrete rapidly many different cytokines in response to stimulation. The origin, development, and function of NK cells are reviewed in this chapter.

ORIGIN AND DEVELOPMENT

Like all hematopoietic cells, NK cells arise from a bone marrow–derived hematopoietic stem cell. Studies tracing NK cell development along the branching paths of hematopoietic differentiation indicate that these cells share a common progenitor with lymphoid cells but do not develop from myeloid precursors (reviewed in 8). NK cells share a common progenitor with T lymphocytes (8). However, unlike T cells, NK cells do not require a thymus for their development; they develop normally in athymic nude mice. Moreover, NK cells also are present in mice lacking the recombinase enzymes, encoded by the *RAG-1* and *RAG-2* genes, necessary for the maturation of B and T lymphocytes. Thus rearrangement of somatic genes apparently is not required for assembling the receptors used by NK cells for antigenic recognition. Studies from the *in vitro* culture of NK/T-cell progenitors have indicated that their development is promoted by stem cell factor, interleukin (IL)-7, IL-2 (or IL-15), and unknown factors contributed by stromal feeder cells (reviewed in 8 and 9). How this relates to the physiologic conditions *in vivo* is uncertain.

Studies from gene "knock-out" mice have revealed that NK cell development requires IL-15 and the receptor for IL-15 (comprising the IL-15 receptor-a, IL-2 receptor-b, and IL-2 receptor-g subunits), the IRF-1 transcription factor (necessary for the production of IL-15), Jak3 (necessary for IL-15 receptor signaling), and the Ets-1 and Ikaros transcription factors (affecting unknown pathways; reviewed in 9). Disruption of other cytokine genes or their receptors [e.g., interferon-g (IFN-g, IL-2, IL-7] has a minimal effect on NK cell development. Because of the close developmental relationship between NK and T cells, all of the genetically deficient mice that lack NK cells also have T-cell abnormalities. Therefore conclusions about the role of NK cells in immune responses based on studies of these animals must be interpreted with caution.

In humans NK cells arise very early during ontogeny, being detected in the fetus by 8 weeks of gestation, before to the appearance of T cells at ~16 weeks. NK cells are abundant in the fetal liver, spleen, and blood and express a phenotype similar to that of NK cells in the adult (8). In contrast, NK cell development in mice occurs more slowly. The NK cells in newborn mice lack many of the NK cell receptors (e.g., Ly49) and are generally less functional, requiring 4–6 weeks for their complete maturation (10). The lifespan of NK cells was originally considered to be quite short (i.e., a few days or weeks); however, recent studies are challenging this view and indicating that at least some NK cells might have long lives.

TISSUE DISTRIBUTION

NK cells are abundant in the blood (comprising 5%–20% of circulating lymphocytes) and are present in spleen, liver, and bone marrow at a frequency of a few percentage of the total lymphocytes (1). They also are found in lower numbers in the thymus, lung, and gut lymphoid tissues. However, unlike B and T lymphocytes, NK cells are rarely encountered in lymph nodes and do not recirculate in the lymphatics. In mice certain viral infections result in the recruitment of NK cells from the bone marrow into infected liver tissues by a process that involves interferon-a/b (7).

The decidual tissues of the placenta contain maternally derived lymphocytes and macrophages. Most of these lymphocytes are NK cells, which were initially referred to as uterine granular lymphocytes or granulated metrial gland cells (11). The uterus is the only anatomic location in the body where NK cells predominate over other types of lymphocytes. What attracts and maintains NK cells in this site is not known. The phenotype of decidual NK cells also differs from that of the mother's peripheral blood NK cells (11). The physiologic significance of maternal NK cells in the decidua has not been determined, although a role in fetal sustenance and protection of the fetus against pathogens has been proposed (12).

EFFECTOR FUNCTIONS

The effector functions of NK cells are quite similar to those of $CD8^+$ cytotoxic T lymphocytes (CTLs). As their names imply, both cells mediate cell-mediated cytotoxicity. Killing of target cells by NK cells involves predominantly the directional secretion of perforin (a protein related to the C9 subunit of complement) and granzymes (a class of proteases) (13). In addition, NK cells and CTLs express Fas ligand and membrane or soluble tumor necrosis factor (TNF) that also can lyse susceptible cells. Unlike naive, resting $CD8^+$ T cells that require activation before synthesizing perforin or TNF, mature NK cells constitutively express these molecules. Hence, NK cells do not require proliferation, transcription, or protein synthesis before mediating cytotoxicity. This provides a rapid response, the hallmark of innate immune reactions. Mice lacking *perforin* genes fail to reject certain transplantable tumors, indicating a role for cytotoxicity in tumor immunity (14,15).

The cytokines secreted by activated NK cells are similar to those made by $CD4^+$ and $CD8^+$ T cells. Abundant IFN-g is produced by activated NK cells, and this has been shown to be essential in innate immunity against several intracellular bacteria (e.g., *Listeria monocytogenes*) (16,17), parasites (e.g., *Toxoplasma gondii*) (18,19), and viruses (e.g., mouse cytomegalovirus) (7). Whereas T cells also produce IFN-g, the kinetics of this response are slower than those of NK cells and require clonal expansion of antigen-specific T cells. IL-12 and IL-18 produced in inflammatory sites are required for the induction of IFN-g by NK cells. The prevailing view is that activated macrophages produce IL-1, IL-6, IL-12, IL-15, and TNF, which may augment NK cell synthesis of IFN-g. In turn, IFN-g potentiates macrophage stimulation that promotes their antimicrobial functions. Although innate immunity alone may be unable to eradicate these pathogens, it buys time for the development of a sterilizing adaptive immune response and the generation of immune memory.

Although NK cells predominantly produce cytokines characteristic of Th1 and $CD8^+$ T cells (including lymphotoxin, TNF-a, and IFN-g), under certain conditions, they can also secrete IL-5 and IL-13, typically made by Th2 cells (20,21). Activated NK cells synthesize granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and several different chemokines that may influence the migration and differentiation of other hematopoietic cells (1,7). However, unlike T cells, NK cells do not produce IL-2 or IL-4. Cytokines produced by NK cells also may promote a subsequent Th1-dominated T-cell response, affect the isotypes of antibodies generated, and influence bone marrow hematopoiesis (1).

RECEPTORS AND RECOGNITION

NK cells discriminate between normal and transformed or pathogen-infected tissue by a complex process that involves both activating and inhibitory receptors. Unlike B or T cells, no single receptor provides for self- versus non–self-discrimination. Rather, NK cells apparently use many different activating receptors to initiate cell-mediated cytotoxicity and cytokine production (reviewed in 5 and 22). The best-defined activating receptor on NK cells is CD16, a low-affinity receptor for the Fc region of IgG. CD16 allows NK cells to kill IgG-coated targets by antibody-dependent cellular cytotoxicity (ADCC). CD16 has no intrinsic signaling capacity but is noncovalently associated with the membrane adaptor proteins CD3z and the high-affinity Fc receptor for Ige (FceRIg). These adaptors have immunoreceptor tyrosine-based activation motifs (ITAMs) that, on CD16 binding to IgG, result in tyrosine phosphorylation of the ITAM in CD3z or FceRIg and subsequent stimulation through the protein tyrosine kinases Syk or ZAP70 (22). CD3z and FceRIg also are associated with the 1C7 (NKp30) and NKp46 NK cell receptors (23,24); however, the ligand specificities of these receptors are not known. DAP12, another ITAM-bearing adaptor protein on NK cells, is associated with several different NK cell receptors, some of which recognize classic or nonclassic major histocompatibility complex (MHC) class I ligands. For example, the Ly49D/DAP12 complex on mouse NK cells recognizes H-2D^{d}, the human CD94–NKG2C/DAP12 complex binds human leukocyte antigen (HLA)-E, and the human KIR2DS/DAP12 complex recognizes HLA-C ligands (22).

NK cells also express a receptor, designated NKG2D, for the human nonclassic MHC class I chain–related A and B molecules, MICA and MICB (25) (Fig. 19.1). The NKG2D receptor associates with DAP10, another membrane adaptor protein with a YxNM motif in its cytoplasmic domain that permits recruitment and activation of the p85 subunit of phosphatidylinositol 3 (PI3)-kinase (26). The NKG2D/DAP10 receptor complex is expressed on all NK cells, CD8^{+} T cells, and gd-TcR^{+} T cells (25). MICA and MICB are not expressed in substantial amounts on normal tissues but are overexpressed on carcinomas (27). NK cells are able to kill MICA/B-bearing tumors, suggesting a role for the NKG2D/DAP10 receptor complex in immune surveillance. In addition to these multisubunit receptor complexes linked to the Syk/ZAP70 or PI3-kinase signaling pathways, activation of NK cells also may involve other adhesion molecules, integrins, and costimulatory receptors [e.g., CD2, lymphocyte functional antigen (LFA)-1, 2B4, DNAM-1, CD44, CD28, and others) (reviewed in 5,22,28).

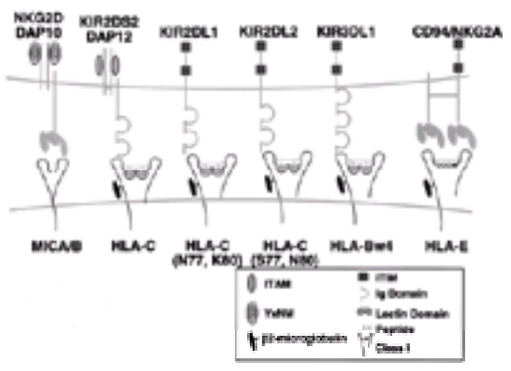

Figure 19.1. Activating and inhibitory natural killer (NK) cell receptors for major histocompatibility complex (MHC) class I. Diagrammatic representation of activating and inhibitory NK cell receptors and their MHC class I ligands. Killer cell Ig-like receptor (KIR)2DL1 and KIR2DL2 are inhibitory members of the KIR family that express two immunoglobulin (Ig)-like domains (2D) in the extracellular region and have a long (L) cytoplasmic domain containing two immunoreceptor tyrosine-based inhibition motif (ITIM) sequences. These receptors recognize a polymorphism of human leukocyte antigen (HLA)-C at residues 77 and 80 in the a1 domain. Peptides within the HLA-C binding groove can affect KIR recognition. KIR3DL1 is an inhibitory receptor with three Ig-like domains (3D) in the extracellular region and two ITIMs in the long (L) cytoplasmic tail. KIR3DL1 recognizes the Bw4 serologic motif in the a1 domain of the HLA-B heavy chain. The C-type lectin-like CD94 protein is disulfide-bonded to NKG2A, which has an ITIM in its cytoplasmic domain. This heterodimer binds HLA-E. Expression of HLA-E on the cell surface requires that it contain nine amino acid peptides derived from the leader segments of certain HLA-A, -B, and -C molecules in its peptide-binding groove. The KIR2DS2 receptor has two Ig-like domains (2D) in its extracellular region, but a short cytoplasmic domain without intrinsic signaling capacity. It noncovalently associates with the DAP12 membrane adaptor protein, which is a disulfide-bonded homodimer containing an IT activation motif (ITAM) in the cytoplasmic region of each subunit. KIR2DS2 interacts with HLA-C, but the precise specificity has not been defined. DAP12 also associates with the activating CD94/NKG2C receptor for HLA-E and with the activating isoforms of the rodent Ly49 receptor family (not shown). NKG2D is a C-type lectin-like receptor that associates noncovalently with DAP10. DAP10 is a membrane adaptor protein, expressed as a disulfide-bonded homodimer that contains a YxNM sequence in its cytoplasmic domain that can recruit and activate the p85 subunit of phosphatidylinositol-3 (PI3) kinase. NKG2D bind the nonclassic class I proteins MICA and MICB. Nomenclature for the KIR molecules and their ligand specificities are described at http://www.ncbi.nlm.nih.gov/prow/guide/679664748g.htm.

Because many of the ligands for these activating NK cell receptors are present on normal cells but are overexpressed in infected, inflamed, or transformed tissues, it is important to prevent NK cells from causing autoimmune destruction. This function is achieved by the presence of inhibitory receptors on NK cells that oppose the action of the activating receptors. In particular, NK cells express inhibitory receptors that recognize self MHC class I molecules and prevent or dampen NK cell attack against cells that express normal or elevated levels of class I. In humans, the killer cell Ig-like receptor (*KIR*) gene family on chromosome 19q13.4 encodes about ten receptors, many of which recognize polymorphic HLA-B or HLA-C ligands (Fig. 19.1) (29). *KIR* genes apparently do not exist in rodents; however, in mice, the Ly49 gene family, encoding receptors for H-2D and H-2K, serve the same function as the KIR molecules in primates. In addition, both human and rodent NK cells express heterodimeric receptors, composed of CD94 and NKG2A subunits, that recognize the nonpolymorphic human HLA-E molecule or the mouse counterpart called Qa1^{b} (Fig. 19.1). NKG2A and several receptors in the *KIR* family contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic domains. When NK cells expressing these inhibitory receptors encounter potential target cells bearing the relevant MHC class I ligand, the ITIMs are tyrosine phosphorylated, resulting in the recruitment of the cytoplasmic protein tyrosine phosphatases Src-homology 2 domain–containing tyrosine phosphatase (SHP-1) or SHP-2 (29). These phosphatases are responsible for inactivation of the NK cells.

Within the KIR, Ly49, and NKG2 families, some of these receptors do not have ITIMs in their cytoplasmic domains but instead associate with the membrane adaptor protein DAP12 and activate NK cells (Fig. 19.1). Therefore both activating and inhibitory receptors exist for MHC class I. To understand this complex system of regulation, it is important to appreciate that an individual NK cell clone within the total population expresses only a subset of these receptors. For example, within the NK cell population are cells that express an activating receptor (e.g., KIR2DS/DAP12) for HLA-C and an inhibitory receptor (e.g., CD94/NKG2A) for HLA-E. Because normal tissues express both HLA-C and HLA-E, the inhibitory receptor dominates and prevents autoimmunity. However, if on infection or transformation, expression of HLA-E, but not HLA-C, were downregulated, NK cells would be able to recognize this HLA-E–negative, HLA-C–positive cell as abnormal and eliminate it. Therefore, by differentially expressing arrays of these various NK cell receptors within the total population, it is possible to generate a very diverse repertoire for the detection of abnormal cells. The process by which repertoire diversity is achieved is not understood. However, it appears that every NK cell clone has at least one inhibitory receptor (and sometimes more) for a self class I ligand, probably to prevent autoimmunity (30,31).

The ability to detect loss of MHC class I has likely evolved to protect against viruses, and possibly transformed cells. Many viruses [including adenovirus, herpes simplex virus, cytomegalovirus, human immunodeficiency virus (HIV), and others] have devised mechanisms to block MHC class I synthesis (reviewed in 32). For example, the Nef gene product of HIV preferentially downregulates HLA-A and HLA-B, but spares HLA-C, possibly attempting to evade T-cell recognition and at least partially avoid NK cell attack (33). Expression of MHC class I is often abnormal in tumors (34), again suggesting the possibility of immune evasion.

NATURAL KILLER CELLS IN TRANSPLANTATION

The classic laws of transplantation state that tissue grafts from either parent (A and B) to an (AxB) F_1 offspring will not be rejected by the recipient. Whereas this fact is generally true for solid organs, it does not apply to bone marrow transplantation. In this case, there is not only the problem of graft-versus-host disease if mature lymphocytes are transferred into an incompatible recipient, but also a host-versus-graft reaction that can result in failure of the stem cells to engraft. One component of the host-versus-graft response is the rejection of the parental bone marrow by radiation-resistant NK cells in the F_1 recipient. In the (AxB) F_1 recipient, some NK cells will coexpress inhibitory receptors for both parental A and B MHC class I haplotypes; however, a substantial proportion will have an inhibitory receptor for only A or only B. This situation presents no problem for the host because all tissues will codominantly express A and B. However, stem cells from the parent expressing only A or only B will be rejected by the subset of NK cells that is not inactivated. This mechanism of parental bone marrow rejection was originally observed in mouse studies, where it was called “hybrid resistance” (35). As allogeneic stem cell transplantation becomes a more frequently used therapy in humans, the potential for hybrid resistance must be considered.

NATURAL KILLER CELL IMMUNODEFICIENCIES AND DISEASES

Immunodeficiency patients totally lacking NK cells are quite rare. However, several cases of this disease have been reported, and the clinical features invariably involve an inability to resolve certain viral infections (36,37). The genetic basis for the absence of NK cells has not been determined in these patients; however, they have normal B and T lymphocytes, implying a lineage-specific defect. Most viruses, such as influenza and adenovirus, are resolved efficiently in these patients; however,

herpesviruses cause life-threatening disease in these individuals. There are similar findings in mice depleted of NK cells, in which control of mouse cytomegalovirus is compromised, but responses to mouse influenza and lymphocytic choriomeningitis virus are apparently normal (reviewed in 7).

Malignancies of NK cells also are infrequent, unlike the situation with lymphomas and leukemias of the B- and T-cell lineages. The clinical disorder known as “large granular lymphocyte lymphoproliferative disease” is used to describe abnormal expansions of either NK cells (with a phenotype $CD3^-$, $CD56^+$), or T cells (typically with a phenotype $CD3^+$, $CD8^+$, and $CD56^-$ or $CD56^+$) (38). Although this disease is often benign, it can be associated with severe neutropenia and anemia.

NK cells (as well as other leukocytes) are affected in the Chediak–Higashi syndrome (39). In this disease, normal numbers of NK cells are present but are characterized by the presence of one large granule in the cytoplasm, and the cytolytic activity of these cells is therefore impaired.

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20 NEUTROPHILS

Helene F. Rosenberg, M.D., and John I. Gallin, M.D.

Neutrophils are the most abundant of the leukocytes, comprising about two thirds of the circulating white blood cell pool. They are short-lived cells whose major physiologic role is the destruction of invading microorganisms. Mature neutrophils are highly motile cells; they respond to a variety of specific chemotactic stimuli and contain cell-surface antigens that mediate transfer and adherence to other cell surfaces. Neutrophils engulf microbial targets by a process known as phagocytosis; they contain numerous storage granules with antimicrobial peptides and oxidative enzymes that participate in the destruction of ingested microinvaders. Neutrophils also secrete products that modulate the inflammatory process. This chapter reviews the development and physiology of neutrophils; Chapter 29 discusses the pathophysiology and disorders of neutrophil function.

HISTORY

In 1882 Metchnikoff conceived the phagocytic theory of host defenses on observing that motile cells from starfish larvae clustered around a foreign body inserted under its skin. Although Metchnikoff was not the first to note this association between host cells and bacteria—in 1880 Koch and others described cells filled with anthrax bacilli—his experiments were the first to suggest a role for them. Metchnikoff also discovered that motile host cells were capable of engulfing, or phagocytosing, certain fungal pathogens, thereby rendering them benign. In contrast, fungi that were not attacked by these phagocytic cells caused disseminated fatal disease. From these and other studies, Metchnikoff predicted that defects in these phagocytic cells would result in a compromised host; his work provided the basis for many of our current concepts of host defense.

NEUTROPHIL STRUCTURE AND DEVELOPMENT

Neutrophils develop from undifferentiated precursors into cells capable of responding to and destroying a variety of microbial pathogens. Neutrophilic precursors proliferate and differentiate in the bone marrow; 55%–60% of the hematopoietic cells in the marrow are dedicated to the neutrophil lineage (1). Although differentiation is believed to be a continuous process, a number of distinct neutrophil precursors have been identified both morphologically and biochemically. The process of differentiation is mediated by growth factors, or cytokines, and also is influenced by bone marrow stromal elements.

Pluripotent hematopoietic stem cells are defined as cells capable of unlimited self-renewal and the generation of all hematopoietic lineages. For many years, this cell population was believed to exist in theory, but today, a stem cell population has been defined biochemically by the presence of the cell-surface protein known as CD34 (2). CD34 is a 110-kd glycosylated protein of unknown function; isolated $CD34^+$ cells can generate numerous hematopoietic lineages (including neutrophils) *in vitro* (3) and can reconstitute all hematopoietic lineages in lethally irradiated hosts (4). This observation has taken on recent clinical significance, as $CD34^+$ progenitor cells circulating in the blood are now in use in bone marrow transplantation and in gene therapy trials for chronic granulomatous disease, a genetic aberration of neutrophil oxidative metabolism (5,6; see Chapter 29).

In the current model of hematopoiesis, the CFU-GEMMs (colony-forming unit–granulocyte/erythrocyte/macrophage/megakaryocytes) are the first committed precursor cells of the myeloid lineage (Fig. 20.1). CFU-GEMM cells are no longer $CD34^+$; they express the cell-surface antigen CD33 (67-kd glycoprotein) and thus can be distinguished biochemically from both stem cell precursors and the terminal deoxynucleotidyl transferase (TdT)–positive precursors of the lymphoid lineages. CFU-GEMM cells generate CFU-GM cells (colony-forming unit–granulocyte-macrophages), which express additional surface antigens (CD13 and CD15) and are committed to the granulocyte and macrophage lineages.

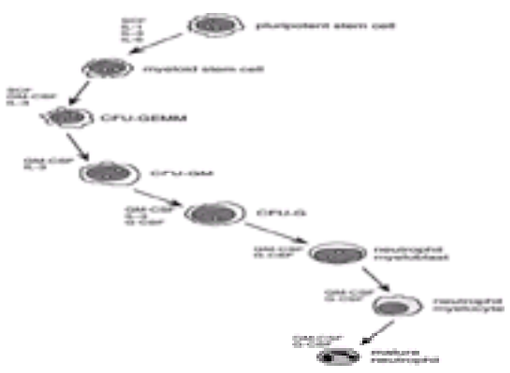

Figure 20.1. Differentiation of the pluripotent stem cell to the mature neutrophil. The cytokines listed are those shown to support the growth of the specific neutrophil progenitors and are described further in the text. Although distinct stages of differentiation have been recognized and defined, hematopoiesis is believed to be a continuous, rather than a stochastic, process. See text for abbreviations.

The myeloblast is the first morphologically distinct cell type of the neutrophil lineage. The process by which the myeloblast differentiates into a mature neutrophil is shown in Fig. 20.2. The myeloblast has a large nucleus, multiple nucleoli, prominent rough endoplasmic reticulum and Golgi apparatus, and numerous mitochondria, all characteristics of a biosynthetically active cell. The myeloblast matures to the promyelocyte. The first granules to appear are synthesized at the promyelocyte stage and are called azurophil, or primary, granules. The azurophil granules contain peroxidase, are rich in acid hydrolases and in the important host defense proteins, defensins, and are classic intracellular lysosomal granules. The azurophil staining characteristic of primary granules is normally observed only in immature cells in the bone marrow.

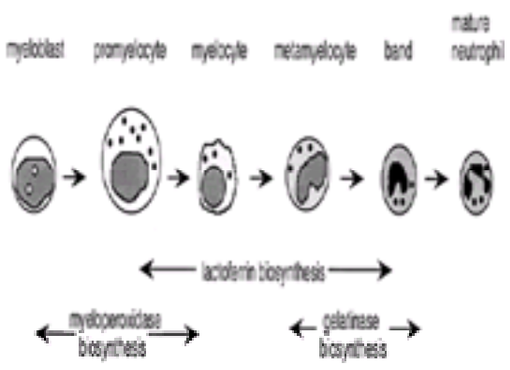

Figure 20.2. Maturation from myeloblast to mature neutrophil in the bone marrow. *Large black circles*, the azurophil granules appearing in the promyelocyte stage; *dark shading*, the specific granules, which first appear in the myelocyte stage. The stages during which myeloperoxidase, lactoferrin, and gelatinase are synthesized are indicated by the *arrows*.

The second group of granules to appear, called specific, or secondary, granules, are formed during the subsequent myelocyte stage. Specific granules contain a number of distinct proteins, including lactoferrin, vitamin B–binding protein, and collagenase, but lack acid hydrolases. The secondary granule membrane serves as a storage pool for several neutrophil plasma membrane proteins, including the adhesion protein Mac-1, the immunoglobulin Fc receptor, the chemotactic f-Met-Leu-Phe (fMLF) receptor, and the reduced nicotinamide adenine dinucleotide (NADPH) oxidase component cytochrome *b*-558, all of which are brought to the cell surface on neutrophil activation. Recent results from several laboratories suggest that the two-granule-populations view of neutrophil granules is somewhat oversimplified. Borregaard and Cowland (7) reviewed these findings and suggested that there are actually four populations of neutrophil cytoplasmic granules. Two additional populations of granules, gelatinase granules and secretory granules, have been identified. Although there is some overlap in protein content, the gelatinase and secretory granules form during the later stages of neutrophil development (Fig. 20.1). Gelatinase granules are defined by their high content of gelatinase, and secretory granules, as the intracellular storage site for alkaline phosphatase. The (overlapping) contents of each of the four distinct populations of neutrophil cytoplasmic granules are listed in Table 20.1.

Azurophil Granules	Specific Granules	Gelatinase Granules	Secretory Granules
Lysozyme	Lysozyme	Lysozyme	Alkaline phosphatase
Myeloperoxidase	Lactoferrin	Gelatinase	Albumin
BPI	Cytochrome b-558	Cytochrome b-558	Cytochrome b-558
Defensins	Procollagenase	Formyl peptide receptor	CD11b
Azurocidin	Mac-1/CR3 receptor	CD11b	CD14
Acid phosphatase	Formyl peptide receptor		CD16
β-Glucosaminidase	B_{12} binding protein		Formyl peptide receptor
α-Mannosidase	Histaminase		
Arylsulfatase	CD11b		
α-Fucosidase			
Cathepsin G			
Cathepsin D			
Elastase			
Phospholipase A			
Deoxyribonuclease			
5′-Nucleotidase			
Collagenase			
β-Glycerophosphatase			
β-Glucuronidase			

* See reference 1 for greater detail.
BPI, bactericidal permeability-increasing protein.

TABLE 20.1. Components of Neutrophil Azurophil, Specific, Gelatinase, and Secretory Granules*

There are no further cell divisions after the myelocyte stage. Primary granules are diluted by cell divisions during the myelocyte stage, and thus mature neutrophils are left with 200 to 300 granules, at a ratio of 1:2 azurophil to specific granules (1,8).

The final stages of neutrophil maturation in the bone marrow include the metamyelocyte and band neutrophil, preceding the mature neutrophil. Differentiation from the myeloblast to the band form requires ~7 days; maturation of the band form to the mature neutrophil requires another 7 days. The mature neutrophil is incapable of cell division and has a segmented, condensed nucleus, no nucleoli, and few mitochondria. Several studies have addressed the mechanism of formation of the condensed, multilobed nucleus of the neutrophil (reviewed in 9). The current working model describes nuclear filaments and filament control factors that interact specifically and hierarchically with distinct regions of genomic DNA. These interactions result in alterations in transcription and ultimately in the observed pattern of nuclear condensation.

Growth factors, or cytokines, interact with one or several of these hematopoietic precursor cells; they induce proliferation and support growth and differentiation (Fig. 20.1). A number of cytokines act on early hematopoietic progenitors; these cytokines include interleukin (IL)-1, IL-6, and stem cell factor (SCF)/*c-kit* ligand (10,11 and 12). Some of their actions with respect to neutrophil growth and development are indirect; for instance, IL-1 induces the production of granulocyte-macrophage and granulocyte colony-stimulating factors (GM-CSF, G-CSF), cytokines with direct effects on neutrophil precursors (13).

Three cytokines have direct effects on neutrophils and their precursors: G-CSF, GM-CSF, and IL-3 (14).

1. *G-CSF* is a 19-kd glycoprotein with actions that are specific for the neutrophil lineage (15,16). G-CSF is produced by macrophages, endothelial cells, and fibroblasts and binds to a single class of high-affinity receptors on neutrophils, normal neutrophil precursors, and leukemic blasts (16,17). Administration of G-CSF results in expansion of the myeloblast compartment, more rapid maturation time, and a dose-dependent increase in circulating neutrophils (18). Mutations leading to structural abnormalities of the G-CSF receptor on neutrophils result in a severe form of congenital neutropenia (19,20 and 21). Signal-transduction pathways through the G-CSF receptor have been elucidated, and include the protein tyrosine kinase families JAK, Src, and Akt. Most recently, G-CSF has been used clinically in combination with antibiotic therapies to combat various infectious diseases in both neutropenic and nonneutropenic individuals (22,23). Betsuyaku et al. (24) showed recently that neutrophils from G-CSF receptor–deficient mice have markedly reduced chemotactic responses to IL-8 both *in vitro* and *in vivo*, despite a normal number of IL-8 receptors, suggesting an otherwise unrecognized interaction between these two signaling systems.
2. *GM-CSF* is a 22-kd glycoprotein produced and secreted by activated T lymphocytes, macrophages, fibroblasts, and epithelial cells (25). In addition to its numerous effects on mature neutrophils (11), GM-CSF enhances survival and proliferation of hematopoietic precursors committed to neutrophil and macrophage lineages. Two distinct classes of GM-CSF receptor have been identified (26). Mature human neutrophils express class I receptors (26), which are downregulated by GM-CSF and also bind IL-3. Class II receptors (not downregulated by GM-CSF, no IL-3 binding) have been detected on leukemic blasts; normal neutrophil progenitors express both receptor classes (15,26).
3. *IL-3* (multi-CSF) is produced by activated T lymphocytes and natural killer (NK) cell, and supports the growth and development of mixed myeloid/erythroid cultures *in vitro*. The actions of IL-3 are directed by high-affinity receptors on early progenitor cells (27). The overlapping biologic activities of GM-CSF and IL-3 led to the discovery that the GM-CSF receptor and the IL-3 receptor, each containing two distinct subunits, have identical b chains (28).

In addition to the soluble growth factors, bone marrow stromal cells play crucial roles in neutrophil development (29,30). The association of maturing neutrophil precursors with bone marrow reticular cells and adherent macrophages has been described. Bone marrow stromal cells not only secrete but can also sequester cytokines into defined hematopoietic microenvironments (31).

The bone marrow serves as a storage pool for ~90% of the total pool of mature neutrophils; the signals initiating release of mature neutrophils from the marrow are not well characterized, although it is believed that IL-1 and a low-molecular-weight cleavage fragment from the third complement component (C3e) (32,33) may participate in this process. Mature neutrophils are more deformable and have lower affinity for the bone marrow adhesion protein hemonectin than do their precursors (34); mature neutrophils leave the bone marrow by passing between discontinuous adventitial cells lining the venous sinuses and between endothelial cells into the circulation. Fever and metabolic stress can enhance the rate of release of mature neutrophils from the bone marrow.

In the steady-state condition, neutrophils circulate in the bloodstream for a brief period ($t_{1/2}$ = 6 hours) before entering tissues. The half-life of neutrophils is shortened greatly in settings of acute inflammation, such as infection. Little is known of the fate of aged neutrophils remaining in circulation; evidence suggests that they leave the circulation primarily via the gastrointestinal tract (35). Senescent neutrophils also are cleared by tissue macrophages in the lung and spleen.

Although homogeneous in appearance, peripheral blood neutrophils have been shown to be heterogeneous by several structural and functional parameters (36). Mature neutrophils differ from one another in the expression of cell-surface receptors, in their ability to produce superoxide, in membrane potential, and in their ability to carry out protein synthesis. Although monoclonal antibodies have been developed that recognize distinct subsets of peripheral blood neutrophils (37,38), the significance of neutrophil heterogeneity remains unclear.

At the molecular level, the intracellular events resulting in the development of the neutrophil phenotype are controlled at the level of transcription by several specific families of transcription factors (reviewed in 39,40,41 and 42). Prominent among the factors involved in the regulation of neutrophil gene expression are members of the Myb, CDP, GATA, AML, Ets, and C/EBP families of transcription factors. None of these factors functions independently to define neutrophil lineage commitment, but the interplay among them functions in the coordinate regulation of genes expressed during neutrophil hematopoiesis. The roles of the C/EBPs (CCAAT enhancer-binding proteins) in neutrophil differentiation have been the best characterized to date (43,44 and 45). Smith et al. (46) and Zhang et al. (47) have reported that C/EBPa expression is critical for the upregulation of G-CSF receptors during normal hematopoiesis, and Zhang et al. reported impaired neutrophil development secondary to loss of G-CSF signaling in engineered C/EBPa-null mice. Similarly, Yamanaka et al. (49) reported that C/EBP-e-null mice were unable to generate

functionally mature neutrophils. Lekstrom-Himes et al. (50) recognized this abnormality as phenotypically and functionally analogous to the human disease syndrome known as neutrophil specific granule deficiency (see Chapter 29).

NEUTROPHIL FUNCTIONS

Several structures and functions distinguish the mature peripheral blood neutrophil in its role as a “professional” phagocytic cell. Neutrophils contain cell-surface receptors that sense signals in their immediate environment. These extracellular signals are translated into intracellular messages that mediate neutrophil motility, adherence, phagocytosis, degranulation, and production of superoxide. The functions of the activated neutrophil are shown in Fig. 20.3.

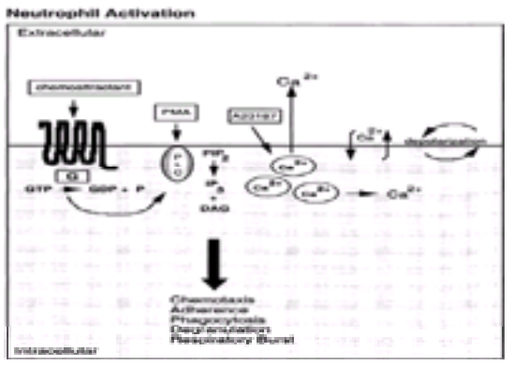

Figure 20.3. Events involved in activation of the peripheral blood neutrophil. Activation begins with binding of a chemoattractant to its specific G protein–coupled receptor. It results in the activation of phospholipase C (*PLC*) and production of inositol triphosphate (*IP*) and diacylglycerol (*DAG*), calcium flux, and membrane depolarization. Phorbol myristic acid (*PMA*) can bypass the receptor by activating PLC directly. The calcium ionophore A23187 likewise causes calcium flux in the absence of receptor-mediated activation. Neutrophil chemotaxis, adherence, phagocytosis, degranulation, and production of toxic oxygen metabolites occur as a result of neutrophil activation.

Neutrophil Chemoattractants

Neutrophils are chemotactic cells; they can respond with directed movement along concentration gradients of specific chemoattractant substances. They respond to a number of chemoattractant agents; among the best characterized of these ligands is the formylated peptide fMLF. This peptide and other similar formylated peptides are by-products of bacterial metabolism. Other agents include complement factor C5a, a proteolytic product of the complement cascade, leukotriene B_4 (LTB_4), and platelet-activating factor (PAF), the latter two being lipids released by neutrophils and platelets, respectively. Neutrophils also recognize peptides from the intecrine families, which include IL-8 and its homologs neutrophil-activating peptide 2 (NAP-2) and GRO-a, as well as macrophage inflammatory peptides (MIP-1a, MIP-1b) (51,52). Neutrophils also synthesize and secrete a significant quantity of IL-8 (53,54).

The neutrophil chemoattractant agents exert their effects by binding to specific cell-surface receptors. The mechanism by which the extracellular binding event translates into intracellular signaling has been a subject of intensive investigation. Initial studies with several of these receptors suggested the involvement of guanine nucleotides (55). The cDNA sequences of PAF (56,57), fMLF (58), IL-8 (59,60 and 61), and C5a receptors (62) are members of a extensive family of seven-transmembrane heterotrimeric guanosine triphosphate (GTP)-binding protein (G protein)-coupled receptors. The members of this receptor family all have guanine nucleotide binding sites, GTPase activity, with specificity (albeit with significant promiscuity) for a given ligand. The ligand-receptor-G protein complex initiates a cascade of events beginning with the activation of membrane-bound phospholipase C (55). Phospholipase C hydrolyzes a specific phospholipid, phosphoinositide-4,5-diphosphate (PIP_2), to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG are themselves second-messenger molecules; IP releases calcium from intracellular storage organelles (calciosomes) into the cytoplasm, and DAG is an activator of protein kinase C, which activates the neutrophil respiratory burst (see Neutrophil Antimicrobial Systems, later). Chemoattractant stimulation also results in changes in the plasma membrane potential (depolarization followed by hyperpolarization), which are likely to play an essential role in signal transduction.

Several of the receptors for the intecrine family of signaling molecules, which are primary agents responsible for differential trafficking of leukocytes, are expressed by human neutrophils. Most prominent of this group are CXCR1 and CXCR2, which are receptors for the ligand IL-8, and CCR1, the receptor for the ligands MIP-1a, RANTES, and MCP-2 and -3 (reviewed in 63, 64).

Cytoskeletal Responses

One immediate result of neutrophil activation by these agents is the chemotaxis of neutrophils toward the source of the attractant. The neutrophil travels by “crawling” across a surface; broad lamellopodia are seen at the leading edge and a short tail, or uropod, at the rear. Retraction fibers projecting from the rear of the cell are often broken off as the neutrophil moves forward (65).

On a subcellular level, the neutrophil cytoskeleton must respond to the chemoattractant signals by making structural alterations that result in net movement of the cell in a given direction. The neutrophil cytoskeleton is composed of actin filaments; cytochalasin B, a fungal product that interferes with actin polymerization and inhibits filament formation and likewise neutrophil chemotaxis and phagocytosis (66,67). The mechanism by which a chemoattractant signal is transmitted to the cytoskeleton is not clear; however, a number of actin-binding proteins can actively alter the cytoskeletal structure. Profilin (68) is a protein in leukocytes that binds to actin monomers and thus prevents their polymerization into filaments; the phosphoinositol metabolite PIP_2 reduces the affinity of profilin for actin. Cofilin and destrin also are actin-binding proteins that bind phosphoinositides (65,69) and may be linked to neutrophil activation. Gelsolin is yet another actin-binding protein that binds to and blocks the growing end to polymerizing actin filaments and severs actin–actin bonds in preexisting filaments (65,70). Intracellular calcium promotes the interaction of gelsolin with actin (71), whereas PIP_2 (72) inhibits it, suggesting a way in which the cytoskeleton might respond to neutrophil activation. Witke et al. (73) reported that mice with a targeted disruption of the gelsolin gene exhibited delayed neutrophil migration both *in vitro* and into peritoneal exudates *in vivo*.

Neutrophil Adherence

For neutrophils to leave the peripheral bloodstream and enter the site of active tissue inflammation, they must first pass through the endothelial cell layer lining the postcapillary venule. Neutrophil adherence to endothelial cells is mediated by two distinct families of cell-surface receptors: the leukocyte integrins and the selectins (Fig. 20.4).

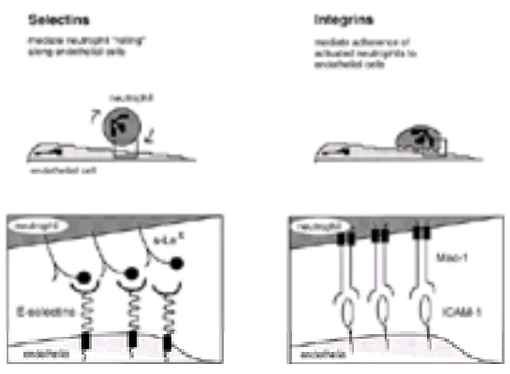

Figure 20.4. Comparison of the features characteristic of selectins and integrins. The areas within the boxes in the upper two panels are “magnified” in the lower two panels. Endothelial cell selectins bind to the sialylated Lewis X (*s-Le*^{x}) antigen on the neutrophil cell surface, thereby mediating neutrophil “rolling” along endothelial cells. In contrast, the adherence of activated neutrophils is mediated by the integrin Mac-1 (CD11b/CD18) and its ligand intercellular adhesion molecule-1 (ICAM-1) on endothelial cells.

Leukocyte Integrins

The leukocyte integrins represent a subclass of a large family of proteins that mediate interactions between cells and their extracellular environment. The leukocyte integrins—known as leukocyte function–associated antigen 1 (LFA-1) (CD11a/CD18), Mac-1 (CD11b/CD18 or CR3), and p150, 95 (CD11c/CD18)—mediate the interactions of leukocytes with each other, with extracellular particles, and with endothelial cells. Each of the three leukocyte integrins is a heterodimeric cell surface protein that contains a distinct alpha chain (CD11a, CD11b, or CD11c) and a common beta chain (CD18) (74,75,76 and 77). The alpha and beta chains each have an extracellular, a transmembrane, and a cytoplasmic domain; distinctive features include an extracellular cysteine-rich repeated motif on the beta chain and a putative cation-binding site on each alpha chain. Intact leukocyte integrins are absolutely crucial for neutrophil function; individuals with leukocyte adhesion deficiency (LAD) have recurrent bacterial and fungal infections associated with defects in neutrophil motility and adherence (78) (see Chapter 29).

The leukocyte integrin Mac-1 plays a major role in neutrophil-mediated activities. The complete, glycosylated heterodimer is stored in the specific granule compartment; on stimulation with any one of a variety of inflammatory mediators, Mac-1 is translocated along with the specific granule membrane to the neutrophil cell surface. In addition to mediating neutrophil adherence to endothelial cells, Mac-1 binds to the complement component IC3b (79) and directs phagocytosis and intracellular lysis of complement-opsonized microorganisms. Mac-1 also recognizes fibrin and fibrinogen and can mediate neutrophil adherence to the extracellular matrix (80). In addition, monoclonal antibodies to Mac-1 can block neutrophil aggregation and adhesion-dependent chemotaxis (80) and peroxide production (81,82). A number of these monoclonal antibodies can block a single Mac-1 function while leaving the other functions unaffected; it is believed that Mac-1 is a modular protein, with different domains subserving a variety of independent activities. Lu et al. (83) described a strain of Mac-1–deficient mice obtained by disruption of the CD11b gene. Neutrophils from Mac-1–deficient mice demonstrated defects in adherence, phagocytosis, and aggregation *in vitro*, although neutrophil accumulation *in vivo* in response to peritoneal thioglycollate proceeded normally.

The leukocyte integrin LFA-1, which is crucial to lymphocyte adherence, also is present on the neutrophil surface and plays a role in antibody-dependent killing (75,84). In contrast to Mac-1–deficient mice, LFA-1–deficient mice exhibited marked reduction in neutrophil extravasation in response to TNF-a, leading the authors to conclude that the contributions of LFA-1 to the adhesion process were more prominent than those of Mac-1 (85). Given these findings, it is curious to note that Mac-1–deficient mice develop spontaneous infections, whereas LFA-1–deficient mice do not. The third leukocyte integrin, p150, 95 is also found in neutrophils, also binds to complement component IC3b (86), and appears to play a role in neutrophil–endothelial interactions (75,80). Intracellular signaling through integrin-initiated pathways mediates virtually all “downstream” neutrophil functions (87). These signaling cascades proceed by the Src family of tyrosine kinases, with specific kinases Hck, Fgr, and Lyn identified through studies with specific gene-deleted mice (88).

Intercellular cell-adhesion molecule (ICAM)-1 has been characterized as a ligand for Mac-1 and LFA-1 on the endothelial cell surface. ICAM-1 is a member of the immunoglobulin gene family and contains five extracellular immunoglobulin-like domains (74,75,76 and 77). Endothelial ICAM-1 expression is upregulated by a number of cytokines, including IL-1, tumor necrosis factor-a (TNF-a), and interferon-g (IFN-g). Similar to the results with LFA-1–deficient mice, neutrophils from mice deficient in ICAM-1 display reduced neutrophil migration (89,90). However, Eppihimer et al. (91) have shown that deficiency of ICAM-1 alters expression of several other adhesion molecules, suggesting that these data must be interpreted with caution.

Selectins

The selectin proteins are similar to the integrins in that they too are transmembrane proteins that mediate cell–cell interactions; however, unlike the leukocyte integrins, selectins are uniquely leukocyte proteins. They function independent of the activation state of the neutrophil, and they recognize carbohydrate, rather than protein, ligands on their target cells (92,93). Three unique selectins have been characterized. L-selectin (MEL-14, LAM-1, Leu-8) was first described as a “homing receptor” on lymphocytes but has since been detected on all leukocytes. E-selectin (ELAM-1) is a cell-surface antigen on activated endothelial cells that mediates adhesion of neutrophils (94). P-selectin (PADGEM, CD62, GMP-140) is found in the membranes of both platelet and endothelial cell storage granules and mediates adhesion of neutrophils to each of these cell types (95,96).

All selectins have a similar multidomain structure. The structural components include an extracellular region with features similar to C-type animal lectins, a second domain with an epidermal growth factor (EGF)-like repeat, and a third domain containing a motif common to complement regulatory proteins; similar to the integrin a and b subunits, the selectins also contain a single transmembrane region and short cytoplasmic tail.

The endogenous ligand of E-selectin is a sialylated form of the myeloid specific carbohydrate known as the Lewis X antigen (97,98); this distinct carbohydrate moiety is commonly found in both glycolipids and N- and O-linked glycoproteins of myeloid cells. P-selectin also recognizes the sialylated Lewis X antigen (99). Although the endogenous ligand of the L-selectin has not yet been determined, it has been shown that L-selectin itself contains a sialylated Lewis X glycosyl group and binds through this moiety to the E- and P-selectins on endothelial cells (100,101). Interestingly, with activation *in vitro*, neutrophils shed L-selectin; and *in vivo*, exudative neutrophils have no L-selectin (102). Shedding of L-selectin may be an important requirement for neutrophil detachment from the endothelium during diapedesis.

Evidence suggests that selectins play a role in neutrophil “rolling,” a process in which blood-borne neutrophils slow before adhering to endothelial cells. Two cases of putative selectin dysfunction secondary to the absence of the sialylated Lewis X antigen have recently been described (103) (see Chapter 29).

Several groups have generated specific selectin gene–deleted mice (reviewed in 104). In P-selectin–deficient mice have more circulating neutrophils than are observed in wild-type mice, but there is no leukocyte rolling early in the inflammatory response (105). In contrast, neutrophils from L-selectin–deficient mice exhibit normal rolling early in inflammation but reduced rolling responses at later times (106), suggesting complementary functions for these two proteins. The inflammatory responses of E-selectin–deficient mice appeared to be normal at baseline (107), although aberrant responses to TNF-a have been reported (108).

Phagocytosis

Phagocytosis is the process by which neutrophils engulf invading pathogens and isolate them in membrane-bound compartments, or phagosomes (Fig. 20.5) (109). Neutrophil cytoplasmic granules containing toxic and cytolytic agents (described in later sections) fuse with the phagosome and mediate the destruction of the ingested material.

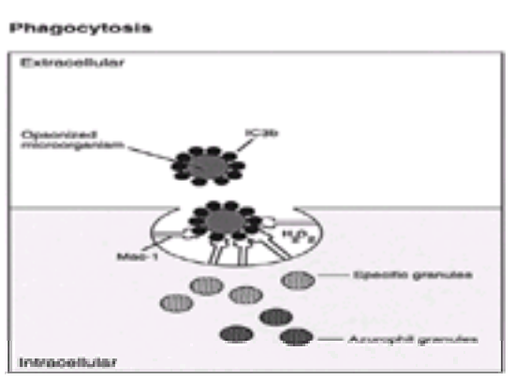

Figure 20.5. Ingestion, or phagocytosis, of a foreign particle by an activated neutrophil. The microorganism is coated, or opsonized, by a proteolytic product of the complement cascade known as IC3b. The complement receptor CR3 (Mac-1) recognizes the IC3b ligand and mediates formation of the phagosome around the opsonized particle. Peroxide within the phagosome results from activation of the respiratory burst (see text). Specific and azurophil granules fuse with the phagosome and release their contents of microbicidal enzymes and proteins (see Table 20.1).

Most invading pathogens or foreign particles are labeled, or “opsonized” by a coating of IgG or metabolites of complement component 3 (C3) known as C3b and IC3b. IgG-opsonized particles can interact with three distinct types of Fc receptors (RI, RII, RIII), all of which recognize the non–antigen-binding (Fc) end of the IgG molecule (110,111). The cDNA sequences of these receptors indicate that they belong to the Ig superfamily (112). Cross-linking of cell-surface Fc receptors (FcRII) by polyvalent immune complexes activates the neutrophil, resulting in phagocytosis and triggering of the respiratory burst. Although the signaling mechanism has not been clarified, receptor cross-linking also results in an increase in intracellular calcium and the production of inositol phosphates. The signal appears to be transmitted via the cytoplasmic tail of the Fc receptors (113). Neutrophils also have receptors for IgA-coated particles (114) and are capable of ingesting some nonopsonized particles,

such as latex beads.

Activation of the complement cascade by either classic (immunoglobulin-dependent) or alternative (immunoglobulin-independent) pathways results in the covalent binding of C3 to the surface of the target particle. As part of the binding process, C3 undergoes cleavage to become C3b, which is recognized by complement receptor 1 (CR1; CD35). C3b can then undergo proteolytic cleavage to become IC3b, which is recognized by complement receptor 3 (CR3). [Note that CR3 is the same as Mac-1 (CD11b/CD18), described earlier as a leukocyte integrin.]

CR1 is found on neutrophils, monocytes, and macrophages and exists in four distinct allelic forms in the human population. All forms have short cytoplasmic tails, a single transmembrane region, and a long extracellular region consisting of multiple small consensus repeats (SCRs) that in combination form long homologous repeats (LHRs); the allelic forms differ in their numbers of LHRs. The SCRs are interesting in that they are shared by a variety of related proteins, including a number of complement-related proteins, the IL-2 receptor, and, as mentioned briefly earlier, E-selectin (115,116); their functional significance is not yet clear.

CR3, as noted earlier, is a multifunctional protein that subserves several neutrophil functions and recognizes at least two distinct ligands. CR3, in its role as an adhesion protein and member of the integrin family, has been discussed earlier; with respect to phagocytosis, CR3 recognizes particles coated with IC3b (but not C3b). Experiments with blocking peptides suggested that CR3 recognizes the sequence arginine–glycine–aspartate (RGD) within the sequence of IC3b (117); recognition of the RGD sequence is a feature shared by other members of the integrin family of proteins.

The engulfment step of phagocytosis proceeds as the neutrophil extends cytoplasmic processes, or pseudopodia, along the edges of the opsonized target. Actin filaments appear locally at the site of engulfment. Pseudopodia can extend only so long as the leading edge encounters cell surface–bound ligand; incompletely opsonized particles cannot be engulfed (118). The pseudopodia meet, fuse, and thus isolate the particle in (plasma) membrane-bound organelles known as phagosomes. The specific granules are the first to fuse with the phagosome and release their contents. After specific granule discharge, primary granules fuse with the phagosome and release microbicidal peptides and myeloperoxidase. The individual components of both types of granule are described in more detail in the following section.

NEUTROPHIL ANTIMICROBIAL SYSTEMS

The two major antimicrobial systems of neutrophils can be classified as oxygen dependent and oxygen independent. The oxygen-independent systems rely on the antimicrobial activities of the cationic peptides and proteins that are released from azurophil and specific granules into the phagosome. The oxygen-dependent systems produce reactive oxygen metabolites that are toxic to invading microorganisms.

Oxygen-dependent Antimicrobial Activity

Baldridge and Gerard (119) were the first to describe the effect known as the “respiratory burst” when they noted that phagocytes incubated with microorganisms used oxygen at a more rapid rate than did their resting counterparts. Others noted that hypoxic conditions reduced (but did not eliminate) the antimicrobial activity of neutrophils (120,121 and 122). The respiratory burst is a process by which neutrophils reduce molecular oxygen to superoxide; superoxide is ultimately converted to hydrogen peroxide and other toxic oxygen metabolites. The respiratory burst is crucial to host defense; defects in any one of several components result in a life-threatening disorder known as chronic granulomatous disease of childhood (see Chapter 29).

The individual components catalyzing the respiratory burst have become the subjects of intensive investigation (Fig. 20.6) (123,124). Two of these components comprise the membrane-bound heterodimer known as cytochrome *b*-558, which contains a 91-kd glycosylated b subunit found in a noncovalent complex with a 22-kd a subunit (125,126). The low midpoint potential of this cytochrome (–245 mV) enables it to perform the direct single-electron reduction of molecular oxygen (127). The 91-kd subunit contains a binding site for the electron donor NADPH and a region homologous to FAD-binding domains of other proteins (128,129 and 130). There are two heme groups per heterodimer, which are thought to be coordinated jointly by both polypeptides (131,132). In the resting neutrophil, cytochrome *b*-558 is found primarily within the membrane of the specific granule (133,134). On neutrophil activation, the vesicles containing membrane-bound cytochrome *b*-558 fuse with the emerging phagolysosomes and with the plasma membrane (135).

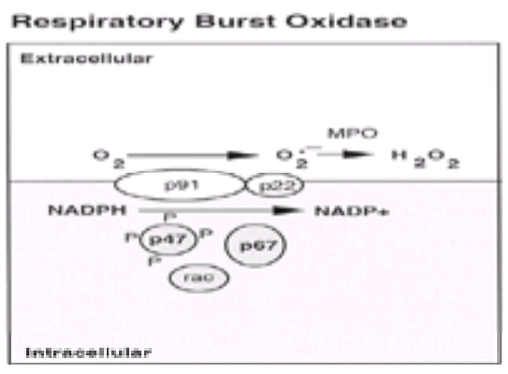

Figure 20.6. Components of the respiratory burst oxidase include two membrane-bound proteins (p91 phox and p22 phox) and three cytoplasmic proteins (p47 phox, p67 phox, rac). Neutrophil activation results in translocation of the cytoplasmic components to the membrane; together, these components catalyze the reduction of molecular oxygen to superoxide at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The precise location at which the superoxide is produced is not known. Superoxide is then converted to the toxic oxygen metabolite hydrogen peroxide.

The respiratory burst requires the activity of three independent cytosolic components known as p47 phox (phagocyte oxidase) (136), p67 phox (137), and a low-molecular-weight G (GTP-binding) protein known as rac (138,139 and 140). Neutrophil activation results in phosphorylation of p47 phox (141) and the translocation of both p47 phox and p67 phox to the plasma membrane (141,142 and 143). The 91-kd membrane component contains a carboxyl-terminal domain essential for interaction with the translocated p47 phox (143,144). Although both of these cytosolic components are crucial for superoxide production, their mechanism(s) of action remains unclear. The third cytosolic component, rac, has been isolated from neutrophils as a complex with an inhibitory protein known as rho-GDI (145). The role played by rac/rho-GDI in the production of superoxide is likewise unclear.

Another source of oxygen-dependent toxicity is the peroxidase–peroxide–halide system (146,147 and 148). Myeloperoxidase, a major component of the primary neutrophil granules, catalyzes the conversion of hydrogen peroxide (derived primarily from the respiratory burst) and hydrogen chloride to hypochlorous acid, molecular chlorine, and chloramines. Native myeloperoxidase is composed of two glycosylated identical heavy subunits (55–63 kd) and two identical light (10–15 kd) subunits, with two iron (Fe^{3+})-containing heme groups covalently linked to each of the heavy subunits.

The production of nitric oxide by nitric oxide synthase is another oxygen-dependent antimicrobial system. Nitric oxide synthase has been characterized primarily in mouse macrophages (149,150 and 151), but nitric oxide also may function as an antimicrobial agent in human neutrophils (152). Nitric oxide plays an important role in the macrophage-mediated killing of intracellular parasites and may assist in neutrophil-mediated bacterial killing. Although there seems to be more support in the literature documenting nitric oxide synthesis by human neutrophils (153,154 and 155), Holm et al. (156) reported that indicators such as nitrate and nitrite are produced by human neutrophils in response to appropriate stimuli, but that there is no production of nitric oxide itself.

Oxygen-independent Antimicrobial Activity

The azurophil and specific granules store a number of antimicrobial proteins, including lysozyme, lactoferrin, and a number of distinct cationic proteins, as described later and in Table 20.1. The contribution of each individual granule component to the overall antimicrobial activity of the neutrophil is difficult to measure, as single protein defects (analogous to CGD) have not yet been identified; the activities described later result from experiments done with purified proteins *in vitro*. More details on neutrophil granule proteins can be found in references 7 and 157,158 and 159.

Lysozyme

Lysozyme is a small (14–15 kd) hydrolytic enzyme whose target is bacterial cell wall peptidoglycan. Lysozyme has been found in both primary and secondary granules of the human neutrophil, as well as in numerous tissues and secretions (e.g., saliva). Although lysozyme can destroy bacterial cell wall peptidoglycan *in vitro*, its role as

an antibacterial agent *in vivo* is unclear for two reasons. First, other antimicrobial proteins released simultaneously with lysozyme activate endogenous bacterial muramidases that are equally if not more effective at hydrolyzing their own cell-wall peptidoglycan; second, neutrophils from several nonhuman species lack lysozyme with no apparent ill effect.

Lactoferrin

Lactoferrin is a large (78 kd) glycosylated protein, is a member of the transferrin family of iron-binding proteins, and is found in neutrophil specific granules as well as in other body tissues as secretions (160,161 and 162). The antibacterial properties of lactoferrin may be due in part to its ability to bind free iron, which is necessary for bacterial viability; high concentrations of plasma lactoferrin may contribute to the anemias associated with chronic inflammatory diseases. Two of the three known isoforms of lactoferrin have ribonuclease activity, which may or may not be related to its antipathogen functions. Lactoferrin has antiviral activity against RNA viruses (163), a striking parallel to the antiviral activity described for the otherwise unrelated ribonucleases present in human eosinophils (164). Individuals with a condition known as neutrophil specific granule deficiency (see Chapter 29) have neutrophils that are devoid of lactoferrin and are subject to recurrent bacterial infections (165,166); however, the absence of a number of other granule proteins (including defensins; see later) precludes any conclusions as to the significance of lactoferrin as an antibacterial agent *in vivo* from these findings.

Cationic Proteins

The neutrophil primary granule contains a number of cationic antimicrobial proteins. They include defensins, cathepsin G, azurocidin/CAP37, and bacterial permeability–increasing protein/CAP57 (167).

Bacterial Permeability–Increasing Protein

Bacterial permeability–increasing protein (BPI) is a single-chain protein (50–60 kd) with potent antibacterial activity *in vitro* (168); other isolates include CAP57 (169), which is identical to BPI. In contrast to the other primary granule cationic proteins that are arginine rich, BPI has an elevated lysine content. There are two interesting features of BPI: (a) its toxicity is remarkably specific for gram-negative bacteria; and (b) all the antibacterial activity resides in the cationic amino-terminal half of the protein. The mechanism of BPI's toxicity has been delineated: BPI inserts into the outer bacterial membrane, resulting in permeabilization of the bacterial cell and subsequent growth arrest. This event results in activation of bacterial enzymes, which eventuates in autolytic destruction of bacterial cell wall peptidoglycan and phospholipids (157).

Defensins

The defensins human neutrophil proteins (HNPs) are a group of small (29–34 amino acids) antimicrobial cyclic peptides that are rich in arginine and contain six cysteines in three invariant disulfide bonds (170,171,172,173 and 174). Defensins are active against a variety of gram-positive and gram-negative bacteria as well as against some fungi and viruses. HNP-1, HNP-2, and HNP-3 differ by only a single N-terminal amino acid; the amino acid sequence of HNP-4 differs at many points, but the cysteine components are conserved. Defensins comprise ~50% of the total primary granule protein content. Experiments with purified proteins have shown that defensins form voltage-gated ion channels in artificial lipid membrane systems (175). This finding suggests that, similar to BPI, the antimicrobial activity of the defensins likely begin with a membrane-perturbation event. Defensins also function as monocyte and T-cell chemoattractants (176,177).

Cathepsin G

Cathepsin G is a serine protease (25–29 kd) with activity primarily against gram-negative bacteria. Cathepsin G has significant sequence homology to neutrophil elastase and to a serine protease from cytotoxic T lymphocytes (178). The antibacterial activity of cathepsin G appears to be unrelated to its serine protease activity; elevated temperatures, protease inhibitors, and proteolysis of cathepsin G itself did not alter its bactericidal properties (179). One group has shown that short peptides derived from isolated regions of cathepsin G have antibacterial activity and may in part account for the antibacterial activity of the protein as a whole (180).

Azurocidin/CAP37

Azurocidin/CAP37 is a member of the group of proteins known as serprocidins. It is similar in structure to a serine protease, but it has mutations in the catalytic site precluding serine protease activity (181,182). Similar to cathepsin G and elastase, azurocidin/CAP37 is highly cationic owing to a high content of arginine residues and, similar to BPI and the defensins, probably acts by interfering with intact bacterial membranes. This protein is also a monocyte and T-cell chemoattractant (176,183).

PRO- AND ANTIINFLAMMATORY MEDIATORS PRODUCED BY NEUTROPHILS

The assumption that neutrophils are terminally differentiated, biosynthetically inactive cells has been shown to be incorrect; stimulated neutrophils synthesize and secrete a variety of inflammatory mediators that affect the activity of neutrophils themselves as well as that of other inflammatory cells (184). Cassatella (185) recently published an extensive review of this subject. In brief, a variety of external stimuli, including bacteria and bacterial products, fungi, protozoa, viruses, and some cytokines and growth factors (including TNF-a, IL-1b, IL-2, IL-15, and IL-13) and chemotactic agents (fMLF, C5a, and PAF) can induce the production of both pro- and antiinflammatory mediators. There have been reports documenting the production of the proinflammatory cytokines TNF-a, IL-1b, IL-1b, IL-12, and interferon-g, the antiinflammatory cytokines IL-1 receptor agonist and TGF-b; and numerous CC and CXC chemokines (including IL-8) and hematopoietic growth factors. Future research will be needed to elucidate the various independent and interdependent pathways leading to production of these immunomodulatory proteins as well as to determine the biologic significance of these findings in given physiologic settings and conditions.

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21 MONOCYTES AND MACROPHAGES

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Ilya Metchnikoff's description of ameboid cells in starfish larvae ingesting a rose thorn led to the realization that there exist specialized hematopoietic cells that are able to recognize and ingest foreign material. This fundamental cellular process of phagocytosis is a feature of a specialized lineage of myeloid cells that originates in the bone marrow. During embryogenesis at the time circulation is established, macrophages populate tissues where they reside for the life of the animal. A circulating pool of precursor cells, the monocytes, continually renews these resident macrophages. Macrophages are among the body's most versatile cells in that they adapt to different roles according to their local milieu and play distinct roles at different stages of development. During development, they clear apoptotic cells, an essential process that allows the developing embryo to proceed to the next stage of maturation. In the fully developed animal or human, macrophages play a key role in tissue remodeling and in first-line host defense, and they are an essential interface between innate and adaptive immunity. Macrophages secrete >100 biologically active metabolites, thereby instructing the homeostatic cascades of the clotting and complement systems as well as interfacing with vital functions of the organs in which they reside. Our focus is the origins of mononuclear phagocytes and their role in innate immunity.

ORIGIN AND DEVELOPMENT OF MONOCYTES AND MACROPHAGES

In the mammalian embryo, the monocytes and macrophages that are destined to make up the mononuclear phagocyte system appear first in the fetal yolk sac and then subsequently derive from fetal liver precursors. In the mammalian adult, monocytes are produced from precursors in the hematopoietic bone marrow. Circulating monocytes provide a cellular pool that can be mobilized to deliver large numbers of inflammatory cells on demand to sites of inflammation. This circulating pool also gives rise to the more stable, long-lived populations of resident tissue macrophages scattered throughout the body. This population of resident cells acts as a first line of host defense against invading pathogens.

The cells that make up the mononuclear phagocyte system are in a continual state of differentiation and adaptation. The extensive repertoire of cell-surface receptors expressed by these cells facilitates their interaction with the extracellular environment (other cells, pathogens, humoral factors, and substrates). Some of the receptors are coupled to the cytoskeleton in a way that enables these cells to internalize particles (i.e., >0.5 µm diameter) with great efficiency from the extracellular environment. These transcriptionally active cells are also endowed with a prodigious array of secretory products, ranging from signaling molecules (such as cytokines and chemokines) to effector molecules able to destroy invading pathogens (such as reactive species of oxygen and nitrogen).

In the adult, monocytes originate in the bone marrow. Monocytes and neutrophils are thought to share a common progenitor, known as the colony-forming unit, granulocyte-macrophage (CFU-GM) (1). Morphologically identifiable members of the monocytic lineage in the bone marrow include monoblasts and promonocytes, which in turn give rise to mature monocytes. The newly formed mature monocytes remain in the bone marrow for £24 hours before entering the peripheral blood, where they distribute between a circulating and a marginating pool (2,3). In normal human adult peripheral blood, monocytes account for ~1%–6% of the total leukocytes. The half-life of these cells in the circulation has been estimated at ~17 hours under resting conditions in the adult mouse (4) and £70 hours in normal human adults (5).

Monocytes are recruited from the peripheral blood under two broadly defined circumstances. The first is a poorly understood, low-level, constitutive process that gives rise to mature resident macrophages that populate most tissues. One old idea is that this process is a random one that signals the tissues to entice the passing cells to adhere and take up residence. It seems likely, at least in higher animals, that constitutive expression of chemokines in organs plays an instructive role in the baseline coaxing of monocytes from the circulation into the tissues. The egress of monocytes from the blood to the tissue is greatly amplified in the context of acute inflammation. This amplification response is clearly orchestrated by a number of chemokines that serve not only to regulate chemotaxis and transmigration, but also to modify cell function. This latter process is manifest by both an increase in bone marrow monocytopoiesis and by a decrease in the monocyte circulating half-life (6). The molecular signals mediating this process of recruitment are well known and are discussed further later.

The majority of resident tissue macrophages are thought to derive from circulating monocytes (7,8). A minority of these mature cells, however, may be produced by local division of precursor cells within the tissues themselves (9,10).

Resident Macrophage Populations

Mature tissue macrophages may be found embedded in the connective tissues and interstitia of many organs (often referred to as histiocytes in this setting). Additionally, they may be located in prominent subendothelial locations (e.g., hepatic sinusoidal Kupffer cells, adrenal sinusoidal macrophages), subepithelial locations (choroid plexus macrophages, salivary gland macrophages), or freely within tissue spaces (e.g., pulmonary alveolar macrophages, synovial macrophages, resident peritoneal macrophages) (reviewed in 11,12). Early histologists described many of these cells before their hematopoietic origins became known; and thus they carry unique names, including the microglia of the brain, the osteoclasts of bone, and the Kupffer cells of the liver.

The phenotypes of these resident cells vary greatly and are influenced by the microenvironment. The ability of macrophages to respond to the local milieu imparts a heterogeneity and plasticity. The versatility of macrophages to adapt and modify their phenotypes has sometimes led to the mistaken premise that there are subsets of macrophages that are predetermined in the bone marrow. Macrophages represent a single lineage of bone marrow–derived cells that are capable of undergoing extensive regional differentiation, and this capacity accounts for their marked heterogeneity.

The strategic subepithelial and subendothelial locations of resident macrophages suggest a critical role in first-line host defense. Resident macrophages are adorned with a wide array of cell-surface receptors that might be considered pattern-recognition receptors. Prototypic examples include the mannose receptor, which is able to recognize directly a broad range of carbohydrates that adorn infectious agents. Pathogen recognition may involve cooperation between receptors. Receptor ligation triggers release of a variety of antimicrobial molecules either extracellularly or into the nascent phagosome. The ability of these resident cells to limit the infection depends on the susceptibility of the microbe to this repertoire of antimicrobial products that compose the cells' armamentarium. For example, a certain gram-positive bacterium that is susceptible to lysozyme, a product of resident macrophages, would be killed, whereas other gram-positive bacteria or gram-negative organisms might not be killed. Resident macrophages therefore have a limited armamentarium and lack the full array of microbicidal capabilities characteristic of their inflammatory and activated counterparts (13,14).

However, the ability of resident macrophages to contain an infection is augmented by their ability to initiate vasodilatation and to increase blood vessel permeability. These processes facilitate the egress of serum factors and active inflammatory cells that act as rapidly deployed reinforcements in restricting the infectious nidus. Another example is a murine model of listeriosis, in which Lepay et al. (15) showed that resident Kupffer cells were incapable of generating reactive oxygen intermediates (ROIs), and that the total hepatic production of H_2O_2 could be accounted for by recently recruited monocytes. In another study of murine listeriosis, Rosen et al. (16) demonstrated that inhibition of myelomonocytic cell recruitment interfered with the ability of the mice to resist the infection. These data indicate that the resident macrophage populations lacked the ability to adequately contain serious infectious challenges, and that successful resistance to these infections was contingent on the host's ability to recruit an inflammatory cell population containing monocytes. This same population of Kupffer cells has been shown, however, to play a significant role in hepatic cytokine secretion and liver regeneration, indicative of a trophic rather than a microbicidal role. In a rat model, selective ablation of Kupffer

cells before partial hepatectomy resulted in impaired liver regeneration and was accompanied by reduced hepatic cytokine secretion (17).

Homeostatic functions have been ascribed to resident macrophage populations, but with a few notable exceptions, they remain poorly understood. Mature macrophages are found in the bone marrow where they interact with developing erythroid and myeloid precursors, forming a functional unit within the bone marrow (18,19 and 20). In this setting, the central mature macrophage contributes to a variety of hematopoietic functions (21), including erythroblast iron and ferritin metabolism (22), phagocytosis of extruded erythroid nuclei (23), phagocytosis of effete erythrocytes (24), and production of hematopoietic growth factors (25 and 26,27). In the central nervous system, the resident microglial population undergoes metabolic changes after physiologic stress (such as water deprivation) that are clearly different from those seen in response to infections (28), again suggesting a role in tissue homeostasis.

Cytokines, Colony-Stimulating Factors, and Monocyte Maturation

The colony-stimulating factors (CSFs) play critical roles in hematopoiesis by virtue of their ability to support the survival, proliferation, differentiation, and end-cell functions of myeloid cells (29,30 and 31) (see Chapter 17). Two CSFs thought to be critical to the monocyte/macrophage lineage are macrophage colony-stimulating factor (M-CSF) (also known as CSF-1), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Several studies, many of them using mice genetically deficient in one or more CSFs, have contributed greatly to our understanding of the physiologic contributions made by these CSFs.

The osteopetrotic (op/op) mouse is a naturally occurring mutant animal that has congenital osteopetrosis secondary to a severe osteoclast deficiency. Early studies of these mice established that they lack the ability to produce M-CSF (32). These mice were found to be severely lacking in a variety of macrophage populations: decreased CFU-GM, decreased circulating monocytes, and decreased serosal cavity and tissue macrophages. Resident macrophage populations of the liver, spleen, and thymus appeared to be less severely affected by this deficiency (33). The residual macrophages in the op/op mice were capable of normal phagocytic function *in vivo* but appeared to be less competent to release cytokines in response to inflammatory stimuli such as lipopolysaccharide (LPS) (34). Studies such as these have given rise to the concept of M-CSF–dependent and –independent populations of macrophages.

In an extension of these observations, Witmer-Pack et al. (35) used immunohistochemistry to identify subpopulations of macrophages and dendritic cells in the M-CSF–deficient mice. They found that in addition to osteoclasts, macrophage populations from the peritoneal cavity, splenic marginal zone, and subcapsular sinuses of lymph nodes were particularly affected by the absence of M-CSF. Macrophage populations in the thymic cortex, splenic red pulp, lymph node medulla, intestinal lamina propria, liver (Kupffer cells), lung (alveolar macrophages), and brain (microglia) were less affected and reached significant levels even in the absence of M-CSF. Cells of the dendritic cell lineage were not morphologically affected in the mutant mice. Additional studies have confirmed these observations and have further reinforced the concept of M-CSF–dependent and –independent macrophage subpopulations (36,37). Endogenous levels of GM-CSF, and interleukin (IL)-3 are elevated in the op/op mice and are thought to compensate partially for the M-CSF deficiency in the animals. The administration of exogenous GM-CSF to op/op mice corrected the macrophage deficiency, but not the osteoclast deficiencies in these animals, indicating an absolute requirement of osteoclasts for M-CSF (38).

Functions ascribed to GM-CSF include enhancement of antigen presentation (39), phagocytosis (40,41), microbicidal activity (42), and leukocyte adhesion (43).

The effects of GM-CSF on macrophage development and activity were demonstrated dramatically in a study in which transgenic mice were made to express the GM-CSF gene under the control of a retroviral promoter (44). Elevated levels of GM-CSF were detected in the serum, urine, peritoneal cavity, and eyes of these animals. Massive accumulations of macrophages in the eyes led to ocular opacity and retinal damage, and similar accumulations of macrophages were found in the peritoneal and pleural cavities, as well as in skeletal muscle. Additionally, these macrophages had an activated phenotype. A more refined analysis of the physiologic functioning of GM-CSF became possible with the advent of gene-targeting techniques in mouse embryonic stem cells.

In the first study of GM-CSF–deficient mice to be published, a critical and unexpected role for GM-CSF in pulmonary homeostasis was described (45). The mice deficient in GM-CSF developed a progressive accumulation of surfactant lipids and proteins within their alveolar spaces (similar to that in the human disease, alveolar proteinosis) but had no appreciable reduction in alveolar macrophage number. No abnormalities in steady-state hematopoiesis were detected. In a second study published by another group, the pulmonary phenotype and normal hematopoiesis were confirmed, and the additional finding of focal areas of subclinical lung infections in some animals was noted (46). Longer-term follow-up of these animals revealed a modest impairment in their reproductive capacity as well as a propensity to develop pulmonary and soft tissue infections (47).

More recent studies with these mice have further defined a role for GM-CSF in host defense. When infected with the intracellular pathogen *Listeria monocytogenes*, GM-CSF–deficient mice were less capable of containing the infection, had severely depleted levels of bone marrow hematopoietic cells, and demonstrated deficient peritoneal cavity inflammatory responses when compared with their wild-type counterparts (48). In an alternative model, LeVine et al. (49) studied a model in which GM-CSF–deficient mice were challenged with intratracheally inoculated group B streptococci. The clearance of these organisms from the lungs was reduced in mice deficient in GM-CSF compared with that in wild-type controls. This decreased clearance was not associated with a decrease in phagocytosis in the GM-CSF–deficient mice but was associated with a marked decrease in production of ROIs by alveolar macrophages. These studies suggest that GM-CSF may play a more significant role as an activator of monocyte-macrophage functions than as a mediator of monocyte-macrophage growth and differentiation.

Chemokines and Monocyte Development, Chemotaxis, and Recruitment

A diverse array of stimuli have been reported to induce the chemotaxis of monocytes and other leukocytes (see Chapter 16). These mediators include formylated bacterial peptides (50), cleaved complement components such as C5a (51), platelet-activating factor (PAF) (52), and leukotrienes (53). In addition, many cytokines and other biologically active molecules have been described to mediate chemotaxis of monocytes. These include fragments derived from extracellular matrix components such as elastin (54), fibronectin (55), and collagen (56); cytokines such as platelet-derived growth factor (PDGF) (57), GM-CSF (58), and transforming growth factor type b (TGF-b) (59); and collectins such as pulmonary surfactant protein A (60). The physiologic importance of the *N*-formylpeptide receptor (FPR) in host defense was defined by Gao et al. (61), who deleted the gene encoding this receptor to create FPR-null animals. These mice developed normally and did not develop infections when housed under specific pathogen-free conditions. Challenge of these mice with the intracellular pathogen *Listeria monocytogenes* resulted in increased mortality rates, however, and was associated with increased hepatic and splenic bacterial burdens (61).

The most recent attention has focused on the chemokines, a large family of peptides that control leukocyte migration (62,63) and that have been implicated in the development, homeostasis, and functioning of the immune system (64). Chemokines reported to act on monocytes and/or macrophages include the monocyte chemoattractant proteins (MCP) -1, -2, -3, -4, and -5; macrophage inflammatory proteins (MIP)-1a and -1b, I309, hemofiltrate CC chemokines (HCC) -1 and -4; fractalkine; growth-related oncogenes (GRO)-a, -b, and -g; IFN-inducible protein (IP)-10; leukotactin 1 (Lkn-1); lymphocyte and monocyte chemoattractant (LMC); macrophage-derived chemokine (MDC); monokine induced by IFN-g (MIG); myeloid progenitor inhibiting factor 1 (MPIF-1); regulated on activation normal T-cell expressed and secreted (RANTES); stromal cell-derived factor (SDF-1); and platelet factor 4 (PF4). The molecular diversity of chemokines and their promiscuity of receptor–ligand interactions poses challenges in clearly identifying the precise role for each of these molecules in macrophage/monocyte homeostasis, although clues do exist (see later).

MCP-1 is a CC chemokine that is capable of attracting monocytes, memory T cells, and natural killer (NK) cells (65). Studies of its activity in transgenic mice overexpressing the chemokine attest to its monocyte-recruitment capabilities (66,67 and 68). More recent investigations with MCP-1 knock-out mice have defined further the physiologic role of this chemokine. Lu et al. (69) demonstrated normal levels of circulating leukocytes and of resident macrophage populations in their studies of MCP-1–null mice. These knock-out mice demonstrated decreased recruitment of monocytes to the peritoneal cavities of animals injected locally with thioglycollate broth and showed a decreased accumulation of monocytes in experimentally induced delayed-type hypersensitivity reactions. These data indicate a unique role for MCP-1 in the inflammatory recruitment of monocytes, and suggest that this chemokine does not play an essential role in baseline monocytopoiesis or in the baseline recruitment of resident macrophage populations.

MIP-1a is another CC chemokine with monocyte chemoattractant activity (70). Studies using neutralizing anti-MIP-1a antiserum to block the activity of this chemokine *in vivo* in mice showed that it plays a critical role in the recruitment of monocytes to sites of wound injury (71). These studies also demonstrated that appropriate tissue repair (as assayed by local collagen production and angiogenic activity) after wound injury was critically dependent on MIP-1a–mediated monocyte recruitment. The role of MIP-1a in viral infections was analyzed in the first report describing the phenotype of the MIP-1a knock-out mouse (72). These mice, unable to make MIP-1a, demonstrated decreased levels of mononuclear cell infiltrates in the heart after infection by coxsackievirus and in the lung after infection by influenza virus. In a neutropenic mouse model of invasive pulmonary aspergillosis, MIP-1a was depleted by means of antibody before intratracheal instillation of *Aspergillus fumigatus* conidia (73). The mice in whom the chemokine had been depleted demonstrated a six-fold increased mortality rate, which was associated with a 12-fold increase in lung fungal burden. Taken together, these data point to a critical role in host defense for MIP-1a, and indicate that this chemokine mediates unique activities that cannot be compensated for by other chemokines.

MDC was identified by random sequencing of cDNA clones from human monocyte-derived macrophages (74). This CC chemokine was found to be highly expressed in macrophages and monocyte-derived dendritic cells but not in monocytes or cell lines. Studies with purified MDC revealed it to be chemotactic for monocyte-derived dendritic cells, and for NK cells, as well as for monocytes. The activity of MDC has recently been extensively investigated in a murine cecal ligation and puncture model (75). Exogenously administered MDC protected the mice from death, whereas blockade of endogenous MDC activity with neutralizing antibodies resulted in decreased

survival in this model. Furthermore, MDC induced respiratory burst activity and stimulated the release of lysosomal hydrolases from peritoneal macrophages exposed to this chemokine *in vitro*. These data indicate that chemokines can modulate monocyte/macrophage end-cell functional activities in addition to inducing cellular recruitment from the circulation.

PF4 is a CXC-chemokine (76) that is one of three chemokines known to be released by activated platelets. Whereas most CXC chemokines act predominantly on the polymorphonuclear cell lineage, PF4 acts on a wide range of cell types. In addition to its activity on mature cell lineages, PF4 also sustains the survival of hematopoietic progenitor cells (77). Recently, purified human PF4 was shown to prevent peripheral blood monocytes from undergoing spontaneous apoptosis, and it was also able to induce the differentiation of macrophages from monocytes (78). In these studies, the phenotype of PF4-induced macrophages differed from that of GM-CSF–induced macrophages in that major histocompatibility complex (MHC) class II molecules were not upregulated on the macrophage cell surface in the presence of PF4. These findings suggest a means by which platelets are able to modify the cellular phenotype of recently recruited inflammatory cells such as monocytes and provide an example of how macrophage phenotypes may be differentially regulated, depending on the cellular environment to which they are recruited.

Reverse Transmigration of Monocytes and the Formation of Dendritic Cells

After granulocytes are recruited to extravascular inflammatory sites, they undergo apoptosis and are cleared locally by macrophages by means of a mechanism that does not result in a proinflammatory response (79,80,81 and 82). The fate of recruited monocytes has been less well defined. Studies using adoptive transfer of macrophages have shown that these cells emigrate from sites of inflammation to draining lymphoid organs in a nonrandom fashion (83,84). In the *in vitro* studies with human umbilical vein endothelial cells cultured on amniotic tissue, the majority of monocytes that migrated transendothelially into the amniotic tissue subsequently emigrated back across the endothelium in a basal-to-apical direction (85). This process of reverse transmigration was mediated in part through the P-glycoprotein (MDR-1) transmembrane transporter (86), and by transmembrane tissue factor (87). In contrast, the initial (i.e., forward) transmigration of leukocytes is mediated by leukocyte integrins of the CD11/CD18 family (88,89) and by CD31 on both leukocytes and endothelial cells (90,91).

Transendothelial migration may be an important determinant in the differentiation of members of the mononuclear phagocyte system. Human monocytes can be differentiated into functional, immunostimulatory dendritic cells when cultured *in vitro* in the presence of IL-4 and GM-CSF (92,93). In an *in vitro* model of monocyte transendothelial migration, Randolph et al. (94) showed that monocytes cultured with endothelium (in the presence of serum, but without supplemental cytokines) differentiated into dendritic cells. Of the $CD14^+$ monocytes that migrated through the endothelium and into the underlying collagen matrix, those that remained in the subendothelial matrix differentiated into macrophages, whereas those that transmigrated out of the matrix developed into dendritic cells. The differentiation and transmigration of dendritic cells in this system were significantly enhanced if phagocytosable foreign material (latex beads or yeast cell walls) was present in the subendothelial matrix (94).

These studies were further extended to an *in vivo* model in which latex microspheres were injected into mouse skin sites (95). The monocytes contained in the resultant inflammatory infiltrate phagocytosed all of the locally injected particles, with a majority differentiating into mature macrophages. Approximately 25% of the monocytes that had phagocytosed the injected particles migrated out of the injection sites and localized in the T-cell areas of the draining lymph nodes, where they exhibited a phenotype characteristic of dendritic cells and not of macrophages (95). These authors have postulated that dendritic cells that derive from monocytes may play a unique role in the acquisition and presentation of particulate (as opposed to soluble) antigens, and that they therefore occupy a unique niche in host defense.

Taken together, these findings indicate that the fate of monocytes after their migration from the circulation is significantly more complex than previously believed, and that these cells retain a high degree of developmental plasticity even after they have left the circulation. Although many data have implicated cytokines in this differentiation process, the physical acts of phagocytosis and cellular transmigration are clearly also important factors, although their exact mechanism(s) of action remains to be determined.

Genes That Regulate the Differentiation of Monocytes and Macrophages

The development of monocytes and macrophages is determined by the expression of lineage-specific transcription factors that cooperate with more ubiquitously expressed factors. Some of the transcription factors that are thought to play a key role in myeloid cell development include PU.1, CCAAT displacement factors, AML-1B, Sp-1, GATA-1 and -2, and c-jun. Of these, the role of PU.1, which is a member of the Ets family of transcription factors, has been particularly informative, as it seems to form a focal point for the assembly of transcription factors that specify myeloid cell differentiation. PU.1 is expressed in a hematopoietic-specific fashion in B cells and myeloid cells, where it is required for the development of these lineages. Its DNA recognition sequence is purine rich with a core motif of 5'-GGAA/T-3'. The amino terminus serves as the activation domain. The molecule also contains a PEST [region rich in proline (P), glutamate, (E), serine, (S) and threonine,(T)], which is an instability sequence, and a carboxyl terminus that contains Ets homology winged helix-turn-helix motif that binds DNA. Numerous PU.1 target genes have been identified, including those encoding the M-CSF receptor (96), the GM-CSF receptor (97), class A scavenger receptors (98), the CD11b leukocyte integrin (99), the macrophage mannose receptor (100), FcgR1b (101), phagocyte oxidase (phox) subunits (102,103), c-*fes*(104), and serine proteases (105).

PU.1 appears to play an important role in macrophage differentiation. A proposed paradigm for the role of PU.1 as a bridging factor emerged from studies on CD11b, FcgR1b, and macrophage mannose receptor promoters. A consensus from this work indicates that PU.1 interacts with the ubiquitous transcription factor, Sp-1, and that this complex is able to interact with the basal transcription machinery. Of interest is that most macrophage-restricted genes lack a classic TATA box and that the preinitiation complex is specified by a so-called transcription initiator (Inr). The relative strength of the Inr and the absence of a TATA box, in conjunction with PU.1 and enhancers like the interferon (IFN)-g response elements, all appear to be key variables in directing macrophage-specific gene expression.

Gene-targeting experiments revealed that PU.1 is a critical factor for the expression of genes associated with terminal myeloid development and also affects other hematopoietic lineages. The first report characterizing PU.1–knock-out mice demonstrated that PU.1-null embryos died at a late gestational age (embryonic days 16.5–18), and that the mutant embryos were unable to generate progenitors for monocytes, granulocytes, and T and B lymphocytes (106). These embryos were also anemic because of impaired maturation of erythroblasts (in the face of normal erythroblast numbers), but demonstrated normal megakaryocytopoiesis. Other investigators independently generated PU.1–knock-out mice and reported the birth of homozygous null animals that then died of severe septicemia within 48 hours of birth (107). Analysis of these mice confirmed the absence of mature macrophages, neutrophils, and B and T lymphocytes, as in the earlier study. When these neonatal mutant mice were maintained with prophylactic antibiotics, they were able to develop T lymphocytes and neutrophil-like cells, but failed to develop B lymphocytes or macrophages.

These PU.1-null animals have now been used extensively in further studies of hematopoiesis and have helped to refine our understanding of the physiology of this important transcription factor. Tondravi et al. (108) demonstrated that the development of both macrophages and osteoclasts is defective in PU.1-null mice, and that the resultant defect in osteoclastogenesis gives rise to an osteopetrotic phenotype. These authors also showed that PU.1 mRNA accumulated in bone marrow–derived macrophages as they matured to osteoclasts *in vitro*. More recent studies have shown that PU.1 is also essential for the production of myeloid-derived, but not lymphoid-derived, dendritic cells (109).

Although PU.1-null mice were described to develop neutrophil-like cells, these cells were subsequently shown to be arrested in their differentiation and incapable of becoming functionally competent (110). This finding suggested that PU.1 was not required for neutrophil lineage commitment but was essential for neutrophil development, maturation, and end-cell function. Hematopoietic cells derived from PU.1-null mice failed to proliferate *in vitro* in response to M-, G-, or GM-CSF, and this finding correlated with minimal expression of the receptors for G- and GM-CSF, and absence of receptor for M-CSF (111). Subsequent transfection of cDNAs for the receptors for M-CSF or GM-CSF into PU.1-null cells allowed the cells to survive and grow in the relevant growth factor, but failed to rescue the cells sufficiently to give rise to mature macrophages (112). In an extension of these findings, Henkel et al. (113) reported that PU.1-null hematopoietic progenitors were able to commit to the monocytic lineage, but their differentiation was arrested at a stage before mature macrophage development (113). In a recently published study, DeKoter and Singh (114) demonstrated that low intracellular concentrations of PU.1 protein induced a B-lymphocyte fate on the hematopoietic progenitor cell, whereas high concentrations of PU.1 promoted macrophage development and blocked B-lymphocyte differentiation. These studies demonstrate how the graded expression of a transcription factor is able to specify hematopoietic cell fate in a mammalian model of differentiation.

Lichanska et al. (115) published a comprehensive analysis of macrophage differentiation in the mouse embryo, using a number of differentiation markers, including PU.1 and the M-CSF receptor (also known as c-*fms*). A striking finding was the presence of normal numbers of phagocytically active, c-*fms*–positive embryonic macrophages at embryonic day 11.5 in mice lacking PU.1. Furthermore, they showed that PU.1-null embryonic stem cells gave rise to macrophage-like cells when cultured *in vitro*. These data confirm that fetal macrophages (i.e., those derived from the yolk sac before the initiation of fetal liver hematopoiesis) are ontologically and functionally distinct from macrophages arising later in life. The authors of this study suggested that the PU.1-independent macrophages could represent the same population as the M-CSF–independent macrophages described in the op/op mouse.

The specification of myeloid cells clearly involves the interplay of a number of transcription factors that are not necessarily myeloid restricted in their expression but, when titrated with other factors, provide unique complexes that instruct stem cells to commit to the myeloid lineage. Superimposed on this commitment is the regulation of genes in response to external regulators like cytokines and chemokines that confer the functional heterogeneity that is the hallmark of macrophages.

EFFECTOR FUNCTIONS OF MONOCYTES AND MACROPHAGES

Macrophages secrete >100 biologically active substances. These substances include natural proteinases, lysosomal enzymes, all the components of the alternative pathway of complement, tissue factor, plasminogen activator, and plasminogen activator inhibitor. The secretion of this vast array of substances allows macrophages to respond to and influence their local environments. One such example is that macrophages are able to secrete the third complement component (C3), which is cleaved by an alternative pathway convertase that is also macrophage derived. This macrophage production of complement allows for local opsonization of particles, which may then be recognized by macrophage complement receptors (116).

The generation of ROIs is a specialized function of myeloid cells and a key component of their host defense armamentarium. For this reason, we focus on this aspect of macrophage secretion.

Reactive Oxygen Intermediates and Reactive Nitrogen Intermediates as Effector Molecules

Much of the ability of phagocytes to mediate antimicrobial and tumoricidal activity resides in their ability to generate large amounts of toxic molecular species such as ROIs and RNIs (reviewed in 117). Macrophages and neutrophils are the key phagocytic cells able to generate these intermediates at high enough levels to mediate their host defense functions.

REACTIVE OXYGEN INTERMEDIATES

ROIs result from the activity of phagocyte NADPH oxidase (phox), a molecular complex assembled at the plasma membrane after translocation of its constituent cytoplasmic components (118). This enzyme complex reduces molecular oxygen to the superoxide free radical by the addition of a single electron (119). The antimicrobial effects of ROIs were suggested by the observation that activation of macrophages to enhance their antimicrobial activity was accompanied by an increased macrophage capacity for respiratory burst activity (120).

The physiologic significance of this effector pathway was elucidated well before the advent of gene targeting in mice. In this case, naturally occurring human mutations in the genes encoding the subunits of the NADPH oxidase lead to the disease known as chronic granulomatous disease (CGD) (see Chapter 29). In CGD, the inability of phagocytes to kill ingested microorganisms causes recurrent infections with abscess formation in the liver, lungs, and lymph nodes of affected individuals. Histologically, these lesions contain characteristic granulomas. The most common human mutations (in two thirds of all patients with CGD) affect the X-linked $gp91^{phox}$ subunit (121). It is clear that some patients with CGD retain some oxidase activity and have a less severe clinical phenotype. Interestingly, as little as 5%–10% of normal respiratory burst capacity may be sufficient to generate a normal phenotype (122). Mouse models in which the genes encoding either the $gp47^{phox}$ (123) or $gp91^{phox}$ (124) subunits of the NADPH oxidase are deleted accurately recapitulate the human CGD phenotype, and these animals have been used to define further the roles played by NADPH oxidase in host defense (125,126 and 127).

REACTIVE NITROGEN INTERMEDIATES

Nitric oxide synthases (NOSs) catalyze the reaction of L-arginine plus molecular oxygen to form L-citrulline and the short-lived, but highly biologically active gas nitric oxide (NO). Three major isoforms of NOS have been identified. Two, NOS1 (also known as nNOS, or neuronal NOS) and NOS3 (also known as eNOS, or endothelial NOS), are constitutively expressed in the appropriate cell types and generate low levels of NO. The third isoform, NOS2 (also known as iNOS, inducible NOS, or independent NOS) is inactive under resting conditions and is induced in macrophages with the appropriate cytokine or microbial stimulation to produce large quantities of NO (reviewed in 128). The antimicrobial activity of NO has been most convincingly demonstrated by genetic deletion of NOS2 from mice (see later).

For some years, the inducible NOS pathway was thought to be restricted to the phagocytes of rodents and thought not to be present in human cells (see 129 for example). This view was reinforced by the ready detection of inducible NOS activity in rodent tissue and inflammatory macrophages and by the apparent lack of inducible NOS activity from monocytes prepared from the peripheral blood of healthy humans. In retrospect, it seems apparent that this situation arose in part from the lack of studies (for technical reasons) with monocytes derived from the blood of healthy mice or with tissue or inflammatory macrophages derived from humans. Such studies have since been conducted, and the existence of the inducible NOS pathway in human cells is no longer in question. For example, Nicholson et al. (130) demonstrated the presence of NOS2 activity in alveolar macrophages of humans with tuberculosis. The evidence for NOS2 activity in human cells is reviewed in (131).

Deletion of the murine gene encoding NOS2 to create NOS2–knock-out mice has been reported independently by several groups (132,133 and 134). In one of these mutant mouse strains, the intended NOS2 deletion did not occur, and an alternative NOS2 transcript was expressed (133). Reanalysis of these animals showed that functional NOS2, albeit at low levels, was indeed expressed (135), suggesting that some of the conclusions drawn from infectious models using these particular animals may need to be reevaluated. To date, no NOS2 mutations have been described in humans, but polymorphisms detected in the NOS2 promoter have been associated with susceptibility to more severe forms of malaria (136).

In NOS2-null mice, macrophage activation and ROI production were unimpaired; and both lymphocyte ontogeny and leukocyte migration were comparable to those of wild-type control animals. When these animals were used in a variety of infectious models by these and other authors (reviewed in 117,137), they showed decreased survival (frequently associated with increased microbial burdens) for a number of pathogens, including Ectromelia virus, *Chlamydia pneumoniae*, coxsackievirus, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Trypanosoma cruzi* [this list does not include experiments with the mutant mouse strain created by Wei et al. (133)]. Infection of mice with influenza A virus resulted in protection from pneumonitis in the mice lacking functional NOS2 activity (138), suggesting that NO production in response to infection is not always of ultimate benefit to the host. It should be noted that although NOS2-null mice are poorly equipped to deal with many exogenous infectious challenges, they have not (over several generations) developed spontaneous infections by endogenous bacterial flora (such as enteric gram-negative bacteria) (139).

The traditional concept of NO mediating antimicrobial and cytotoxic effects in the immune system continues to undergo reevaluation, and more activities are being attributed to this effector molecule (reviewed in 117). NOS2-derived NO affects the production of several cytokines, including IL-1, IL-6, IL-8, IL-10, IL-12, IFN-g, tumor necrosis factor (TNF)-a, and TGF-b (reviewed in 140). The differentiation of Th1 lymphocytes is also influenced by NOS2-derived NO (135). Diefenbach et al. (141) demonstrated the activity of NO as an immune signaling molecule. These authors showed that NO derived from NOS2 was required for IL-12 or IFN-a/-b–induced activation of NK cells and documented the NO dependence of IL-12 responsiveness *in vivo* (141). Thus NO may play an important role very early in the host response to pathogens (i.e., before the generation of specific immunity) and may thus be considered an effector of innate immunity. Other examples of nonmicrobicidal activities for NOS2-derived NO include liver regeneration (142), induction of apoptosis through inhibition of the proteosome (143), and induction of T-cell receptor (TCR)-mediated apoptosis in T lymphocytes (144). The ability of NO to inhibit caspase activity may protect macrophages from undergoing apoptosis and also prevents the release of soluble IL-1 and IL-18 from macrophages, thereby reducing the circulating levels of these cytokines in NOS2-null mice and leading in turn to decreased IFN-g levels (145).

REACTIVE NITROGEN INTERMEDIATE AND REACTIVE OXYGEN INTERMEDIATE DOUBLE KNOCK-OUT MICE

The availability of the knock-out mice mentioned earlier has facilitated breeding of strains to produce “double knock-outs”—mice lacking the means to generate both ROIs (i.e., $gp91^{phox}$ –/–) and RNI (i.e., NOS2 –/–) (146). The prevailing view before the publication of this study was that these two pathways were biochemically parallel and functionally nonredundant (i.e., single-gene knock-out mice lacking either one of the two pathways are markedly more susceptible to a variety of infections, in spite of the preservation of the other pathway). However, the double–knock-out mice proved to be significantly more susceptible to infection with endogenous microorganisms than were mice singly deficient in either of the pathways. Indeed, the roles of these two pathways are at least partially redundant, and preservation of one pathway in the absence of the other provides some host defense capability. The double–knock-out mice showed evidence of some macrophage-mediated killing, indicating that NOS2- and phox-independent microbicidal mechanisms are present, albeit at functionally low levels.

In a detailed analysis of peritoneal macrophages derived from $gp91^{phox}$ –/– and NOS2 –/– single- and double–knock-out mice, Vazquez-Torres et al. (147) demonstrated that these two effector pathways act sequentially, are functionally distinct, and are essential for limiting the growth of *Salmonella typhimurium in vitro.* NADPH oxidase activity peaked shortly after phagocytosis of the bacteria and was required for an initial, rapid antibacterial phase. In contrast, NOS2-dependent pathways provided a subsequent, more sustained bacteriostatic effect. In their model, IFN-g augmented macrophage antibacterial activity primarily via the NOS2-dependent pathway (147). In an extension of these studies, Mastroeni et al. (148) studied the course of *Salmonella* infection *in vivo* in the same knock-out mice. Their findings demonstrated that both the NADPH oxidase and the NOS2-dependent pathways were required for host resistance to infection by *Salmonella*, but that each pathway acted at different temporal stages of the infection. In the $gp91^{phox}$ –/– mice, increased bacterial burdens were present as early as the first day after infection, and all mice had died by the fifth day after infection. In contrast, the NOS2 –/– mice demonstrated increased bacterial burdens only after the first week of infection (148). The implication of these studies is that the NADPH oxidase acts early in an infectious challenge, helping to keep bacterial burdens low from the outset, whereas the NOS2-mediated pathway comes into force later, providing a more sustained antibacterial response.

Other Antimicrobial Effector Mechanisms of Monocytes and Macrophages

PHAGOSOMAL ACIDIFICATION

Phagocytosis of a number of microorganisms by macrophages leads to phagosomal acidification that coincides with killing of the internalized organisms. This mechanism has been demonstrated for pathogens as diverse as *Escherichia coli* (149), *T. gondii* (150), and *Candida albicans* (151). Several microorganisms have evolved mechanisms to evade host defenses by inhibiting phagosomal acidification. Examples include *Legionella pneumophila* (149), *T. gondii* (150), and *Mycobacterium avium-intracellulare* (152). *M. avium-intracellulare* acts to exclude vesicular proton–adenosine triphosphatase (ATPase) from the maturing phagosome (152); but in macrophages that have been immunologically activated, this evasion mechanism is overcome by the host, and phagosomal acidification and microbicidal activity are restored (153).

RESTRICTION OF IRON

Most microorganisms require iron for various essential functions and have evolved mechanisms to chelate iron actively from the extracellular environment (154). Host cells such as macrophages correspondingly actively control iron availability during infections in an attempt to deprive pathogens of an essential element (155). For example, immunologically activated human macrophages can limit the intracellular multiplication of *L. pneumophila* by limiting the availability of iron (156). At least two mechanisms for host-mediated iron restriction have been described: a downmodulation of transferrin receptors, restricting intracellular iron delivery (156), and destruction of ferritin by IFN-g–activated macrophages (157). Deprivation of iron by host phagocytes may be detrimental to host defense functions, as both phox and NOS2 activity are in part iron-dependent processes; and this situation may represent an important predisposition to infection in patients receiving long-term iron chelation therapy (158). A critical balance between host cell iron requirements and exclusion of iron from pathogens therefore must be maintained.

NATURAL RESISTANCE–ASSOCIATED MACROPHAGE PROTEIN 1, IRON, AND MACROPHAGE-MEDIATED RESISTANCE TO INFECTION

One of the best-characterized mouse host resistance loci is that which encodes the protein known as natural resistance–associated macrophage protein 1 (Nramp1) (reviewed in 159). Studies that led to the identification of this gene product were initiated >25 years ago, when inbred mice were shown to segregate into resistant and susceptible strains, based on their ability to contain an infectious challenge with *Salmonella typhimurium* (160). Subsequent studies reported similar susceptibility/resistance segregation for a number of additional pathogens including *Leishmania donovani* (161), *Mycobacterium bovis* BCG(162), and *Mycobacterium lepraemurium* (163). In each case, the resistant phenotype behaved as a dominant trait encoded by a single gene named independently *Bcg* (162), *Ity* (160), and *Lsh* (161). The ability of these mice to resist infection was expressed as the ability of macrophages to contain intracellular replication of the respective pathogens.

This genetic locus was ultimately cloned and sequenced in 1993 and was predicted to encode an integral membrane protein (now called Nramp1) with characteristics suggestive of an ion transporter/channel (164). This protein is macrophage-restricted (165), is localized to the membranes of phagosomes (166), and mediates resistance to infection in mice (167,168). Although the molecular mechanism of action of Nramp 1 remains to be fully defined, recently discovered additional Nramp proteins are providing clues. *Nramp2* is another mammalian *Nramp* gene that encodes a protein that shares 78% similarity with *Nramp1*, but is expressed by a number of different cell types (169). The Nramp2 protein is able to transport vectorially Fe^{2+}, Mn^{2+}, Zn^{2+}, and other divalent cations (excluding Ca^{2+} and Mg^{2+}). Studies suggest that Nramp2 acts as an iron-acquisition mechanism, both in the cell where it is able to transport iron from endosomes into the cytoplasm, and in the intestine, where it acts as a transferrin-independent iron-import system (170,171 and 172). A number of investigators have therefore postulated that Nramp1 may function in host defense by providing macrophages with the intracellular iron required for processes such as generation of ROIs and RNIs, while simultaneously depriving microbes within the phagosome of the iron that is essential for their growth and survival.

MACROPHAGES AND APOPTOSIS

The removal of apoptotic cells and microorganisms requires phagocytosis. Phagocytes express pattern-recognition receptors that recognize, on the one hand, pathogen-associated molecular patterns and, on the other, apoptotic cell-associated molecular patterns. The clearance of apoptotic cells is a major phagocytic, noninflammatory function of macrophages (173,174). Thus we have postulated that the primary role for primordial phagocytic receptors is the removal of apoptotic cells during development and that the recognition of microorganisms is a later adaptive function. Findings with model organisms like *Caenorhabditis elegans* and *Drosophila melanogaster* have indicated that some of the recognition pathways are conserved between invertebrates and vertebrates. A good example is the remarkable conservation of the phosphatidylserine receptor between mammals, worms, and flies. Ligating this receptor by apoptotic cells results in an antiinflammatory rather than a proinflammatory response such as that more often associated with macrophage phagocytosis of microorganisms. Some of macrophage pattern-recognition receptors implicated in this process *in vitro* include the following: CD14 (81), complement receptors (175), class A scavenger receptors (176), class B scavenger receptors such as SR-BI (177), CD36 (178), and croquemort (a CD36-like molecule identified in *Drosophila* macrophages) (179,180), vitronectin receptor (79), phosphatidylserine receptor (82,181), and the ATP-binding cassette transporter (182). Evidence for the phagocytic function of these receptors in the clearance of apoptotic cells *in vivo* comes from studies on the *ced* genes in *C. elegans* and of croquemort in *Drosophila*. In mammals, the redundancy of the system has been illustrated by an analysis of mice genetically lacking class A scavenger receptors (SR-A) (183). These mice failed to demonstrate any failure to clear apoptotic cells *in vivo* (184), in spite of convincing evidence for the ability of class A scavenger receptors to mediate clearance of apoptotic cells *in vitro* (176).

The close proximity of macrophages to cells undergoing apoptosis during the tissue remodeling that occurs during embryogenesis has led to speculation that macrophages may be involved in inducing apoptosis in bystander cells, and not simply phagocytosing those cells that have already undergone apoptosis (185). Experimental evidence for this process in mammals is confined to a pupillary membrane model in the developing rodent eye, which fails to undergo apoptosis when local macrophages are eliminated by means of a locally expressed toxic transgene (186) or by local injection of toxic liposomes (187). Recent evidence from this research group indicates that the ability of macrophages to induce apoptosis is in part dependent on the cell-cycle phase of the target cell (188). These authors have suggested that macrophages may induce apoptosis by subverting the external signals that normally promote cell-cycle progression. Although the evidence for macrophages playing a role in the induction of apoptosis in this model system is clear, this mechanism has not been validated in other situations of physiologic apoptosis, and caution should be applied when generalizing this mechanism to other situations in the body.

Macrophages undergo apoptosis after infection by a range of bacterial pathogens *in vitro* (reviewed in 189). Whether this may be classified as a host defense mechanism remains to be determined. This mechanism could be viewed as depriving the pathogen of an intracellular niche from which it would otherwise replicate secluded away from the rest of the immune system. Additionally, this mechanism could facilitate antigen processing by the host, and assist in the generation of an enhanced specific immune response against the pathogen. Alternatively, this apoptotic process could be viewed as a pathogen-induced mechanism to kill the host cells that would otherwise act to destroy the pathogen. The demonstration of pathogen-expressed antiapoptotic mechanisms (e.g., 190) supports the view that host cell apoptosis after infection is primarily a host defense mechanism.

MONOCYTE AND MACROPHAGE ACTIVATION AND DEACTIVATION

The modern concept of macrophage activation as an acquired cellular resistance to intracellular parasites was expounded in the 1960s by Mackaness (191), who demonstrated a requirement for sensitized lymphocytes (later shown to be T cells) in this process. Subsequent studies showed that activating macrophages in this fashion (i.e., to enable killing of pathogens) also activated the ability of the cell to kill tumor cells (192). These concepts evolved into a step-wise model of activation in which certain products from T-cell supernatants could confer an inflammatory and an activated phenotype on homogeneous populations of resident macrophages (193,194). This work paved the way for the characterization of the responsible cytokines, most notably macrophage-activating factor, later identified as IFN-g (195,196). The activated state has been characterized by enhanced phagocytosis, processing and presentation of extracellular antigens, and an increased ability to lyse intracellular parasites and to destroy tumor cells (197). Many of these concepts still hold true today, although much more is now known about the molecules that mediate these events.

Interferon-g

Many studies with gene knock-out mice and some with naturally occurring human mutations have contributed to our understanding of the mechanisms of macrophage activation. IFN-g (originally described as macrophage-activating factor) is the major responsible cytokine. IFN-g is a dimeric glycoprotein that is produced in immune responses predominantly by activated T lymphocytes and NK cells, although macrophages also make this cytokine in an autocrine fashion (198). The cell-surface receptor complex for IFN-g is composed of two chains, a and b. Although the b chain does not bind the ligand directly, it is required for IFN-g–mediated signaling as well as for formation of the receptor complex (199).

Disruption of the gene encoding IFN-g in mice did not interfere with their development and did not give rise to spontaneous infections when the mice were housed in the absence of pathogens (200). These mice, however, were deficient in their production of macrophage antimicrobial products, had reduced expression of MHC class II antigens, and succumbed to ordinarily sublethal doses of *Mycobacterium bovis* BCG. In particular, macrophages isolated from these mice after infection with BCG produced very little NO in response to LPS stimulation, but were able to produce some ROIs, albeit at low levels when compared with wild-type littermates. These mice

are unable to contain infection with *Mycobacterium tuberculosis*, in association with a failure of their macrophages to produce RNIs (201,202). Similar studies with these mice have indicated that IFN-g is essential for the resolution of enteric *Salmonella* infections (203). A similar overall phenotype was observed when the receptor for IFN-g (IFN-g-Ra, or -R1) was knocked out in mice, this mouse strain demonstrated susceptibility to infection with both *L. monocytogenes* and vaccinia virus (204). Likewise, knock-out of the IFN-g-Rb (or -R2) gene resulted in severe immune defects and susceptibility to infection by *L. monocytogenes* (205). Taken together, these results indicate that IFN-g is not essential for embryogenesis or for development of the immune system, but that a variety of immune cell functions are significantly impaired when this cytokine is absent, rendering affected animals very susceptible to certain infections. Furthermore, these results suggest that priming for generation of RNIs (i.e., via NOS2 activity) is more dependent on IFN-g than is priming for ROI production.

After the description of the knock-out mice discussed earlier, human mutations in the IFN-g receptor axis began to be delineated. The initial clinical description was of six children who developed disseminated infections with atypical mycobacteria, three of whom died of the infection (206). Laboratory analysis showed that their production of IFN-g and TNF-a was defective. The molecular defect responsible for this phenotype was identified by means of a genetic screen involving four of the affected children, their unaffected family members, and healthy control individuals (207). All four affected children had mutations in the gene encoding IFN-g-R that resulted in an absence of receptors from the cell surface; this absence was associated with a functional defect in macrophage activation *in vitro*. At the same time as this report, another group reported a similar mutation in the IFN-g-receptor gene in a child who had died at age 10 months from idiopathic disseminated *M. bovis* BCG infection (208). The demonstration that affected humans and knock-out mice have otherwise preserved Th1-type immune responses (with normal delayed-type hypersensitivity responses in the humans) suggests strongly that this locus mediates susceptibility to infection largely by its effect on macrophage activation. Furthermore, in a model of experimental influenza virus infection with the IFN-g knock-out mice, IFN-g was not required for the development of effective humoral or cellular immune responses (209).

Since the initial reports of human mutations in the IFN-g-receptor gene, several novel mutations with variable phenotypes have been uncovered. Jouanguy et al. (210) reported a mutation that gave rise to a partial receptor deficiency in a child with disseminated BCG infection, whose younger sister had pulmonary tuberculosis after having had BCG vaccination withheld in infancy. Cells from these patients responded functionally to IFN-g *in vitro*, but only if high concentrations of the cytokine were used. Dorman and Holland (211) described a patient with disseminated atypical mycobacterial infection who was shown to have a truncating mutation in the gene encoding the IFN-g-receptor signaling chain (IFN-g-Rb or -R2). Most recently, Jouanguy et al. (212) described four cases of novel mutations in the IFN-g-receptor ligand-binding chain (IFN-g-Ra, or -R1) in which normal numbers of receptors could be detected at the cell surface by antibodies. Radiolabeled IFN-g failed to bind to these receptors, and cells expressing these receptors were unable to respond functionally to exogenous ligand. An important implication of this study is that demonstration of immunoreactive IFN-g-receptors at the cell surface does not preclude the diagnosis of complete functional receptor deficiency.

Interleukin 12

IL-12 is a heterodimeric cytokine composed of 35-kd and 40-kd subunits known as p35 and p40, respectively. It is produced by a variety of cells (predominantly macrophages and dendritic cells) and is thought to mediate resistance to intracellular organisms by virtue of its ability to induce IFN-g production by NK and T cells, as well as by promoting the generation of specific Th1 immune responses (213,214 and 215). Production of this key cytokine by mononuclear phagocytes is thought to be an early event in the generation of an immune response, and IL-12 is therefore regarded as a central mediator of innate immune responses. Because IL-12 acts upstream of IFN-g and is a major inducer of IFN-g, it might be predicted that deficiency of IL-12 would give rise to a phenotype similar to that seen for IFN-g-deficiency. Knock-out mice in which the genes encoding either the p35 subunit (216) or the p40 subunit (217) of IL-12 develop normally are fertile and have no obvious abnormalities. These IL-12–deficient mice were, however, unable to contain infections with *Leishmania major* and were unable to mount *Leishmania*-specific delayed-type hypersensitivity responses. When lymph node cells derived from these mice were stimulated with *Leishmania* antigen *in vitro*, decreased levels of IFN-g and increased levels of IL-4 were produced. IL-12 was therefore confirmed to be essential for the production of IFN-g in response to infections and for the facilitation of Th1-type immune responses. In an alternative model of IL-12 deficiency, mice were treated with a neutralizing anti–IL-12 monoclonal antibody and then infected with *S. typhimurium* (218). As expected, these mice were unable to contain the infection and were found to have impaired upregulation of MHC class II molecules, decreased NOS2 activity, decreased IFN-g production, impaired granuloma formation, and upregulated IL-10 activity. Administration of recombinant IFN-g to these mice reversed these defects, confirming that IL-12 indeed acts upstream of IFN-g in the immune response (218). Mice generated with a deficiency in the b1 chain of the IL-12 receptor yielded a similar immunologic phenotype; their ability to produce IFN-g in response to a variety of stimuli was significantly impaired (219).

In 1998, Altare et al. (220) reported the finding of human IL-12 receptor deficiency in four children with disseminated mycobacterial infections (either BCG or *Mycobacterium avium*), two of whom had also had non-typhi *Salmonella* infections (220). Mutations in IFN-g, its receptor, and in IL-12 itself were all excluded during the evaluation of these individuals. Mutations in the b1 chain of the IL-12 receptor resulting in IL-12 receptor deficiency were found in these patients, with resultant IFN-g deficiency. At the same time as this report, three other unrelated individuals (aged 19, 22, and 26 years) with severe mycobacterial and *Salmonella* infections were found to lack expression of the IL-12 receptor b1 chain, with resultant lack of IL-12–mediated signaling and IFN-g production (221). In another individual with disseminated *M. bovis* BCG and *Salmonella enteritidis* infections, a large homozygous deletion was found in the gene encoding the IL-12 p40 subunit, with resultant absence of functional IL-12 activity (222). Taken together, the results of these investigations involving both humans and mice demonstrate that IL-12 is a critical mediator of resistance to infection by both mycobacteria and *Salmonella*, that IFN-g is a key downstream mediator of IL-12 function, and that impaired macrophage function is the major determinant of susceptibility to infection by these organisms (223).

Interleukin 18

IL-18 was originally identified as an IL-12–independent IFN-g–inducing cytokine expressed by a range of cells including macrophages (224). Although it shares considerable biologic activity with IL-12, IL-18 is structurally unrelated and has additional biologic functions (225). Whereas IL-18 alone is not able to induce IFN-g from naive T cells (because of their lack of IL-18 receptors), IL-18 and IL-12 act synergistically in IFN-g induction (226). This finding has been confirmed with IL-12– and IL-12–receptor-deficient mice, which have minimal ability to produce IFN-g in the face of normal IL-18 production and activity. IL-18 does, however, appear to play a role in host defense mediated by macrophages. The administration of recombinant IL-18 to mice infected with highly virulent *Cryptococcus neoformans* resulted in enhanced host defense against this pathogen, in a manner that was reversible by administration of IFN-g neutralizing antibodies (227).

Mice deficient in IL-18 have been generated and have confirmed a role for IL-18 in IFN-g production, NK cell activity, and the induction of Th1-type responses (228). The defects in these IL-18–deficient animals were not so severe as those in their IL-12–deficient counterparts; however, mice deficient in both cytokines demonstrated a more severe phenotype than that in either single–knock-out animal (228). When challenged experimentally with mycobacteria, these IL-18–deficient mice developed higher mycobacterial burdens than did their wild-type counterparts, but did not progress to acutely disseminated disease (229). Although their IFN-g levels were reduced when compared with those of wild-type mice, their IL-12 levels were similar, confirming an independent function for IL-18 in the induction of IFN-g and demonstrating that IL-12 does not appear to compensate for the lack of IL-18 in these animals.

Tumor Necrosis Factor a

TNF-a is a proinflammatory and immunomodulatory cytokine produced predominantly, and in large quantities, by activated macrophages (reviewed in 230,231). This cytokine is also the chief mediator of septic shock induced by bacterial LPS or superantigens. In contrast to the cytokines mentioned earlier, TNF-a is an effector molecule produced by activated macrophages, rather than an activator of macrophages. The ability of macrophages to release TNF-a depends in part on the differentiation state of the macrophage (recruited cells being more competent than resident macrophage populations) and in part on the nature of the signals inducing the activating response (232).

Recently there has been an explosion of interest in the signal-transduction pathways that couple pathogen recognition to host-cell responses (particularly focusing on production of TNF by macrophages) (233,234). The mammalian Toll-like receptors (named for their homology to a receptor first described in *Drosophila*) are the critical cell-surface molecules involved in the initial recognition of pathogens, and they initiate the subsequent signaling cascade that leads ultimately to activation of the transcription factor nuclear factor-kB (NF-kB) (235,236 and 237). Although many of the details of the recognition events mediated by the Toll-like receptors remain to be elucidated, it appears that they are able to confer some degree of pathogen-specific recognition (238), which may direct the subsequent innate and adaptive immune responses in the appropriate directions.

TNF-b (also known as lymphotoxin b, or LT) is structurally related to TNF-a but is produced primarily by lymphocytes rather than by monocytes or macrophages. A mouse knock-out model in which the TNF-b gene was disrupted (with preservation of that encoding TNF-a) demonstrated a critical role for TNF-b in lymphoid organogenesis rather than in host defense (239), and this cytokine is not discussed further here.

The first knock-out mice involving TNF-a had deletions of the receptor rather than of the cytokine itself. The biologic effects of TNF are mediated by two receptor chains, a 55-kd chain (TNFRp55 or TNFR1) and a 75-kd chain (TNFRp75 or TNFR2). Pfeffer et al. (240) targeted the gene encoding the p55 chain to generate mice that lacked detectable p55 protein but expressed normal levels of p75. These TNFRp55-null animals developed normally, but their cells lost the capacity to respond to TNF *in vitro*. In addition, these mice were resistant to the toxic effects of LPS and bacterial superantigen but were severely compromised in their ability to deal with experimental *L. monocytogenes* infection. Rothe et al. (241) generated independently a mouse strain lacking TNFRp55 and found essentially the same defect in host defense as that reported by Pfeffer et al. In addition, this group reported that although the mutant mice were resistant to the effects of low doses of LPS after

D-galactosamine sensitization, they remained sensitive to high doses of LPS.

Mice lacking the TNFRp75 were subsequently generated and shown to exhibit wild-type sensitivity to low-dose LPS challenge after D-galactosamine sensitization, but were relatively resistant to the effects of high doses of TNF and to local cytotoxicity mediated by TNF (242). The ability of these mice to clear *L. monocytogenes* was slightly impaired relative to that of wild-type mice, but was not so severely affected as the ability of the mice lacking TNFRp55. Subsequent studies with these mice have assessed their ability to handle infection with *C. albicans* (243). Mice lacking TNFRp55 were highly impaired in their ability to clear this infection and died readily of it. Mice lacking TNFRp75 were also deficient in their ability to clear the yeast from their tissues but did not have increased mortality rates.

TNF-a knock-out mice (i.e., those unable to produce the cytokine) were fertile, viable, and without obvious phenotypic abnormality, but they were particularly susceptible to infection with *L. monocytogenes*, confirming a role for TNF-a in antibacterial host defense (244). Additional immunologic features of these mice included a relative resistance to LPS-mediated septic shock, reduced delayed-type hypersensitivity responses, and disordered development and architecture of splenic follicles.

Although there is ample evidence to suggest that TNF is a prime effector in septic shock in humans, the use of TNF antagonists (monoclonal antibodies or soluble receptors) in this clinical setting has not been shown to be of benefit (245). These reagents are, however, finding a therapeutic niche in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (246). Reports of an increased incidence of bacterial infections in some patients receiving these agents concern us (247) but are not surprising in view of the role that TNF plays in the host antimicrobial response as demonstrated in mice.

Macrophage Deactivation

Just as the host must be able to activate cellular defense mechanisms rapidly in the face of a pathogenic challenge, so must the host be able to limit the physical extent and temporal duration of the activation; otherwise, unwanted inflammatory consequences may arise. Similarly, microbes and tumor cells have evolved mechanisms for inhibiting the activation of cellular defense mechanisms, this time as a means of ensuring their survival.

The transforming growth factors (TGF-b1 and TGF-b2) were among the first macrophage-deactivating factors described (248). Subsequently, IL-10 was found to potently suppress the ability of mouse macrophages to release TNF-a, to produce oxidase-generated ROIs, and to weakly inhibit the NOS2-mediated generation of RNIs (249,250). This cytokine, a product of Th2 lymphocytes, monocytes, and macrophages (among other cells), downregulates a number of monocyte and macrophage activation-dependent functions (reviewed in 251). In a review of the information gained from studies with IL-10 knock-out mice, Rennick et al. (252) concluded that IL-10 acts to protect the host from the harmful side effects of an “overly zealous” immune/inflammatory response. A not unexpected consequence of this activity is that the presence of excess IL-10 is associated with an increased susceptibility to a number of infectious organisms, including *C. albicans* (253), *Klebsiella pneumoniae* (254), *M. avium* (255), and *S. pneumoniae* (256).

Intracellular pathogens such as *M. avium* have evolved mechanisms that both interfere with routine cellular activities and promote the functional deactivation of their macrophage hosts. Macrophages infected with *M. avium* increased their production of TGF-b, which reduced the microbicidal activity of the cells after stimulation with IFN-g (257). Likewise, infection of macrophages with *M. avium* resulted in a downregulation of cellular receptors for IFN-g, which in turn led to decreased expression of IFN-g–inducible genes and a decreased ability of the host cells to kill their invading pathogens (258). Similarly, macrophages infected with *M. avium* produce increased amounts of IL-10, which in turn inhibits the production of TNF-a by macrophages, and also induces the release of soluble TNF-a receptors by macrophages, leading to inactivation of the already reduced amounts of TNF-a that are produced (259).

Recent studies have implicated the peroxisome proliferator-activated receptor g (PPARg) as an important regulator of macrophage function, particularly in macrophage deactivation (reviewed in 260). These receptors are ligand-dependent transcription factors of the nuclear hormone receptor superfamily and have well-documented roles in the regulation of adipocyte differentiation and in glucose homeostasis. These receptors are known to be expressed at high levels in macrophages (261), are induced in monocytes exposed to oxidized low-density lipoprotein (oxLDL), and are expressed at high levels in the macrophage-derived foam cells present in atherosclerotic lesions (262,263). These observations have been followed by studies suggesting that PPARg agonists mediate macrophage deactivation. Ricote et al. (264) reported that PPARg activity was markedly upregulated on activation of monocyte-derived macrophages *in vitro* and that PPARg agonists inhibited macrophage expression of NOS2, gelatinase B, and class A scavenger receptors. This group also reported that activation of PPARg resulted in antagonism of the transcription factors AP-1, STAT, and NF-kB (which are all implicated in the transcription of proinflammatory genes). At the same time, Jiang et al. (265) reported that PPARg agonists inhibited the production of proinflammatory cytokines by monocyte-derived macrophages activated *in vitro*. A more recent study with novel PPARg agonists failed to show any inhibition of proinflammatory cytokine production by human monocytes, human monocyte-derived macrophages, or murine macrophage-like cell lines, calling into question the “deactivating” capabilities of PPARg-mediated stimulation (266). Further studies are needed to delineate the true physiologic functioning of this receptor in monocyte and macrophage biology.

Interleukins-4 and -13: Alternative Activation States of Macrophages

Although macrophage activation and deactivation have traditionally been conceived as relating to the acquisition and loss of macrophage ability to kill tumor cells or microorganisms, alternative states of macrophage “activation” are possible. IL-4, which is produced predominantly by Th2-type lymphocytes, inhibits the production of proinflammatory cytokines and ROIs by macrophages (i.e., “deactivation”), while simultaneously upregulating MHC class II expression and upregulating the expression and function of the macrophage mannose receptor (i.e., “activation”) (267). When mouse macrophages were treated with IL-13, the release of NOS2-mediated RNI and proinflammatory cytokines was suppressed, whereas MHC class II and macrophage mannose receptors were significantly upregulated (268). These authors reported a preservation of oxidase-mediated ROI production in macrophages treated with IL-13. Whereas IFN-g decreases fluid-phase pinocytosis and receptor-mediated endocytosis by macrophages, IL-4 and IL-13 enhance both of these processes in actin-dependent and -independent fashions (269). IL-4 and IFN-g both independently and in combination act to upregulate mannose receptor–mediated phagocytosis by macrophages, even though these cytokines have divergent activities on mannose-receptor expression levels (270).

Data such as these have suggested that individual macrophage activities may be selectively upregulated (i.e., activated) in a fashion distinct from that mediated classically by IFN-g. Additionally, cytokines such as TGF-b, usually thought of as promoting macrophage deactivation, do have monocyte/macrophage activating ability that is in part mediated by the physiologic state of the target cell (271). It may therefore be more appropriate to refer to the specific phenotypic characteristics or activities of a given monocyte or macrophage, than to use the blanket terms “activated” or “deactivated.”

CONCLUSIONS

The mononuclear phagocyte system is part of an essential framework of tissue homeostasis. The versatility and plasticity of monocytes and macrophages reveal that the role of these cells extends well beyond gatekeepers that guard the portals by which pathogens may enter. The idea that model systems like *Drosophila* may be a fertile ground to dissect out necessary genes that may be involved in fundamental myeloid cell processes like phagocytosis and the application of gene-array technology to delve further into the interplay between pathogen and macrophage are examples of the work that may lie ahead.

Chapter References

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22 EOSINOPHILS

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Eosinophils are a distinct class of bone marrow–derived granulocytes that normally are present in low numbers in the blood. After leaving the circulation, eosinophils preferentially localize in tissues with mucosal epithelial surfaces. Increased numbers of eosinophils in the blood or tissue eosinophilia typically accompany allergic diseases and helminth parasite infections and may occur with various other, often idiopathic, disorders (see Chapter 35). Because eosinophils are terminally differentiated after leaving the marrow, the more acute, “effector” functions of these end-stage granulocytes have historically been the focus of studies of eosinophil function, including the means by which eosinophils participate in the immunopathogenesis of allergic diseases and the activities of eosinophils as helminthotoxic cells. In addition to these conventional effector responses, pertinent to diseases commonly marked by eosinophilia, other findings indicate that eosinophils have functional roles in host responses not conventionally associated with eosinophilia, such as wound healing (1,2), fibrosis (3), and graft rejection (4). Eosinophils express receptors for a multitude of cytokines and other immunologic mediators and contain in their cytoplasmic granules preformed stores of diverse cytokines. Eosinophils can engage in cognate cell–cell interactions with other cell types, including lymphocytes (5), and may function as class II major histocompatibility complex (MHC)-dependent antigen-presenting cells to elicit responses of $CD4^+$ T cells (6). Eosinophils, as recently reviewed (7,8,9 and 10), therefore, are more than end-stage effector cells and can participate in host immune and inflammatory responses not necessarily distinguished by quantitatively extensive eosinophil infiltration.

DEVELOPMENT AND MATURATION OF EOSINOPHILS

Eosinophils arise in the bone marrow from interleukin (IL)-5–responsive $CD34^+$ progenitor cells. Allergen challenge of sensitive asthmatic patients elicits the expression of the IL-5 receptor a chain on human marrow $CD34^+$ cells (11). The signal for this IL-5–receptor expression may well be IL-5 itself, because it induces a change in the expression of IL-5 receptors on progenitor cells from a soluble, antagonistic isoform to a functional membrane-associated isoform (12). In addition to IL-5, two other cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3, also can stimulate eosinophilopoiesis. In contrast to IL-3 and GM-CSF, which principally promote the development of other lineages, IL-5 in humans uniquely promotes the early development and terminal differentiation of eosinophils (and basophils). [In mice, IL-5 also regulates B-1 cell development (13)]. Although IL-5 may be produced by $CD8^+$ T cells, natural T cells, natural killer (NK) cells, mast cells, eosinophils themselves, and bone marrow endothelial cells, IL-5 is a prototypical cytokine elaborated by Th2 $CD4^+$ T cells. IL-5 production by Th2 lymphocytes accounts for the lymphocyte dependence of eosinophil production presciently demonstrated by Baston and Beeson (14) in experimental animals and for the eosinophilia accompanying Th2 T-cell–mediated immune responses characteristic of allergic diseases and helminth infections.

Receptors for GM-CSF, IL-3, and IL-5 are composed of distinct cytokine-specific, low-affinity a chains, which combine with a common b chain to constitute high-affinity ab receptors for each of these cytokines (15). Mice with genetic deletions of IL-5, the IL-5 receptor a chain, or the common receptor b chain fail to develop augmented eosinophilia in response to allergic or helminthic challenges (13,16,17 and 18). Nevertheless, each of these three genetic knock-out mice still exhibits basal eosinophilia, indicating that other cytokines or mediators also contribute to normal eosinophilopoiesis.

Eosinophil maturation within the marrow develops over about a week to generate a pool of mature, yet marrow-retained, eosinophils. IL-5 alone and in concert with the chemokine eotaxin can release this pool of already developed eosinophils from the marrow into the circulation to immediately increase blood eosinophilia and facilitate eosinophil recruitment to sites of specific inflammation (19,20). This process that releases intramedullary eosinophils involves the migration of eosinophils from marrow sinusoids into the sinus lumen. During this transmigration, eosinophils shed L-selectin and increase b_2-integrin expression. Eosinophil a_4 integrins promote retention of eosinophils within medullary sinusoids, whereas b_2 integrins facilitate eosinophil release from the marrow (19). Eotaxin also promotes the release from the marrow of eosinophil progenitor cells (20). Sites of allergic inflammation, such as the bronchial mucosa, have been shown to contain $CD34^+$ eosinophil progenitor cells (21). Whether these cells arise from the marrow or from circulating progenitor pools is unknown (22).

TISSUE DISTRIBUTION OF EOSINOPHILS

Eosinophils released into the blood circulate with a half-life of ~8–18 hours. Eosinophils normally leave the circulation to localize in tissues, especially those with mucosal interfaces with the external environment, notably the respiratory, gastrointestinal, and lower genitourinary tracts. Although the mechanisms governing this normal localization of eosinophils to mucosal tissues are not fully known, the chemokine eotaxin is involved in the homing of eosinophils to the gastrointestinal, but not respiratory, tract (23). Eosinophils are principally tissue-dwelling cells, and for every eosinophil present in the circulation, there are estimated to be 300 to 500 in the tissues (24). Eosinophils live longer than neutrophils and persist in tissues for at least several days, if not several weeks. The eosinophilopoietic cytokines, GM-CSF, IL-3, and IL-5, also act on mature eosinophils to prolong their longevity and prevent their apoptosis (25).

Eosinophil Adherence Mechanisms

In addition to the normal homing of eosinophils to mucosal tissues, in specific forms of inflammation, eosinophils accumulate in tissue sites in relatively greater abundance than do neutrophils. Contributing to this eosinophil localization are multiple adherence molecules expressed on eosinophils that help regulate their egress from the marrow, their circulation through the bloodstream, and their subsequent entry into tissues. As for other leukocytes, the recruitment of eosinophils into tissue sites of inflammation uses the combinatorial interactions involving specific adhesion molecules (via their expression and altered affinity states) that mediate cellular interactions with the vascular endothelium and the actions of chemoattractant molecules (26,27). Eosinophils express several adhesion molecules broadly shared with other leukocytes that mediate their initial rolling and subsequent adherence to endothelial cells. Similar to neutrophils, eosinophils can adhere via CD11/CD18 heterodimeric b_2 integrins to intercellular adhesion molecules (ICAM)-1 and -2. Likewise, specific sialoglycoproteins mediate adherence between eosinophils and endothelial E-selectin and P-selectin. Eosinophils express less of the E-selectin ligand, sialyl-Lewis X, than do neutrophils, perhaps explaining the weaker binding of eosinophils to E-selectin (27). Eosinophils express the P-selectin glycoprotein ligand-1 (PSGL-1) (28) and exhibit greater binding to P-selectin than do neutrophils, especially at lower P-selectin densities and under shear-flow conditions (29,30 and 31). Unlike neutrophils, but similar to lymphocytes, eosinophils are able to bind to vascular cell adhesion molecule (VCAM)-1. Eosinophils express two a_4 integrins, very late activation antigen (VLA)-4 (a_4b_1) and a_4b_7, that bind to VCAM-1 (32). Moreover, a_4b_7 can bind to the mucosal addressin, mucosal addressin cell adherence molecule (MadCAM), that is principally expressed in vascular beds in the gut (32). Another b_2 integrin, a_db_2, which binds ICAM-3 and is expressed on other leukocytes, is an additional integrin that preferentially mediates eosinophil adhesion to VCAM-1 (33). Both IL-4 and IL-13 enhance expression of VCAM-1 and P-selectin on human endothelial cells (26,31). Eosinophils, but not neutrophils, adhere to human umbilical vein endothelial cells under shear stress, using both the P-selectin–PSGL-1 and VLA-4–VCAM pairs of receptors (31,34). Thus IL-4 or IL-13 stimulation of the endothelium may participate in contributing to the preferential localization of eosinophils in specific sites of inflammation (26).

Eosinophil adherence molecules, by their interactions with extracellular matrix components, also modulate the activities of eosinophils that have exited the bloodstream (35). Eosinophil VLA-6, a_6b_1, binds laminin. Both a_4b_1 and a_4b_7 interact with specific domains of tissue fibronectin, and these interactions can enhance eosinophil functional responses (32). Eosinophils express CD44, which binds hyaluronic acid (36). Siglec-8, a sialic acid–binding immunoglobulin-like lectin, is expressed on eosinophilic leukocytes, as well as basophils and mast cells, and mediates binding to sialoglycoconjugates (37,38).

Eosinophil Chemoattractants

Mobilization of eosinophils into tissues, in addition to involving interactions with vascular lining cells, is governed by specific receptor-mediated chemoattractant stimuli. Chemoattractants promote the directed migration of eosinophils, and some chemoattractants may also enhance the adhesion of eosinophils and subsequent migration through the vascular endothelium. Many compounds have been identified as eosinophil chemoattractants (39), including humoral immune mediators like platelet-activating factor (PAF) and the complement anaphylatoxins, C5a and C3a; certain cytokines; and several chemokines, most notably the eotaxins. None of these is specific solely for eosinophils, but eotaxin and the more recently recognized eotaxin-2 and eotaxin-3 exhibit the most restricted specificity for eosinophils (40,41 and 42). Each of these eotaxins signals through the CCR3 chemokine receptor that is expressed on eosinophils as well as on basophils, some Th2 cells, and some mast cells (43,44 and 45). Other chemokines that attract eosinophils include RANTES (regulated on activation normal T-cell expressed and secreted), which signals via CCR3 and CCR1 receptors, and macrophage inhibitory protein 1a (MIP-1a), which signals through CCR1 (46,47). Thus the mobilization and recruitment of eosinophils to sites of immunologic reactions are governed by both (a) the responsiveness of eosinophils to chemoattractants (chemokines, cytokines, and other mediators) that facilitate eosinophil emigration from the vasculature and elicit directed migration of eosinophils, and (b) the expression and functional states of eosinophil adherence molecules and the differential expression of endothelial cell adherence ligands.

STRUCTURE OF EOSINOPHILS

The human eosinophil, unlike the neutrophil, typically has a bilobed nucleus. The distinguishing morphologic feature of the eosinophil is its content of large, distinctive cytoplasmic granules (48). These specific granules are morphologically unique because each contains one or more crystalloid cores. This crystalloid core, present only in the specific granules of eosinophils, is recognizable by transmission electron microscopy and usually appears electron dense. Both the core and the surrounding matrix of these granules contain a number of cationic, positively charged, proteins that provide the basis for their staining with eosin. Eosinophils at sites of inflammatory responses can exhibit morphologic changes in their specific granules, including loss of either the matrix or core components from within intact granules, compatible with the mobilization and extracellular release of these major granule components (48).

A second population of cytoplasmic granules, the primary granules, arises early in eosinophil maturation. These granules are present in eosinophilic promyelocytes and may persist in lesser numbers in mature eosinophils (48). Another distinct population of smaller cytoplasmic granules has been delineated morphologically with cytochemical identification of acid phosphatase and arylsulfatase B in these granules (48). The cytoplasm of eosinophils participating in inflammatory reactions in tissues has increased numbers of these smaller granules as well as increased numbers of vesicular and membranovesicular structures (48).

Lipid bodies are cytoplasmic structures distinct from granules (48). Lipid bodies are roughly globular in shape and range in size from minute to the size of large cytoplasmic granules; by electron microscopy, they appear dark with osmium fixation due to their lipid content. Lipid bodies lack a delimiting membrane but contain an internal honeycomb-like membranous structure that is often obscured by the overlying lipid. Lipid bodies are found in neutrophils and other cells, especially in association with inflammation, but eosinophils typically contain more lipid bodies than do neutrophils (49) Whereas normal blood neutrophils contain approximately one lipid body per cell, normal eosinophils contain from approximately five to ten; eosinophils in inflammatory reactions may contain approximately two dozen lipid bodies (50). Lipid body formation in eosinophils is rapidly inducible within minutes (51). Both exogenous PAF and endogenous PAF, elicited by stimuli such as IL-5, activate signaling pathways that lead to lipid body formation (52,53). In eosinophils, key enzymes involved in eicosanoid formation, including prostaglandin H synthase, the 5- and 15-lipoxygenases, and leukotriene (LT) C_4 synthase are localized, in part, at lipid bodies (54,55).

CELL-SURFACE RECEPTORS AND PROTEINS

Eosinophils express cell-surface receptors for a multitude of immunologic ligands (Table 22.1) (9). Receptors for immunoglobulins include those for IgG, IgE, and IgA. The receptor for IgG on eosinophils is principally the low-affinity FcgRII (CD32) (56), whereas neutrophils have FcgRII and FcgRIII (CD16), and monocytes have FcgRI (CD64) and FcgRII. Human eosinophils, unlike murine eosinophils, do not usually express CD16. Exposure of human eosinophils to interferon-g and other stimuli, including PAF and C5a, however, can elicit expression of a phosphatidylinositol-linked form of CD16 on eosinophils (56,57). CD16 may be expressed on eosinophils from some patients with eosinophilic disorders.

TABLE 22.1. Eosinophil Plasma Membrane Proteins and Receptors

Eosinophils also have three potential receptors for IgE. Human, but not murine (58), eosinophils may express the high-affinity IgE receptor, FceRI, typically found on basophils and mast cells (59). Although the FceRI a-chain protein is clearly detectable within eosinophils, its surface expression can be low or undetectable (60). Moreover, whether engagement of eosinophil FceRI can elicit degranulation of eosinophils, as it does on basophils and mast cells, remains the subject of controversy among investigators because of conflicting experimental findings (59,60 and 61). Thus the functional roles of eosinophil FceRI remain to be fully delineated. Eosinophils express FceRII, the low-affinity IgE receptor, like CD23 found on lymphocytes, monocytes, and other cells (62). Eosinophils also express a lectin-like protein, eBP/Mac-2, which binds IgE with low affinity (63).

Eosinophils express FcaRI (CD89), which, because of its greater glycosylation, has a greater molecular weight than does FcaRI on neutrophils (64). In addition, eosinophils, like neutrophils, may contain both splice variants and alternative isoforms of FcaR, although the functional roles of these alternative FcaRs are not certain (65,66). Eosinophil FcaRI binds secretory IgA more potently than other forms of IgA, and engagement of FcaRI triggers eosinophil release of granule proteins (67).

Eosinophils have receptors for complement components including C1q (CR1), C3b/C4b (CR1), iC3b (CR3), C3a, and C5a (68,69 and 70). Eosinophil C5a receptors differ in posttranslational modification and ligand affinity from neutrophil C5a receptors (70). The C3a and C5a receptors are members of the G-protein–coupled seven transmembrane–spanning family (71). Both C3a and C5a are eosinophil chemoattractants and stimulate oxygen radical production by eosinophils. Eosinophils express selected receptors for chemokines. CCR1 is a receptor for MIP-1a, macrophage chemotactic protein (MCP)-3, and RANTES, whereas CCR3 is a receptor for eotaxin, eotaxin-2, eotaxin-3, MCP-3, and RANTES (43). Macrophage-derived chemokine (MDC) induces eosinophil chemotaxis via an as yet unidentified receptor, different from CCR3 or CCR4 (72). Although eosinophils from atopic patients or eosinophils stimulated with IL-5 have been reported to respond to IL-8 (73,74), neither normal nor cytokine-stimulated eosinophils appear to express functional IL-8 receptors (75). Eosinophils also express CXCR4 and can respond (e.g., migration, calcium fluxes) to the ligand for this receptor, stromal cell–derived factor-1a (SDF-1a) (76).

Mature eosinophils, like their immature precursors, express functional heterodimeric receptors for the three cytokines, GM-CSF, IL-3, and IL-5, which promote eosinophilopoiesis and stimulate functioning of mature eosinophils. In addition to these eosinophil-active cytokines, eosinophils have receptors for a broad range of other cytokines (Table 22.1), including IL-1a (77), IL-2 (78), IL-4 (79), IL-12 (80), IL-13 (81), interferons (IFNs) a and g (77,82), tumor necrosis factor a (TNF-a) (83), stem cell factor (c-*kit*) (84), and IL-16 (which signals via CD4 on eosinophils) (85). Thus eosinophils are potentially stimulated by a considerable diversity of cytokines, although the consequences of these cytokine receptor–mediated responses for the functioning of eosinophils is yet to be fully established.

Of potential importance to cognate interactions between eosinophils and B and T lymphocytes is the fact that eosinophils can express several relevant plasma membrane proteins. Class II MHC proteins are generally absent on blood eosinophils, but their expression is inducible *in vitro* (86). Moreover, eosinophils recruited into tissues sites of inflammation exhibit *in vivo* induction of human leukocyte antigen (HLA)-DR expression as a consequence of their recruitment from the bloodstream (87). Eosinophils can express CD40 (88), CD154 (CD40 ligand) (89), CD153 (CD30 ligand) (90), CD28 (B7-2), and CD86 (91).

Eosinophils have receptors for several lipid mediators, although whether these receptors are intracellular as well as surface expressed is not known. These lipid mediator receptors include those for PAF and LTB_4, which are chemoattractants for eosinophils and stimulate eosinophil degranulation and respiratory burst activity (9). Both IL-5R– and FcgR-mediated stimulation of eosinophils elicit endogenous generation of PAF, which signals via PAF receptors to augment eosinophil functions,

including the enhancement of LTC_4 generation (52). Eosinophils have receptors for prostaglandin E_2 (although the subtypes have not been defined) (9) and for both currently recognized cysteinyl leukotriene receptors (92,93). The functions of the cysteinyl leukotriene receptors have not been fully defined, although these leukotrienes can augment eosinophil functioning and prolong eosinophil viability (94). Eosinophils have intracellular estrogen (95) and glucocorticoid receptors (9).

CONSTITUENTS OF EOSINOPHILS

Eosinophils are granulocytes, and as such, their granules are stores of proteins packaged and ready for extracellular release. The specific granules of eosinophils contain a range of proteins that include both specific cationic proteins and, as more recently demonstrated, preformed stores of diverse cytokines and chemokines.

Cationic Granule Proteins

Four cationic granule proteins of eosinophils have been extensively studied, in part because of their abundance in the specific granules and their capacities to exert multiple effects on host cells and microbial targets (96). Major basic protein (MBP), named on the basis of its quantitative predominance in the granule and its markedly cationic (basic) isoelectric point of ~11, is a 13.8- to 14-kd protein derived by proteolytic processing from a translated 23- to 25-kd preproMBP precursor. In addition to MBP, a homolog of MBP has been identified that is somewhat smaller (13.4 kd) and less basic (pI 8.7) (97). The activities of the MBP homolog (which was present in prior preparations of MBP), in comparison with MBP itself, have not yet been defined. MBP lacks any enzymatic activity and likely exerts its effects on the basis of its markedly cationic nature.

A second granule protein is eosinophil peroxidase (EPO), an enzyme distinct biochemically and physicochemically from the myeloperoxidase of the neutrophil and monocyte. EPO, which is also cationic (pI 10.8), uses hydrogen peroxide and halide ions to catalyze the formation of hypohalous acids, which are toxic for parasites, bacteria, and tumor and host cells. EPO uses bromide in preference to chloride and is even more active with a pseudohalide, thiocyanate (98,99). Thus EPO synthesizes several oxidant products, including hypobromous acid and hypothiocyanous acid.

The other dominant cationic granule proteins are eosinophil cationic protein (ECP; 18 kd, pI 10.8) and eosinophil-derived neurotoxin (EDN) (18–19 kd, pI 8.9). EDN, not as yet demonstrated to be neurotoxic for humans, is named because it, when injected intracerebrally into test rabbits, elicits a characteristic neuropathologic response termed the “Gordon phenomenon,” which is identical to the response initially elicited by the injection of tissues containing human eosinophils (100). Both ECP and EDN share sequence homology with pancreatic ribonuclease (RNase) (101). EDN expresses 100-fold more RNase activity than does ECP (102), although their toxic effects on bacterial, parasitic, and mammalian target cells apparently are not principally due to their RNase catalytic activities.

In the specific granule, MBP is uniquely localized to the crystalloid core, whereas ECP, EDN, and EPO are localized in the matrix of the granule around the core. MBP also is found in low amounts (~3% of eosinophil levels) in basophils, but whether this reflects endocytosis or endogenous synthesis is not known. Uptake of MBP and EPO into mast cells occurs by endocytosis. Small amounts of EDN and ECP also are found in neutrophils. Because neutrophils contain mRNA transcripts for them, EDN and ECP are likely synthesized by neutrophils (103). Thus eosinophils are the dominant source of these four markedly cationic proteins. The properties of these proteins and their numerous biologic effects have been reviewed (96) because these proteins have major effects not only in potential roles of the eosinophils in host defense against helminthic parasites but also in their contribution to tissue dysfunction and damage in eosinophil-related allergic and other diseases. Because MBP lacks enzymatic activity, one mechanism by which this highly cationic polypeptide may exert its activities is through interactions with lipid membranes. MBP associates with acidic lipids and disrupts liposomes prepared from such lipids (104). Such interactions might contribute to its wide range of toxicity.

The large, specific granule of the eosinophil contains a variety of acid and neutral hydrolases similar to the hydrolytic enzymes in neutrophil granules, although the content of enzymes in eosinophil granules has never been thoroughly studied. Eosinophils are a major source of a 92-kd metalloproteinase, a gelatinase (105).

Cytokines and Chemokines

Eosinophils elaborate diverse cytokines and chemokines (Table 22.2) (106). The potential activities of eosinophil-derived cytokines are protean. Eosinophil-derived cytokines include those with potential autocrine growth factor activities for eosinophils and those with potential roles in acute and chronic inflammatory responses. A notable feature of eosinophils as sources of cytokines is that eosinophils contain preformed stores of these cytokines in eosinophil–specific granules, as first demonstrated by immunogold ultrastructural localization of TNF-a in eosinophil–specific granules (107). Thus, in contrast to most lymphocytes, which must be induced to synthesize *de novo* cytokines destined for release, eosinophils [like natural T cells (108)] can immediately release preformed cytokine and chemokine proteins into the milieu proximate to themselves. Although levels of preformed cytokines in eosinophils may be lower than those of specifically stimulated lymphocytes, local and rapid release of eosinophil-derived cytokines in tissues could readily effect responses of adjoining cell types.

Cytokines	Chemokines
TGF-α, TGF-β1	RANTES
IL-1α	MIP-1α
IL-2	IL-8
IL-3, IL-5, GM-CSF	MCP-3
IL-4	Eotaxin
IL-6	
IL-10	
IL-12	
IL-16	
IFN-γ	
TNF-α	
VEGF	
PDGF-β	
HB-EGF	
MIF	
LIF	
NGF	

GM-CSF, granulocyte-macrophage colony-stimulating factor; HB-EGF, heparin-binding epidermal growth factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; MIF, macrophage migration inhibitory factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; RANTES, regulated on activation normal T-cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

TABLE 22.2. Cytokine and Chemokine Products of Human Eosinophils

Eosinophils synthesize each of the three cytokines that have growth factor and function-enhancing activities for eosinophils, GM-CSF, IL-3, and IL-5 (109,110,111,112 and 113). Eosinophils contain mRNA transcripts for GM-CSF, IL-3, and IL-5. Evidence for active cytokine production by eosinophils includes the demonstration that eosinophil viability-sustaining activities of IL-3 and GM-CSF were present in supernatant fluids of stimulated eosinophils. *In situ* hybridization studies of lesional and elicited eosinophils have documented that eosinophils participating in allergic and other responses in tissues contain transcripts for these cytokines. Thus eosinophils can elaborate three cytokines that promote the survival of eosinophils, antagonize apoptosis, and enhance effector responses of these cells. Roles for adhesion proteins and specific integrins in mediating the enhanced release of GM-CSF in eosinophils have been evidenced by findings that eosinophils adherent to fibronectin have enhanced survival *in vitro* and increased production of GM-CSF and IL-3 proteins, as well as enhanced effector responses (114,115). The increased eosinophil survival was inhibited by antibodies to either IL-3 or GM-CSF, as well as by blocking antibodies to fibronectin and a_4 and b_7, implicating a_4b_1 and a_4b_7 integrins expressed on eosinophils in transducing signals for heightened eosinophil release of IL-3 and GM-CSF (114,115).

Other cytokines elaborated by human eosinophils that may have activities in acute and chronic inflammatory responses include IL-1a (116), IL-6 (117,118), IL-8 (119), TNF-a (120), and MIP-1a (120). Human eosinophils can elaborate other “growth” factors, including transforming growth factor (TGF)-a (121), TGF-b1 (122,123), vascular endothelial growth factor (VEGF) (124), platelet-derived growth factor-b (PDGF-b) (125), and heparin-binding epidermal growth factor (126). These cytokines may contribute to epithelial hyperplasia and fibrosis, as well as have other activities. In addition, eosinophils are recognized as sources of specific cytokines and chemokines capable of stimulating or inhibiting lymphocyte responses, including IL-2 (127), IL-4 (128,129), IL-10 (130,131), IL-12 (80), IL-16 (132), RANTES (133,134), and TGF-b1 (122,123).

ACTIVATED EOSINOPHILS

Blood and tissue eosinophils, in conjunction with eosinophilic diseases or inflammation, may exhibit morphologic, biochemical, or functional alterations indicating that they have been “activated” (135). Morphologically, these activated eosinophils may have cytoplasmic vacuolizations with diminished numbers or sizes of their large specific granules, and often they contain heightened numbers of lipid bodies and extensive numbers of membranovesicular structures in their cytoplasm (48). Electron microscopy of eosinophils can demonstrate absence of the normal electron density of either the crystalloid cores or the matrices in specific granules, indicating that contents of the core or matrix have been released (48,136). “Activated” circulating blood eosinophils are metabolically more active than are unstimulated cells, with increased hexose monophosphate shunt activity and increased superoxide anion production *in vitro* (137). Activated eosinophils centrifuged over density-gradient media are less dense (“hypodense”) than are normal eosinophils (138). Increased numbers of such circulating hypodense eosinophils have been associated with various diseases, including asthma. Activated eosinophils may exhibit enhanced plasma membrane expression of some proteins, including CD69, HLA-DR, and CD25

(139).

The morphologic, biochemical, and functional attributes associated with eosinophils activated *in vivo* can be elicited in part by exposure of eosinophils to specific stimuli, particularly the three eosinophil active growth factor cytokines. GM-CSF, IL-3, and IL-5 activate eosinophils *in vitro*, prolonging their survival in culture, rendering them hypodense, and enhancing their capacity for LTC_4 formation and parasite killing (140,141 and 142). Thus the effector functions of mature eosinophils can be stimulated in tissue sites by these three cytokines and likely by other mediators acting on the many eosinophil-expressed receptors. In addition, interactions with the extracellular matrix components can further contribute to eosinophil activation. Eosinophil activation, however, is not a singularly binary process, and some attributes of activation may be elicited without other attributes by mediators and mechanisms that remain to be fully delineated. In contrast to earlier reports, which suggested that activated eosinophils release an altered form of ECP (detectable with the EG2 monoclonal antibody) that reflected eosinophil activation, staining with this antibody is now known not to reflect a state of eosinophil activation (143,144,145 and 146).

PRODUCTS OF EOSINOPHILS

Eosinophils release a broad range of products (147), including oxidative products and newly formed lipid mediators, as well as the preformed granule-derived cationic proteins and cytokines (Table 22.2) noted earlier.

The oxidative products released by eosinophils include hydrogen peroxide, superoxide anion, hydroxyl radical, and singlet oxygen. In addition, eosinophil peroxidase, as noted earlier, may catalyze the formation of longer-lived hypohalous oxidants, including brominating oxidants (148,149).

When stimulated, eosinophils form and release biologically active lipids. The oxidative metabolism of arachidonic acid by the 5-lipoxygenase pathway in human eosinophils leads to the intracellular production and subsequent release of LTC_4 (150). The elaboration of this eicosanoid by eosinophils differs from the predominant formation of LTB_4 from neutrophils. Neutrophils contain an epoxide hydrolase that forms LTB_4 from LTA_4, but eosinophils possess a specific LTC_4 synthase, which conjugates glutathione to the intermediate LTA_4 to produce LTC_4 (151) (see Penrose and Austen, Chapter 27). Eosinophils also elaborate products from the 15-lipoxygenase pathway of arachidonic acid metabolism (152). Arachidonic acid metabolism via cyclooxygenase pathways in eosinophils forms predominantly prostaglandin (PG) E_2 and thromboxane B_2 (147,153). In addition to perinuclear membranes, sites of eicosanoid formation include lipid bodies in eosinophils; lipid body domains likely contribute to the augmented formation of lipoxygenase and cyclooxygenase products in primed eosinophils (54). Increased numbers of eosinophil lipid bodies correlate with increased capacities to release LTC_4 and PGE_2 (54). Moreover, lipid bodies in eosinophils have been shown to be sites of specific LTC_4 formation in eotaxin- and RANTES-primed and stimulated cells (154). In addition to their roles as paracrine mediators, cysteinyl leukotrienes also have autocrine effects antagonizing eosinophil apoptosis (94).

Eosinophils are a source of PAF, 1-*O*-alkyl-2-acetyl-3-phosphocholine, a potent paracrine mediator with a wide range of biologic activities (147,155). PAF also functions in autocrine signaling in eosinophils, including in pathways stimulated by IL-5R and FcgR engagement (52).

MECHANISMS OF EOSINOPHIL DEGRANULATION

Because eosinophil–specific granules contain four major cationic proteins as well as many preformed cytokines and chemokines (Table 22.2), the processes by which eosinophils selectively mobilize these granule constituents for their extracellular release are fundamental to the regulated functioning of these cells. Unlike mast cells or basophils that undergo acute exocytotic degranulation in response to cross-linking of their high-affinity FcgRI, a comparable physiologic mechanism to elicit comparable exocytotic degranulation of eosinophils has not been identified. Cross-linking of eosinophil IgG or IgA FcR can stimulate release of eosinophil cationic proteins, but this rapid FcR-mediated acute “degranulation” process is not physiologic and is actually cytolytic for eosinophils (156). In contrast to the acute degranulation that can be elicited *in vitro*, electron microscopic observations of tissue samples demonstrate that specific eosinophil granule contents are mobilized and released by mechanisms not involving the wholesale exocytosis of specific granules (48). The ultrastructure of lesional eosinophils provides compelling evidence that eosinophil specific granule contents are mobilized *in vivo* by selective incorporation into small vesicles that traffic to the cell surface and release these granule contents [i.e., by a process of “piecemeal” degranulation based on vesicular transport (48)]. By this process, for instance, interferon-g can rapidly stimulate the extracellular release of eosinophil RANTES and its focal deposition in the microenvironment surrounding viable eosinophils (157,158). How this process of vesicular transport is regulated and functions to selectively mobilize specific eosinophil granule–derived cytokines or cationic proteins is under investigation.

In addition to regulated release of granule contents from viable eosinophils, a common, but often overlooked and enigmatic, occurrence is the apparent lysis of eosinophils. Both cutaneous and pulmonary biopsy specimens from individuals with eosinophil-associated diseases contain free, extracellular, but still membrane-bound, core-containing eosinophil granules. The mechanism of cytolysis in such reactions is undefined and occurs more commonly than heretofore recognized (159,160). Whether this process releases biologically active granule-bound proteins *in vivo* is uncertain.

FUNCTIONS OF EOSINOPHILS

Conventional considerations of the roles that eosinophils may play have been guided by quantitative considerations, so that those diseases characteristically marked by more prominent eosinophilia have occasioned the most interest. Thus studies have focused on roles eosinophils play in host defense against helminth infections and in the immunopathogenesis of allergic and other eosinophilic diseases (Chapter 35). In addition, roles of eosinophils must be considered in immune or inflammatory responses not conventionally recognized to involve abundant eosinophils. Conventional hematoxylin and eosin stains are not always sufficiently distinctive or sensitive to recognize tissue eosinophils. Moreover, in some diseases, morphologically intact eosinophils may not be present, although immunostaining for eosinophil proteins in involved tissues can provide evidence that eosinophils were present during the evolution of the disease (161). Thus eosinophils may be involved in the pathogenesis of some inflammatory and immune responses even without prominent lesional eosinophilia.

As end-stage effector cells, eosinophils can have roles in host defense and disease pathogenesis that are beneficial and detrimental, respectively, to the host. In addition, eosinophils may have roles in immune responses based on their collaborative interactions with other cells.

Roles in Host Defense

In contrast to unicellular protozoan parasites, multicellular helminth parasites characteristically stimulate Th2-mediated blood or tissue eosinophilia (162). In such infections, which are often associated with augmented IgE antibody formation, eosinophils can have a role in killing helminthic parasites, especially during their larval stages. Examples of parasites that may be killed *in vitro* by eosinophils include *Trichinella* and schistosomes, organisms too large to be phagocytosed (163). The initial adherence of eosinophils to the surface of the parasite surface may be mediated by eosinophil CR1 binding to C3b deposited on the surface of the worm and/or by FcgR- or FceR-mediated binding with antiparasite IgG or IgE, respectively (163). *In vitro*, deposition of eosinophil cationic granule contents onto the surface of the parasite follows, accompanied by a tighter binding of the eosinophil to the parasite. Cell products that can contribute to the death of the parasite include MBP, ECP, EDN, and EPO. Oxidative products such as hydrogen peroxide also may contribute to parasite cytotoxicity (163).

More recent studies, in which the administration of neutralizing antibody to IL-5 or infections in IL-5 gene knock-out mice have been used to abrogate the development of eosinophilia in helminth-infected mice, have caused the role of eosinophils as helminthotoxic effector cells to be reevaluated [reviewed in (164,165)]. In these eosinophil-depleted mice, the intensities of primary and secondary infections with some helminths have not been uniformly greater than in eosinophilic mice, nor have IL-5 transgenic mice consistently exhibited increased resistance to infection with some helminth species. Nevertheless, these studies performed in mice must be interpreted with caution. Some helminth infections elicit Th1-biased responses in mice, which differ from the known Th2-biased responses in humans or rats. Differences between killing of adults or larvae and of responses in tissues or the gastrointestinal tract must be considered. Many of the experimental infections involve introducing unnatural helminth infections into mice, in which innate immune responses may be prominent. Natural human infections are usually a consequence of repeated exposures, during which acquired and not innate immunity becomes prominent. Moreover, murine eosinophils, unlike human eosinophils, lack FceRI (58). With these caveats, the mixed results evaluating eosinophil helminthotoxicity in murine models need not simply negate a role for eosinophils in parasite host defense. In humans, eosinophilia has been correlated with protection against schistosome infections (165). Moreover, helminths characteristically coexist in infected hosts for prolonged periods, and helminths have evolved a diversity of mechanisms to modulate innate and acquired immunity in their natural hosts (166). Thus the apparent ambiguities of studies of the roles of eosinophils in experimental murine systems may reflect more on the complexities of helminth–host interactions. Eosinophils likely have roles in killing infective larval stages, but not adults, of most helminth parasites (164); but given that helminths persist in the face of eosinophilia even in naturally infected hosts, eosinophil helminthotoxicity does not totally protect hosts against these parasitic infections.

Eosinophils do not have major roles in host defense against bacteria and most other small microbial pathogens. With most bacterial and viral infections, eosinopenia, rather than eosinophilia, develops (Chapter 35). Eosinophils can phagocytose and kill bacteria *in vitro*, but eosinophils are not effective *in vivo* against infections with small microbial pathogens when neutrophil function is impaired. One viral infection in which eosinophils may play a role is that due to respiratory syncytial virus (RSV). RSV infections elicit eosinophilia and associated bronchial hyperreactivity (167). The eosinophil granule proteins EDN and ECP, on the basis of their RNase activities,

can exert antiviral activity for this single-stranded RNA virus (168,169).

Roles in Disease Pathogenesis

The capacities of eosinophils to release biologically active lipids as paracrine mediators of inflammation and to degranulate and release their preformed cationic and cytokine granule constituents enable eosinophils to contribute to the immunopathogenesis of various diseases. As noted earlier, eosinophils form several classes of biologically active lipids. Eosinophils, like other cells, may liberate PAF. The potent, diverse activities of PAF can be mediated directly or by stimulating other cells to release leukotrienes, prostaglandins, and complement peptides. Stimulated eosinophils also release LTC_4. LTC_4 and its derivative cysteinyl leukotrienes have bronchoconstrictor activity, constrict terminal arterioles, dilate venules, and stimulate airway mucus secretion (170). Thus eosinophils are potential sources of two major types of mediator lipids, the cysteinyl leukotrienes and PAF.

Oxidants released by eosinophils, including superoxide anion, hydroxyl radical, and singlet oxygen, and EPO-catalyzed hypothiocyanous acid and other hypohalous acids have the potential to damage host tissues (148).

Released eosinophil granule proteins are immunochemically detectable in fluids, including blood, sputum, and synovial fluids, and in tissues, including the respiratory and gastrointestinal tracts, skin, and heart, in association with various eosinophil-related diseases (96). The eosinophil cationic proteins, including MBP, ECP, and EPO, can damage various cell types (96). Release of mediators of inflammation from other cell types also may follow stimulation by eosinophil granule cationic proteins. For instance, MBP can activate basophils, mast cells, and neutrophils to release their inflammatory mediators (96). Thus extracellular release of eosinophil granule proteins, by degranulation or cytolysis of eosinophils, could contribute to local tissue damage by causing dysfunction and damage to adjacent cells.

Other Eosinophil Functions

Other functional roles for the eosinophil are likely, but as yet not fully delineated. In addition to the acute release of lipid, peptide, and cytokine mediators of inflammation, eosinophils likely contribute to chronic inflammation, including the development of fibrosis. Eosinophils are the major source of the fibrosis-promoting cytokine TGF-b in nodular sclerosing Hodgkin's disease and idiopathic pulmonary fibrosis (171). Additional roles of the eosinophil in modulating extracellular matrix deposition and remodeling are suggested by studies of normal wound healing. During dermal wound healing, eosinophils infiltrate into the wound sites and sequentially express TGF-a early and TGF-b1 later during wound healing (1). Depletion of eosinophils with anti–IL-5 monoclonal antibody accelerates skin wound closure (172).

Additional functions for the eosinophil are suggested by the findings that eosinophils may be induced to express class II MHC proteins and can function as antigen-presenting cells. Blood eosinophils lack HLA-DR expression, but eosinophils recovered from the airways 48 hours after segmental antigen challenge express HLA-DR (87). Cytokines, including GM-CSF, IL-3, IL-4, and interferon-g, induce eosinophil HLA-DR expression (116). Both murine and human eosinophils can function as HLA-DR–dependent, MHC-restricted, antigen-presenting cells in stimulating proliferation of T cells (116,173). *In vivo*, murine eosinophils can process exogenous antigens in the airways, traffic to regional lymph nodes, and function as antigen-specific antigen-presenting cells to stimulate responses of $CD4^+$ T cells (6). For potential interactions with lymphocytes, human eosinophils express relevant membrane proteins, including CD40 (88), CD28, and CD86 (91), and express receptors for cytokines, such as IL-2, as noted earlier.

Because eosinophils normally become resident in submucosal tissues, they likely have functional roles in ongoing homeostatic immune responses at these sites. Moreover, broader functional contributions are suggested by the multitude of cytokine receptors expressed on eosinophils and by their potential to release cytokines that are stored preformed in their granules. Interactions of eosinophils with other cells, including lymphocytes, are feasible, but not yet fully investigated. Thus eosinophils likely have functions that extend beyond their currently more defined roles as effector cells contributing to allergic inflammation.

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23 BASOPHILS

Bruce S. Bochner, M.D., and John Schroeder, Ph.D.

In 1877, while experimenting with various basic aniline dyes, Paul Ehrlich (1) first described the existence of tissue elements that exhibited metachromasia, staining reddish after treatment with blue–violet dyes. The term “mast cell,” from the German word *mästen* (to fatten), was used to describe the extensive granularity of these cells. Ehrlich (2) subsequently detected the presence of similar metachromatically staining cells in the blood of humans and called them blood mast cells or basophils, even though he suspected that these cells had an independent origin. After its discovery, the importance and function of the basophil remained a mystery for many years. Not until the mid-1950s was the basophil even considered to be a possible source of histamine (3); conclusive demonstration that basophils released histamine (4) and contained essentially all of the histamine in circulating cells (5) occurred years later. Since these discoveries, a great deal of experimentation has occurred with basophils, often viewed as a convenient circulating substitute for the study of the role of tissue mast cells in allergic diseases. Besides the obvious similarities that both cell types contain histamine and histamine can be released through immunoglobulin E (IgE)-dependent mechanisms, this concept was supported by several studies (reviewed in 6,7). For example, a striking correlation was seen between histamine-release responses of basophils exposed to various allergens *in vitro* and the severity of allergic respiratory symptoms experienced by the basophil donor. However, when methods were developed in the 1980s to isolate and purify human mast cells from a variety of tissues, it became clear that the basophil could no longer be viewed as a mast cell surrogate. Indeed, accumulated information suggests that the basophil in many ways may instead be more closely related to the eosinophil.

This chapter focuses on human basophil biology, with a review of information on basophil growth and differentiation, morphology, and phenotype. Particular attention is given to mediator release, as it is now known that basophils secrete immunomodulatory cytokines in addition to histamine and leukotrienes. Many advances also have been made in understanding the signal-transduction mechanisms involved in the release of these mediators. Finally, information is presented to emphasize the evidence that basophils infiltrate allergic lesions, along with other inflammatory cells, under the influence of specific cytokines. Although both the mast cell and the eosinophil are subjects of their own chapters, the important similarities and differences between these cells and the basophil are discussed.

GROWTH AND DIFFERENTIATION

The conditions for human basophil growth and differentiation have been extensively studied *in vitro* and have been reviewed (8). Initial studies demonstrated that metachromatically staining, histamine-containing leukocytes can be grown from immature precursors present in peripheral blood, bone marrow, fetal liver, and cord blood in the presence of various soluble stimuli. Among these stimuli, interleukin (IL)-3 has been identified as the primary growth and differentiation factor for the basophil (9,10), although cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, and other tissue factors also may participate. *In vitro* studies show that mature basophils retain responsiveness to IL-3, which has a remarkable ability not only to modulate mediator release but also to maintain basophil viability even after weeks in culture (11). Although relatively little is known about basophil hematopoiesis *in vivo*, it has been demonstrated that treatment of nonhuman primates with IL-3 results in increased numbers of circulating basophils, eosinophils, and their progenitors (12).

In recent years, the relationship between the growth and differentiation of basophils and mast cells has become clearer with the demonstration of common progenitor cells for eosinophils and basophils and unique precursors for mast cells (8,10,13). Human basophils are derived from a common hematopoietic stem cell from which eosinophils also originate (13,14). Increased numbers of basophil precursors have been identified in the blood of patients with allergic rhinitis, nasal polyposis, and asthma, disorders associated with eosinophilia (15). In rare diseases in which strikingly increased numbers of circulating basophils are observed or functional and structural abnormalities in basophils are seen, similar eosinophil abnormalities are often identified, whereas mast cell abnormalities are not (16,17). Likewise, in diseases associated with increased numbers of mast cells, such as systemic mastocytosis, no increases in the circulating numbers of basophils are found. Finally, basophils in peripheral blood have never been shown to be capable of cell division or of conversion into mast cells. Thus basophils in many ways are related to eosinophils, and it seems likely that both circulating basophils and tissue-dwelling mast cells represent separate lineages of terminally differentiated cells.

Human basophils also appear to resemble eosinophils with respect to their duration of bone marrow maturation and half-life in the circulation, where they remain for several days (18). In association with certain myeloproliferative diseases, such as acute and chronic granulocytic leukemia, extensive blood basophilia may occur (19,20). Usually, however, the basophil is the least common blood cell and represents <1% of the total number of circulating leukocytes (21). Increased numbers of basophils have been identified in blood and nasal secretions of individuals with allergic rhinitis and in blood and bronchial secretions of individuals with asthma (22,23 and 24). The circulating numbers of both eosinophils and basophils are extremely sensitive to glucocorticoids, showing profound and prolonged suppression after exogenous glucocorticoid administration (25). Unlike the eosinophil or mast cell, the basophil is not normally a tissue-dwelling cell, although the recruitment of basophils from the circulation into tissues and their subsequent participation in various inflammatory responses is well documented.

MORPHOLOGY

With light microscopy and appropriate dyes, human basophils can be readily identified in most biologic specimens. The metachromatic staining of the intracytoplasmic granules of both mast cells and basophils is seen by light microscopy after the cells have been exposed to dyes such as toluidine blue or Alcian blue. This staining is a characteristic property of both types of cells and continues to be among the standard methods for their identification (26,27). Staining results from the ability of the dyes to bind intracellular proteoglycans. To facilitate the study of the biology of human basophils, methods have been developed for their isolation and purification. Because basophils have a specific gravity similar to that of monocytes and lymphocytes (1.070–1.080 g/mL), purification techniques involving density-gradient centrifugation can be used to separate basophils from most granulocytes and erythrocytes, which have higher densities (28). These methods yield basophil preparations with purities ranging from 1% to 20%. To achieve higher purity, additional steps, such as elutriation, affinity purification, negative selection after antibody labeling, or cell sorting can be used (29).

To ensure consistency in their identification and analysis, specific criteria have been established for the delineation of basophils and mast cells (30). Several basophil-specific monoclonal antibodies have now been developed that recognize intracellular or surface structures unique to basophils (31,32). Although these antibodies have proved useful in detecting basophils in tissue sections (33), the function of these molecules remains to be delineated. The morphologic features of basophils can be examined with transmission electron microscopy (Fig. 23.1) (27). Basophils are typically 5–7 μm in diameter, with a segmented nucleus that exhibits marked chromatin condensation. Multiple irregular, short, thick projections are present on the cell surface. In the cytoplasm, basophils have fewer and larger electron dense granules than do mast cells and lack the typical scroll-like appearance of mast cells. Histamine and chondroitin sulfate, the predominant basophil proteoglycan, are stored in these electron dense granules; quantities of these and other mediators are listed in Table 23.1. In various regions of the cell, major basic protein (34), Charcot–Leyden crystal protein (35), glycogen, lipid bodies, and other substances and structures have been identified (27). On degranulation, basophils appear to undergo direct granule extrusion, an event associated with a number of changes in cell-surface configuration (27,36). Extensive basophil surface membrane ruffling and shedding is often seen, in association with detectable alterations in the expression of various cell-surface markers.

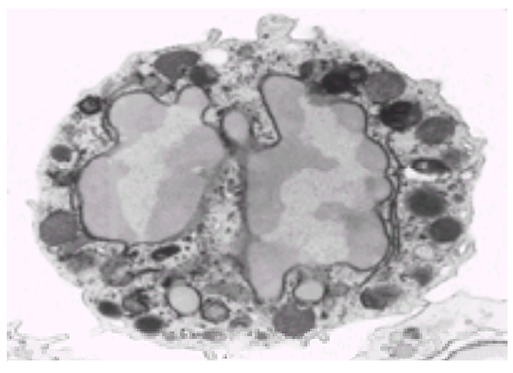

Figure 23.1. Transmission electron micrograph of a basophil isolated from the peripheral blood of a normal donor. The cell is round with short surface processes, is

fully granulated, and has a polylobed nucleus. Small aggregates and individual dense particles of glycogen are present in the cytoplasm, along with smooth, membrane-bound vesicles, either appearing empty or containing dense particles. Golgi areas are poorly developed, and free and membrane-bound ribosomes are minimal. (Reproduced from Dvorak AM, Warner JA, Kissell S, et al. F-met peptide-induced degranulation of human basophils. *Lab Invest* 1991;64:234–253, with permission.)

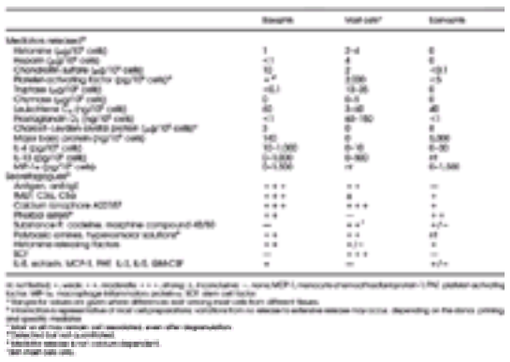

TABLE 23.1. Comparison of Selected Human Basophil, Mast Cell, and Eosinophil Mediators and Secretagogues

SURFACE PHENOTYPE

In addition to evaluation by electron microscopy, basophils have recently undergone extensive phenotypic analysis with respect to the expression of a variety of surface membrane structures. Immunocytochemistry, immunofluorescence and flow cytometry, and binding studies have been used to characterize their surface structures (reviewed in 37,38). Documented similarities and differences among the surface phenotypes of basophils, mast cells, and eosinophils are displayed in Table 23.2. For example, both basophils and mast cells express receptors for IgE that bind Fce with high affinity (so-called FceRI, K_a $<10^{10}$/M). This receptor, which has been cloned, consists of three different subunits (a, b, g), in which two g subunits associate with each a and b subunit; it is the a subunit that binds IgE (39). The active binding site on IgE recognized by the a2 region of the FceRIa subunit is a region in the C3 domain of the epsilon heavy chain (40). Neither basophils nor mast cells express low-affinity IgE receptors (FceRII, CD23). Basophils from different donors express a wide range of IgE-receptor densities (from 10^3 to 10^6 per basophil), which are much greater than those reported for mast cells (typically $<10^4$ per cell) (41). Many years ago, a correlation was found between the number of FceRI receptors per basophil and serum levels of IgE (42). This issue has now been explored in greater detail in recent studies *in vitro* and *in vivo*, including those with humanized anti-IgE antibody in clinical trials. IgE positively and reversibly influences the level of its own receptor (reviewed in 41,43). Although the mechanisms responsible for this action are not yet clear, it requires direct binding of IgE to FceRI (44). In addition, despite the extremely slow rate of dissociation of IgE from its receptor, it appears that a relatively consistent number of unoccupied FceRI receptors exist on basophils (45), thereby permitting passive sensitization of basophils from many donors by incubation with IgE *in vitro*. IgE can be removed *in vitro* by treating the basophils with low-pH solutions, such as lactic acid (46).

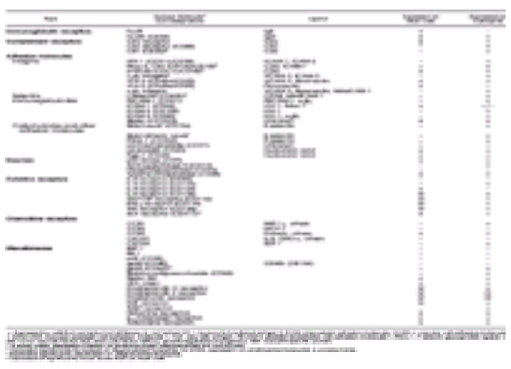

TABLE 23.2. Various Surface Structures On Human Basophils and Their Expression On Mast Cells and Eosinophils

Basophils also express receptors for IgG as well as receptors for complement components, cytokines, chemokines, and other soluble stimuli including lipids, histamine, and low-molecular-weight peptides (Table 23.2). Many more of these characteristics are shared by eosinophils than by mast cells. Basophils express several peptidases of unknown functions. They also express a wide variety of adhesion molecules with biologic functions, including members of the integrin, selectin, and immunoglobulin gene superfamilies.

BIOLOGIC FUNCTIONS

Mediator Release

The ability of human basophils (as well as mast cells) to secrete preformed and newly synthesized mediators underscores their important role in allergic and other inflammatory responses. Several preformed mediators, such as histamine, proteoglycans, and other substances, have been identified in human basophils (Table 23.1), and their rapid release has been documented. Basophils activated with certain stimuli also synthesize and release leukotriene C_4 (LTC_4), which is derived from the metabolism of arachidonic acid. Basophils do not, however, synthesize prostanoid products such as prostaglandin D_2 (PGD_2), whereas mast cells do (47). Basophils generate, *de novo*, large quantities of IL-4 and IL-13, cytokines that play a pivotal role in allergic inflammation (48,49,50,51,52 and 53). Although studies with immunohistochemistry and other methods have identified cytokines in human mast cells (and eosinophils), the quantities appear low, and the parameters involved in their synthesis from these cells are poorly understood (54,55,56 and 57). In contrast, a vast amount of information has been obtained about the generation of IL-4 and IL-13 in basophils. Most notable is the considerably longer time (hours) required for the secretion of these cytokines relative to the time required for the release of histamine and LTC_4 (Fig. 23.2).

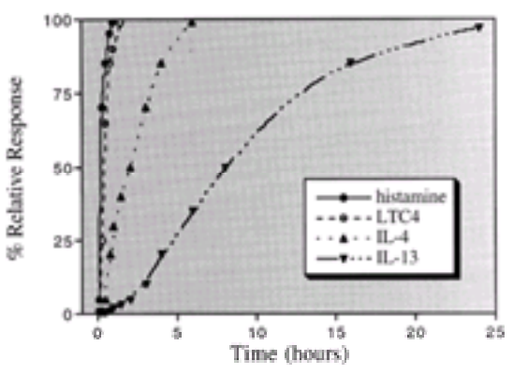

Figure 23.2. Kinetics of mediator and cytokine release from human basophils. Shown is a representative time course of secretion of histamine, leukotriene C_4 (LTC_4), interleukin 4 (IL-4), and IL-13 after immunoglobulin E (IgE)-dependent activation of the cells *in vitro*.

The excitability of the basophil is illustrated, in part, by the fact that it releases histamine concentrations of anti-IgE 100-fold lower than those required to release histamine from most mast cells. This increased sensitivity is not due simply to differences in cell-surface IgE density (58). Basophils also are far more responsive to IgE-independent secretagogues, such as complement products, formylated bacterial products [e.g., f-Met-Leu-Phe (fMLP)] cytokines, chemokines, low-molecular-weight peptides, hyperosmolar solutions, and lipids (Table 23.1) (6,7,59). Although basophils respond to many different stimuli, their activation generally results in the release of histamine and LTC_4. In contrast, their generation of IL-4 and IL-13 seems far more dependent on activation with specific stimuli (50). Both cytokines are made in response to FceRI cross-linking, induced either by a specific interaction with antigen or nonspecifically by anti-IgE or anti-FceRI antibodies. Optimal secretion of IL-4 and IL-13 in response to these stimuli occurs at concentrations nearly 10-fold less than that necessary for optimal histamine release. Surprisingly, most IgE-independent stimuli, such as fMLP, C5a, and various chemokines [e.g., the monocyte chemoattractant protein (MCP) family], although capable of inducing histamine release, fail to induce IL-4 and IL-13 secretion when used alone. Some do appear to synergize or enhance the secretion of these cytokines if combined with other stimuli (60,61 and 62). Two cytokines that activate basophils for cytokine production independent of cross-linking are the histamine-releasing factor (HRF, formerly known as the IgE-dependent HRF) and IL-3. Recombinant HRF directly induces IL-4 secretion and histamine release from basophils isolated from a subset of allergic donors (63,64). It does not appear to stimulate IL-13 release from these cells (unpublished observations). In contrast, IL-3 is an excellent inducer of IL-13 generation in cells from most donors (61,65). However, it does not generally induce the secretion of IL-4 nor does it commonly cause histamine release, unless used at high concentrations and, if so, only from cells of selected donors (66). Basophils treated for just 15 minutes with either HRF or IL-3 secrete increased amounts of IL-4, IL-13, histamine, and LTC_4 on subsequent activation with a cross-linking stimulus (49,50,67,68). For IL-3, this “priming” effect is mediated through binding of its receptor, which, as noted earlier, is expressed at high levels on basophils. Other nonphysiologic agents, such as D_2O and cytochalasin B, also enhance mediator release from human basophils (69).

Compared with the mast cell, the basophil is activated by more types of artificial stimuli such as calcium ionophores (e.g., ionomycin and A23187, both of which increase intracellular calcium), polybasic amines, and the tumor-promoting phorbol esters [which activate protein kinase C (PKC)]. Although all these agents induce degranulation, which results in histamine release, the phorbol esters do so independent of changes in cytosolic calcium (70,71). Changes in cytosolic calcium seem to play an important role in the secretion of IL-13 and IL-14, evidenced by the fact that calcium ionophores are the most potent activators of these cytokines. This statement is particularly true for IL-4. For instance, phorbol myristate acetate (PMA) alone is capable of inducing large quantities of IL-13 but does not activate basophils for IL-4 secretion (65). In fact, when added with ionomycin, PMA inhibits by 70% the IL-4 produced in response to the ionophore alone (72). This finding is surprising in view of the prosecretory effects that PMA and ionomycin have on many cell types, including IL-4 secretion from primary lymphocyte cultures. Inhibitors of PKC activation, like the bisindolymaleimides (Bis), reverse the effect that PMA has on IL-4 secretion while blocking the phorbol from inducing IL-13 secretion in basophils. Thus PKC activation appears to play a pivotal role in regulating the generation of these cytokines in basophils by acting as an inducer of IL-13 and an inhibitor of IL-4.

The basophil secretagogues that are most likely to be clinically important in allergic diseases are those that interact with surface IgE. A great deal of effort has been focused on attempts to elucidate the mechanisms involved in IgE-dependent degranulation of basophils. These secretagogues are multivalent and induce cross-linking of surface IgE, which then triggers calcium-dependent degranulation. Essential to the induction of degranulation is the establishment and maintenance of a critical number of IgE cross-links on the cell surface, estimated to range between 200 and 500, depending on the donor (73,74). Because much of the information pertaining to this sensitivity has been derived from studies of basophil histamine release, it is difficult to say at this time whether similar parameters will apply for the generation of LTC_4 and cytokines. However, studies suggest that there are subtle differences with regard to sensitivity for the release of cytokines as opposed to histamine (75). Thus it seems that the number of receptor aggregates necessary for a half-maximal response of histamine also produces a 50% response in cytokine and LTC_4 secretion.

A substantial amount of information has been obtained in recent years about the sequence of intracellular biochemical events after FceRI cross-linking and how these events relate to mediator release and cytokine generation. Many factors play a role, including tyrosine kinases and phosphatases, phospholipases, guanosine triphosphate (GTP)-binding proteins, PKC, cyclic nucleotides, and calcium (76,77). In human basophils, as in rodent mast cell lines, specific tyrosine kinases are involved in the early events after FceRI aggregation. Both p53/p56lyn (a member of the src family of kinases) and p72syk kinases are expressed in basophils, and both are phosphorylated within minutes after activation (78). These phosphorylation events induced by anti-IgE are blocked by the PP1 and PP2 tyrosine kinase inhibitors, as is the secretion of histamine, LTC_4, IL-4, and IL-13 (79). In contrast, PP1 and PP2 fail to prevent the rapid release of histamine and LTC_4 by fMLP, which is thought to signal through a receptor coupled to a pertussis toxin–sensitive (GTP)-binding protein (80,81).

Despite the presence of IgE on the surface of basophils from every donor, ~10%–15% of individuals fail to release histamine after extensive cross-linking of their basophil surface IgE (82,83). The exact defect responsible for this “nonreleaser” phenomenon is unknown, but it has been suggested that syk kinase is deficient in cells isolated from these donors (84). Another interesting *in vitro* observation is that cross-linking of IgE in the absence of calcium leads to a state referred to as desensitization, in which subsequent rechallenge of basophils with an appropriate IgE-dependent stimulus, even in the presence of calcium, fails to elicit degranulation. This phenomenon is stimulus specific (e.g., antigen desensitizes to anti-IgE but not to fMLP) and is not due to irreversible cross-linking or internalization of surface IgE (73). This approach is often used to demonstrate that a particular histamine-releasing stimulus is acting on the basophil through IgE-dependent or IgE-independent mechanisms (so-called cross-desensitization studies).

Some of the intracellular mechanisms that regulate histamine, LTC_4, and cytokine release from basophils diverge from one another in downstream events (85). The generation of LTC_4 depends on cytosolic phospholipase A_2 ($cPLA_2$) activity, which provides the arachidonic acid substrate through its ability to metabolize phospholipids. The so-called extracellular signal–regulated kinases (ERKs; ERK1 and ERK2), which are downstream of p21ras, can target $cPLA_2$ for activation. Phosphorylation of these ERKs and $cPLA_2$ was found to correlate with IgE-dependent (and -independent) release of LTC_4 from basophils. An inhibitor of ERK phosphorylation, PD098059, prevents the subsequent activation of $cPLA_2$, which results in the inhibition of LTC_4 release (86,87). Both histamine and IL-4 are unaffected, suggesting that the intracellular events regulating the release of these mediators occur independent of the ERK1/ERK2 pathway. Other studies show that phosphatidylinositol-3-(PI3)-kinase activation likely plays a role in the secretion of cytokines as well as histamine and LTC_4. This enzyme initiates signaling pathways that are important for ribosomal activity in the translation of some proteins. Both wortmannin and LY294002 inhibit PI3-kinase, and both compounds also prevent the secretion of all three classes of mediators released in response to IgE-mediated activation (87).

The activation of PKC has long been implicated in the prodegranulatory events that follow IgE-mediated activation, particularly those involved in mediator release. However, much of this belief is derived from studies performed in rodent cell lines, which may not fully translate to human cells. Recent studies show no evidence that the PKC isozymes identified in human basophils, which include b1, b2, and d, are activated after cell stimulation with anti-IgE (88). In contrast, these isozymes are activated after cell stimulation with PMA or fMLP. Although early studies did suggest a prodegranulatory role for PKC activation in the signaling events after FceRI cross-linking in human basophils, these conclusions were derived from experiments with staurosporine. This compound was initially reported to be an inhibitor of PKC but is now known also to inhibit the early and late tyrosine kinase activity associated with FceRI aggregation. Thus pharmacologic studies with staurosporine are difficult to interpret. More selective inhibitors of PKC (e.g., Bis II) show no inhibitory effect on histamine release induced by anti-IgE and, in many instances, enhance mediator release by this stimulus (89). As alluded to earlier, the same is true for IL-4, thereby suggesting that PKC activity in basophils may play a more antagonistic role in FceRI-mediated secretion.

Changes in cytosolic calcium responses play a critical role in the prosecretory events occurring in basophils, and these responses are likely initiated, in part, from the formation of triphosphates, particularly IP_3. Whereas both IgE-dependent and IgE-independent stimuli can induce a calcium response sufficient for degranulation, the duration and magnitude of the response likely determines whether cytokine generation is initiated. Several lines of evidence support this belief. First, the levels of IL-4 secreted in response to anti-IgE are remarkably enhanced with increases in extracellular calcium (50); chelators of this ion [e.g., ethyleneglycol-bis-(b-aminoethylether)-*N,N,N*',*N*', tetraacetic acid (EGTA) completely ablate all generation of IL-4, including the accumulation of its mRNA. As noted, ionophores, which sustain increases in cytosolic calcium, stimulate the secretion of large quantities of IL-4 and IL-13 from basophils. Finally, the concentrations of cross-linking stimuli (i.e., anti-IgE) that are optimal for cytokine secretion, yet suboptimal for histamine release, generally induce prolonged calcium responses in basophils (90). Similar calcium responses are not observed with univalent stimuli (e.g., fMLP or C5a) (68,91), nor is cytokine generated under these conditions.

Pharmacologic studies suggest that a calcium-dependent calcineurin pathway is involved in the IgE-mediated secretion of IL-4 and IL-13. Both FK506 and cyclosporin A (CsA), which are relatively specific inhibitors of calcineurin phosphatase activity, prevent the generation of these cytokines in basophils stimulated by anti-IgE (65). Although FK506 and CsA also inhibit mediator release induced by anti-IgE (92), they are 50- to 100-fold more potent at blocking the secretion of IL-4 and IL-13, and inhibit these cytokines at subpicomolar concentrations. Because calcineurin plays a pivotal role in regulating the nuclear factor of activated T cell (NFAT) family of transcription factors, which are important for cytokine generation in many types of immune cells, members of this family may regulate cytokine generation in basophils.

As noted earlier, the release of IL-13 from basophils, unlike that of IL-4, does not necessarily depend on FceRI cross-linking. IL-13 generation is linked neither to IL-4 nor to histamine. At least three pathways are involved in the generation of IL-13 in basophils, all of which appear pharmacologically distinct (93). Unlike that generated in response to FceRI aggregation or to calcium ionophores, the IL-13 produced after basophil activation with either IL-3 or PMA is completely resistant to FK506. As expected, the IL-13 generated with PMA is blocked with inhibitors of PKC, but these agents have little to no effect on the IL-13 made in response to IL-3 or anti-IgE. Glucocorticoids, which have proven efficacy in the treatment of allergic disease, rapidly inhibit the IL-4 and IL-13 made in response to many different stimuli (94,95).

However, the existence of multiple pathways for IL-13 is supported by the finding that dexamethasone differentially inhibits the secretion of this cytokine. The IL-13 made in response to IL-3 is most affected, being inhibited at subnanomolar concentrations. Nearly 30-fold greater amounts of drug are required to block the release of IL-13 induced by anti-IgE. This finding is unexpected, given that IL-3 lessens the inhibitory effects that glucocorticoids have on histamine and LTC_4 release (96).

Information from the study of many cell types, including basophils and eosinophils, suggests a previously unrecognized state of intermediate activation that has been referred to as *priming*. Basophils exposed to priming stimuli, especially various cytokines, become more sensitive to functional activation through traditional mechanisms such as those mediated through FceRI or complement receptors. As noted earlier, human basophils incubated with IL-3 (or HRF) release markedly greater quantities of IL-4, IL-13, LTC_4, and to a lesser extent histamine, during a subsequent challenge with anti-IgE. IL-3 also primes cells subsequently stimulated with fMLP or C5a for histamine and LTC_4 release (96,97). This priming response occurs with very low concentrations of IL-3. It has recently been proposed that cells from allergic individuals are in a primed state, as assessed by their release to thapsigargin (an agent that increases intracellular calcium) (98). Higher spontaneous or stimulus-induced basophil histamine release occurs in individuals with food allergy and other allergic conditions (99,100,101,102 and 103). Whether these findings result from cytokine-induced priming *in vivo* remains to be determined.

Recent studies have produced information about the mechanisms underlying the early and late effects of IL-3 priming. For example, a transient phosphorylation of $cPLA_2$ is detected in basophils exposed for 15 minutes to IL-3, and this event is associated with increased LTC_4 release from basophils activated with both IgE-dependent and IgE-independent stimuli (104). After 2 hours, $cPLA_2$ is no longer phosphorylated nor is there increased LTC_4 released on activation. $cPLA_2$ is once again phosphorylated by the cells after 18 hours of incubation in IL-3, and LTC_4 release is augmented on activation. An inhibitor of protein synthesis, such as cycloheximide, prevents this late effect of IL-3 (105). Likewise, cycloheximide prevents IL-3, but not anti-IgE, from inducing IL-13 mRNA expression (95). These findings suggest that the late effects of IL-3 on IL-13 and LTC_4 are mediated through the synthesis of as-yet-unidentified proteins.

A great deal of information also has accumulated about the pharmacologic regulation of mediator release. Substances including b_2 agonists (106), H_2 agonists (107), adenosine (108), cyclosporine (109), glucocorticoids (110), and cyclic adenosine monophosphate (AMP)-active agents (111) all prevent *in vitro* mediator release from basophils induced by some but not necessarily all stimuli. Finally, b_2 and H^2 agonists also inhibit cytokine secretion from basophils (112).

Role in Diseases and Recruitment Mechanisms

A major difference between basophils and mast cells is their compartmentalization in the body. Whereas mast cells reside in virtually every organ, basophils are not normally tissue-dwelling cells but instead remain in the circulation. However, during certain inflammatory responses, basophils (like eosinophils) emigrate from the vasculature to the extravascular space. Basophils have been identified in the skin at tissue sites of contact hypersensitivity, bullous pemphigoid, cutaneous basophil hypersensitivity, and late-phase allergic responses (6,22,113 and 114) and also have been identified in the upper and lower airways of patients with allergic rhinitis and asthma (23,24,33,114). Basophils increase in number after localized antigen challenge of the skin or airways in allergic individuals (33,115,116). The numbers of blood basophils correlate with bronchial responsiveness as well as with changes in bronchial reactivity over time (117,118).

The importance of basophils at inflammatory sites has been elucidated by recent studies comparing mediator concentrations with histology and cytology. For example, the short-term release of histamine in the cutaneous antigen challenge model is accompanied by evidence of mast cell degranulation, including the release of PGD_2 and tryptase (119,120). In contrast, during the late-phase response, the number of metachromatic cells increases 30- to 50-fold, and this increase correlates with a second increase in histamine. This event occurs at time points typically beyond 4 hours and is not accompanied by the reappearance of PGD_2 or tryptase, strongly suggesting that the basophil (and not the mast cell) is the source of histamine in the late phase. Various microscopic, functional, and phenotypic analyses reveal that most of the metachromatic cells at these sites are basophils and not mast cells; the same is true of similar studies in the airways (115,121). Glucocorticoids administered systemically or locally are particularly effective in reducing the appearance of mediators along with a dramatic reduction in the influx of basophils (122,123 and 124).

The fact that basophils are found in certain inflammatory sites at percentages that are vastly greater than their percentages in peripheral blood suggests that they are preferentially activated and recruited to these locations. The mechanism traditionally thought to be involved in leukocyte emigration is the local release of chemoattractants that promote adherence and chemotaxis. Cytokines [e.g., interferon (IFN)-g, IL-3, IL-5], complement fragments, chemokines (especially those such as eotaxin that act through the chemokine receptor CCR3), and other factors are chemoattractants for human basophils (reviewed in 59,114,125). However, results of studies examining the *in vitro* binding of purified leukocyte subtypes to cultured human vascular endothelial cells suggest that the endothelium can play an active role in the recruitment of granulocytes (126,127). These responses are the result of interactions between cell-surface structures on both the endothelial cell and the leukocyte. Leukocyte adherence to endothelium and other substrates is mediated through adhesion molecules on the basophil that include the b_2 integrins, leukocyte functional antigen 1 (LFA-1), and Mac-1 (Table 23.2). Agents such as chemotactic factors, IL-3, and IgE-dependent secretagogues directly stimulate divalent cation-dependent adhesiveness of basophils to the vascular endothelium (128,129 and 130). Such events are associated with the upregulation of the expression of b_2 integrins and the intracytoplasmic granule marker CD63, along with the reduced expression of L-selectin and P-selectin glycoprotein ligand 1 (PSGL-1), the major ligand for P-selectin (130,131,132 and 133). The changes in expression of CD11b and L-selectin are identical in basophils extravasated into the lower airways after allergen challenge *in vivo* (134). Neither IL-3 nor IgE-dependent secretagogues have the same effects on neutrophils. For basophils, these effects occur at concentrations of stimulus below the threshold required to induce histamine release. Therefore, the exposure of circulating basophils to low levels of these agents *in vivo* may contribute to their selective adhesion and recruitment during certain inflammatory responses.

The endothelium also plays an active role during the process of leukocyte adherence by expressing adhesion molecules and secreting substances that promote transmigration. For example, the cytokines IL-1, tumor necrosis factor (TNF), and IL-4 can stimulate cultured endothelial cells to acquire adhesive properties, rendering them extremely effective in binding basophils (and other leukocyte subtypes) *in vitro* (128,135,136). With respect to the potential role of adhesion molecules and the preferential recruitment of basophils, recent data suggest that there are differences in adhesion-related responses among granulocytes (137,138). Basophils and eosinophils, but not neutrophils, adhere to the cytokine-inducible endothelial vascular cell adhesion molecule 1 (VCAM-1) (135,136). This circumstance is due to the fact that basophils and eosinophils, unlike neutrophils, express counterreceptors for VCAM-1 (the a_4b_1 and a_4b_7 integrins) and MadCAM-1 (a_4b_7 integrins) (135,137). Because human basophils are capable of interacting with other endothelial adhesion molecules such as P-selectin and E-selectin (133,137), several similarities and differences exist in the recruitment mechanisms used by basophils and other leukocytes. It is not yet known whether the same adhesion molecules or other, as yet unidentified structures are responsible for basophil transendothelial migration or whether there are factors derived from endothelial cells or other tissues (e.g., chemokines) that selectively promote basophil recruitment.

CONCLUSION

Our knowledge of the human basophil has expanded tremendously. The basophil is most closely related to the eosinophil with respect to several characteristics such as growth and differentiation. Both cell types have a remarkably similar pattern of surface markers, and they seem to be recruited and to participate in a parallel way during various disease processes such as allergic inflammation. Nevertheless, the basophil clearly resembles the mast cell in terms of its morphology, mediator profile, and involvement in FceRI IgE-mediated responses. It is anticipated that future studies will shed additional light on the relationships among basophils, mast cells, and eosinophils, and on their respective roles in the pathophysiology of inflammatory responses.

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24 BIOLOGY OF THE MAST CELL

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Mast cells are morphologically and histochemically distinct cells of hematopoietic origin that are widely distributed throughout mammalian tissues. They are particularly abundant in a perivascular distribution in connective tissues and at mucosal surfaces (1). When stained with basic dyes such as toluidine blue, mast cells are easily recognizable under light microscopy by their prominent cytoplasmic granules (Fig. 24.1A). Mast cells have diverse effector functions that are firmly implicated in the pathophysiology of allergic disease (2,3 and 4). Classic mast cell–mediated hypersensitivity (allergic) reactions are initiated by the binding of multivalent allergen to membrane-bound immunoglobulin E (IgE) that is coupled to the tetrameric high-affinity Fc receptor for IgE (FceRI) on mast cells. IgE-dependent activation of mast cells results in their release of preformed inflammatory mediators that are stored in their secretory granules, including histamine, neutral proteases, preformed cytokines, and proteoglycans. Additionally, mast cells activated by FceRI secrete newly synthesized lipid mediators that are the products of endogenous arachidonic acid metabolism, such as prostaglandin D_2 (PGD_2) (5), leukotriene B_4 ($LT\ B_4$) (6), and LTC_4, the parent molecule of the cysteinyl leukotrienes (7). Finally, activated mast cells synthesize and secrete a host of proinflammatory cytokines (8,9 and 10). The net result of tissue mast cell activation thus includes the rapid development of plasma extravasation, tissue edema, bronchoconstriction, leukocyte recruitment, and persistent inflammation, with the clinically recognizable syndromes of anaphylaxis, urticaria, angioedema, and acute exacerbations of asthma. The importance of mast cells and their products in allergic diseases is supported by the finding that pharmacologic pretreatment of susceptible persons with cromolyn, an inhibitor of mast cell exocytosis, attenuates bronchoconstriction in response to allergen challenge (11). The additional recognition that mast cell–derived products regulate fibroblast proliferation (12,13), collagen synthesis (13,14), and angiogenesis (15,16) reflects a likely role for mast cells in wound healing and tissue remodeling (17). Moreover, hyperplasia of mast cells within lesional tissue is a recognized histopathologic feature of rheumatoid arthritis (18), fibrotic lung diseases (19,20), rapidly progressive glomerulonephritis (21), multiple sclerosis (22), and hepatic fibrosis (23). Finally, the findings that mast cells undergo exocytosis and generate lipid inflammatory mediators when these cells are activated by low-affinity Fc receptors for IgG (FcgR) (24) and are activated directly by cell wall proteins of gram-negative bacteria in experimental models of bacterial peritonitis (25,26) indicate potential salutary roles for mast cells in both innate and adaptive immune responses.

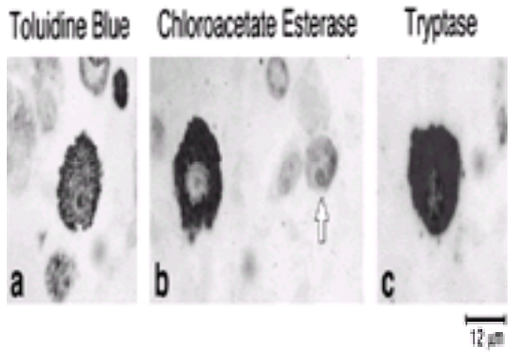

Figure 24.1. Mast cells. Staining and immunohistochemical features of human mast cells in the stroma of an excised nasal polyp. Sections show prominent granule metachromasia with toluidine blue **(A)** and red reaction indicative of chloroacetate esterase **(B).** Neutrophils *(arrow)* also react for chloroacetate esterase activity but much less intensely than mast cells. All human mast cells show immunoreactivity with an antitryptase antibody **(C).** (Photomicrograph courtesy of Daniel S. Friend, M.D., Harvard Medical School, Boston.)

This chapter deals with the current understanding of mast cell development, their effector capabilities, and the relevance of these findings to disease and immunity.

MAST CELL DEVELOPMENT

Mouse

Current knowledge about the molecular basis of mast cell development has come mainly from mouse studies. A mast cell–deficient mouse strain, *W/Wv*(27), proved to have a mutation at the white-spotting (W) locus that encodes the membrane receptor tyrosine kinase, c-*kit* (28). The ligand for c-*kit,* stem cell factor (SCF), also known as KIT ligand or Steel factor (29,30), is a growth factor that is constitutively and abundantly expressed by fibroblasts and stromal cells and exists in both membrane-bound and secretory form. The Sl locus that encodes SCF (31) is mutated in a second strain of mast cell–deficient mice (*Sl/Sld* strain) (32). These observations thus provide molecular evidence that the intact functions of both c-*kit* and SCF are absolutely required for normal mast cell development *in vivo* in mice. The transplantation of normal bone marrow cells from a histocompatible strain populated the tissues of the *W/Wv* mice with normal-appearing mast cells (27), a finding reflecting correction of the defective c-*kit* receptor in the *W/Wv* recipients and confirming the hematopoietic origin of mast cells. Moreover, the finding that recombinant soluble SCF stimulated the growth of mast cells from mouse bone marrow cells *in vitro* further validates the importance of the SCF/c-*kit* interaction in mast cell development from hematopoietic precursors (33).

Although the profound defects in all tissue mast cells in *W/Wv* and *Sl/Sld* mice support the absolute necessity for SCF and c-*kit* in normal mast cell development, other growth factors derived from T lymphocytes are crucial for a mast cell subpopulation that arises in the intestinal mucosa under conditions of helminth-induced inflammation. Athymic nude mice that were infected experimentally with *Trichinella spiralis* failed to mount a reactive intestinal mucosal mast cell hyperplasia, an expected and essential feature of adaptive immunity to helminthic infections (34). These same T-cell–deficient mice had normal numbers of mast cells in the adjacent intestinal submucosal connective tissue. Thus, T-cell–derived growth factors are required for the selective development of “reactive” mast cells in the intestinal mucosa, but not for the development and maintenance of the “constitutive” mast cells of the submucosa. A conditioned medium derived from activated T lymphocytes stimulated primitive mast cells (termed persisting or P cells) to grow from cultures of mouse bone marrow cells or dispersed spleen cells (35,36). The same conditioned medium also elicited the growth of primitive mouse mast cells from cultures of “lymphocyte-like” cells that were isolated from dispersed intestinal mucosa (37), a finding indicating the presence of mast cell–committed progenitors in the mucosal compartment of the intestine. The mast cell growth-stimulating bioactivity of T-cell–conditioned medium was subsequently largely attributed to a hematopoietic cytokine, interleukin-3 (IL-3). Purified IL-3 proved capable of inducing mast cell proliferation from dispersed intestinal mucosa (38), as well as from unfractionated mouse bone marrow (39,40 and 41). The importance of IL-3 for reactive mouse

intestinal mucosal mast cell hyperplasia was confirmed by two *in vivo* studies. In the first, blocking antibodies against IL-3 (or another T-cell–derived cytokine, IL-4) inhibited the hyperplastic mucosal mast cell response in a mouse model of helminth infection (42). In the second, mice with a targeted disruption of the IL-3 gene showed a markedly blunted (but not completely eliminated) reactive intraepithelial intestinal mast cell hyperplasia after they were infected with the nematode *Nippostrongylus brasiliensis* (43), without an effect on constitutive mast cell numbers, including those in the intestinal submucosa. When the IL-3 deficiency was expressed in mice with the c-*kit*-deficient *W/Wv* genetic background, mast cells were lacking in both the intraepithelial and submucosa compartments, and no mast cells developed in response to the infection (43). Taken together, these data support a model of mast cell development in mice that proceeds from bone marrow–derived committed progenitors that traffic to peripheral tissues. In the intestine, these progenitors give rise to constitutive mast cells that reside mostly in the submucosal connective tissues and are c-*kit* /SCF-dependent, but are independent of T-lymphocyte–derived growth factors. Such progenitors also give rise to reactive mast cells in an intraepithelial distribution in the jejunum. These reactive mast cells also require c-*kit* for their derivation *in vivo,* but unlike constitutive mast cells, they are expanded in response to T-cell–derived growth factors, particularly IL-3, in reaction to a mucosal inflammatory stimulus.

The characteristics of the committed precursors of mast cells in mice remained elusive for several decades. The lymphocyte-like cells derived from the intestinal mucosa that gave rise to mast cells *in vitro* were positive for the Thy-1 antigen, a membrane marker that is generally associated with thymocytes (37). Rodewald and colleagues isolated a population of committed mouse mast cell progenitors by sorting rare cells from fetal blood that expressed high levels of c-*kit* and low levels of Thy-1 (44). The c-*kit*^{hi}/Thy-1^{lo} fetal blood cell population, termed promastocytes, lacked FceRI and did not respond to cytokines active for the differentiation of lymphocytes, monocytes, and granulocytes in colony-forming assays; however, they gave rise to pure colonies of primitive FceRI-positive mast cells when they were cultured in the presence of recombinant SCF and IL-3. Both cytokines were necessary for optimal mast cell colony formation, a finding reflecting the apparent synergy between SCF and IL-3 for amplified mast cell growth from their committed progenitors (44). Fetal blood–derived promastocytes injected intraperiteonally into *W/Wv* mice induced the local development of mature mast cells. The failure of promastocytes to engraft bone marrow when injected intravenously likely reflected their commitment to the mast cell lineage. That the derivation of c-*kit*^{hi}/Thy-1^{lo}/ FceRI-negative promastocyte-like cells from uncommitted bone marrow progenitors depends on SCF was subsequently established in an *in vitro* model (45). Mouse bone marrow cells that were selected for their expression of Sca-1 (a membrane marker of uncommitted hematopoietic progenitor cells) developed into promastocyte-like cells and, subsequently, into primitive FceRI-positive mast cells (metamastocytes) when they were cultured in the presence of SCF in combination with two costimulatory growth factors, IL-6 and IL-10 (45). In contrast, the same Sca-1–positive uncommitted progenitors failed to develop a promastocyte-like phenotype in response to IL-3, whereas FceRI-positive mast cells (but not a promastocyte-like population) grew vigorously in response to IL-3 from bone marrow that was depleted of the uncommitted Sca-1–positive fraction. Thus, promastocytes derive from uncommitted hematopoietic precursors in response to SCF, acting through c-*kit.* Unlike IL-3, SCF and its receptor are both necessary and sufficient to provide normal numbers of tissue mast cells *in vivo* in mice. IL-3 serves as a cofactor for mast cell development from SCF-dependent committed progenitors and as a central T-cell–derived factor for mast cell hyperplasia at mucosal surfaces in mice.

Human

The finding that human patients with T-cell immunodeficiencies lack mast cells in the intestinal mucosa but not the adjacent submucosa suggested substantial developmental parallelism for mast cells in mice and humans, with T-cell–dependent and T-cell–independent mast cell subpopulations in both species (46). Recombinant human SCF, like its mouse homolog, supports mast cell growth *in vitro* (47,48 and 49). Furthermore, a gain-of-function mutation in the c-*kit* receptor is associated with an aggressive form of systemic mastocytosis (50). Thus, as in the mouse, SCF and c-*kit* are important for mast cell development in humans. In contrast to the mouse, however, recombinant human IL-3 failed to mediate mast cell growth *in vitro* when it was provided as a single exogenous growth factor to human progenitor cells; instead, it elicited the growth of basophils and eosinophils (51,52 and 53). Furthermore, mature mast cells that were dispersed from human lung tissue lacked binding sites for IL-3 (54). Based on these observations, it was assumed that the capacity for IL-3 alone to expand lineage-committed mast cell progenitors *in vivo* had been deleted during the evolution of higher primates. The potential contribution of IL-3, and of other T-cell–derived factors in human mast cell development, remained unknown until the identification and characterization of human mast cell progenitors and the delineation of their growth factor requirements and responses.

Speculation that human tissue mast cells derived from circulating basophils or monocytes was refuted by the failure of these lineages to give rise to mast cells under a variety of conditions *in vitro* (55,56). Based on cytofluorographic analysis of cell membrane markers, CD34-positive populations of human mononuclear cells identified in peripheral blood (56,57) and bone marrow (58) were found to have mast cell growth potential *in vitro,* based on colony formation in response to recombinant SCF. Although lacking visible secretory granules, these progenitor cells could be distinguished from monocytes by their expression of c-*kit* (56,57,58 and 59), as well as by their lack of CD14 (56), a component of the lipopolysaccharide (LPS) receptor that is considered a marker of the monocyte-macrophage lineage. The mast cell–producing potential of c-*kit*/CD34–positive cells was later shown to be restricted to the subfraction that also expressed CD13 (59), the human homolog of a membrane aminopeptidase previously demonstrated on mouse metamastocytes (45). When cultured *in vitro* with a combination of SCF and IL-6, the c-*kit*/CD34/CD13–positive cell fraction gave rise to mast cells and, to a much lesser extent, monocytes, whereas the c-*kit*/CD34–positive, CD13-negative cell fraction gave rise to multiple lineages under the same conditions (59). Thus, a circulating population of mast cell progenitors in humans has some monocytic growth potential as well. Whether a human promastocyte equivalent, with no monocytic potential, like that of the mouse, also exists is unknown. Nonetheless, the finding that progenitors of mast cells *in vitro* are sorted from peripheral blood of humans, as in mice, is consistent with the origins of tissue mast cells from circulating progenitors in both species. Because mature mast cells are not found in the circulation, these progenitors must acquire their distinctive functional and morphologic characteristics *in situ,* with tissue-specific mast cell features being a function of local microenvironmental factors.

Although human lung mast cells reportedly lack IL-3 receptors, and IL-3 alone fails to stimulate human mast cell growth, SCF-dependent growth of human mast cells *in vitro* from CD34-positive peripheral blood cells was strongly augmented by the addition of IL-3 in one study (57). The apparent disparity was resolved by a study of mast cell progenitors derived *in vitro* from umbilical cord blood mononuclear cells (60). When cultured for 4 weeks in the presence of SCF, IL-6, and IL-10, the cord blood cells gave rise initially to a mononuclear cell population lacking the characteristic metachromatic staining properties of mature mast cells, but with uniform membrane characteristics similar to those reported for human mast cell progenitors of bone marrow or peripheral blood (c-*kit*–positive/CD13-positive/CD14-negative) (56,57,58 and 59). As detected by flow cytometry, these mast cell progenitors expressed the receptor for IL-3, and recombinant IL-3 promoted their proliferation when provided in combination with SCF. With longer periods (9 weeks) of culture, the c-*kit*–positive/CD13-positive mast cell progenitors developed into cytologically mature mast cells, accompanied by a disappearance of the IL-3 receptor from the cell surface, consistent with the reported lack of this receptor on mature human tissue mast cells (54). However, the mature cultured mast cells became IL-3 receptor positive again when they were exposed to either recombinant IL-3 or recombinant IL-5 in conjunction with SCF. Furthermore, although the mature mast cells did not proliferate in response to IL-3 alone, they proliferated vigorously in response to IL-3 when they were provided with SCF (comitogenic response). Thus, even though IL-3 fails to promote mast cell growth by itself, the IL-3 receptor is constitutively expressed by human mast cell progenitors, is inducible on mature mast cells, and mediates an SCF-driven comitogenic effect on human mast cells and their progenitors *in vitro.* Thus, as in the mouse, IL-3 may amplify SCF-dependent mast cell development in humans despite its lack of activity alone as a human mast cell growth factor *in vitro.*

In addition to IL-3, certain other T-cell–associated growth factors augment the SCF-dependent proliferation of mast cells *in vitro* from both human and mouse progenitors (Table 24.1). In mice, IL-4, IL-10 (61), and IL-6 (45) share this property. Mice with tissue-specific overexpression of an IL-9 transgene display hyperplasia of mast cells in the intestinal (62) and lung (63) epithelium, findings suggesting a comitogenic effect. SCF-driven mast cell development from human cord blood–derived pluripotent progenitors is augmented by IL-6 (64); human cord blood–derived c-*kit*–positive/CD13-positive mast cell progenitors and their mature counterparts exhibited SCF-dependent comitogenic responses to IL-5 and IL-6 as well as IL-3 and lesser comitogenic responses to IL-9 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (60). IL-5, previously considered a selective growth factor for human eosinophils (65), was particularly potent as a comitogen with SCF for mast cells originally derived from cord blood with SCF, IL-6, and IL-10. IL-5 was also potently costimulatory with SCF and IL-6 for mast cell development *in vitro* directly from CD34-positive/c-*kit*–positive/CD13-positive human mast cell progenitors sorted from bone marrow and peripheral blood (59). Mature mast cells dispersed from surgically resected human intestines proliferated in response to IL-4 when SCF was present, but not in response to IL-4 alone (66). Furthermore, many of the same T-cell–derived cytokines that support comitogenic responses from human mast cells, such as IL-3, IL-4, IL-5, and IL-6, also attenuate their apoptosis *in vitro* independently of SCF (67).

Cytokine[a]	Mouse		Human	
	Alone	Comitogenic	Alone	Comitogenic
SCF	+	N/A	+	N/A
IL-3	+	+	−	+
IL-4	−	+	−	+
IL-5	−	N/D	−	+
IL-6	−	+	−	+
IL-9	−	+	−	+/−
IL-10	−	+	−	−
GM-CSF	−	−	−	+/−

GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; N/A, not applicable; N/D, not done; SCF, stem cell factor.
[a] SCF is absolutely required *in vivo* in mice and *in vitro* in human models of mast cell development. Although comitogenic effects are observed for GM-CSF (human only) and IL-4 for mast cell growth *in vitro* from committed progenitors, both these cytokines inhibit mast cell derivation from uncommitted progenitor cell sources.

TABLE 24.1. Cytokines Active for Mast Cell Development

Taken together, the development of mast cells and of their committed progenitors is SCF dependent in the human, as in the mouse. Both intestinal mucosal mast cell hyperplasia *in vivo* and SCF-driven mast cell proliferation *in vitro* are augmented by a range of T-cell–derived cytokines that are similar, if not identical, in the two species (Table 24.1). The activity of T-cell–derived cytokines as cofactors for mast cell development and viability *in vivo* may help to explain the prominence of mast cells at sites of allergic inflammation in humans (68,69), as well as in the intestines of mice experimentally infected with nematode parasites (70), in which these T-cell–associated cytokines are abundant. Because mast cell subpopulations in both mice and humans are functionally, morphologically, and biochemically heterogeneous, regional differences in mast cell characteristics and numbers may reflect regional differences in available levels of SCF, the local repertoire of T-cell–derived cytokines, or both. Moreover, the lack of intestinal mucosal mast cells in T-cell immunodeficiencies in both mice and humans likely reflects the requirement for T-cell–derived cytokines in mast cell development at these mucosal surfaces in both species (34,46). Because of the evident parallelisms in mast cell development in mice and humans, the remaining sections of this chapter discuss human mast cell biology unless otherwise specified.

HOMING DETERMINANTS FOR MAST CELLS AND THEIR PROGENITORS

Chemoattractants

Although mast cell development in tissues is regulated by SCF and local T-cell–derived growth factors acting on committed progenitors, the mechanisms involved in the recruitment and distribution of the circulating pool of committed mast cell progenitors, and the movement of mast cells in tissues, have only been recently explored. Several chemoattractants with activity on mast cells have been identified using *in vitro* model systems. Recombinant soluble SCF is chemotactic for a transformed human mast cell line, HMC-1 (71). The chemotactic complement fragments, C5a and C3a, also elicit migration of HMC-1 (72). That complement fragments may support mast cell migration *in vivo* is upheld by the expression of the C5a receptor, CD88, by human mast cells in the synovium of patients with rheumatoid arthritis (73). Several angiogenic growth factors are chemotactic for mouse mast cells (74), a finding potentially relevant to the observed accumulation of mast cells at sites of new blood vessel formation.

The structurally homologous low-molecular-weight chemotactic cytokines known as chemokines are also likely involved in mast cell progenitor homing and in mast cell activation (75). In mice, the intramuscular injection of the recombinant chemokine RANTES (regulated on activation, normal T cell expressed and secreted) resulted in the accumulation of mast cells at the injection site (76). Antibody neutralization suggested that the chemokine macrophage inflammatory protein-1a (MIP-1a) was involved in mast cell degranulation in an *in vivo* mouse model of contact hypersensitivity (77). Although reagents that detect receptors for mouse chemokines are lacking, the development of antibody reagents that recognize human chemokine receptors (CCRs) has facilitated their study on human mast cells and their progenitors. Human mast cell progenitors derived *in vitro* from cord blood with SCF, IL-6, and IL-10 express at least four chemokine receptors based on cytofluorographic and functional analyses (ligand-induced calcium flux or chemotaxis): CCR3, the receptor for eotaxin, monocyte chemotactic proteins (MCP) 2 to 4, and RANTES; CCR5, a receptor for MIP-1a, MIP-1b, and RANTES; CXCR2, a receptor for IL-8, granulocyte chemotactic protein-2 (GCP-2), growth-related oncogene (GRO), epithelial neutrophil-activating peptide-78 (ENA-78), neutrophil-activating peptide-2 (NAP-2), and LPS-induced CXC chemokine (LIX); and CXCR4, a receptor for stromal cell–derived factor-1a (SDF-1a) and SDF-1b (75). The CCR3/CCR5/CXCR2/CXCR4-positive profile of chemokine receptors is unique to human mast cell progenitors from among hematopoietic cells on which these receptors are characterized. The transition of these progenitors into cytologically mature mast cells *in vitro* was accompanied by the disappearance of CXCR2, CXCR4, and CCR5, but the retention of CCR3 (60). The finding that mature human mast cells express CCR3 *in vivo* was recognized by immunohistochemistry, and its function was validated by a chemotactic response of human mast cells dispersed and purified from tissues to recombinant eotaxin (78). In this study, the mast cells expressing CCR3 were primarily those in the submucosa of the airway, whereas mast cells closest to the airway epithelium did not express CCR3; these findings suggest that chemokine receptor expression by mast cells may be regulated by microenvironmental factors. In a study with immunogold electron microscopy, human mast cells dispersed from skin expressed both CXCR2 (localized to their granule membranes) and another IL-8 receptor, CXCR1 (localized to their microplicae) (79). Both CXCR1 and CXCR2 were also expressed on the surface of HMC-1 cells (79), and recombinant IL-8 elicited both chemotaxis and calcium mobilization in HMC-1. Thus, composite evidence holds that mast cells and their committed progenitors express several chemokine receptors, and the profile of chemokine receptors is modified by both the microenvironment and by cell maturation, likely reflecting changes in their requirements for migration or retention. Because SDF-1 is constitutively expressed at multiple tissue sites, the expression of CXCR4 by mast cell progenitors suggests that SDF-1 may be important for their basal homing. The ligands for CCR3, CCR5, and CXCR2 are expressed in a variety of inflammatory circumstances; thus, these receptors are potentially relevant to the accumulation of mast cells in diverse inflammatory diseases (18,19,20,21,22 and 23).

Adhesion Receptors

The process of cell recruitment involves not only specific chemoattractants such as chemokines but also specific adhesive interactions between the trafficking cells and their microenvironment. Mature human mast cells dispersed from several tissues reportedly lack b_2 integrins (80), which are necessary for transendothelial migration of blood-borne leukocytes. Low levels of one b_2 integrin family member, aMb_2 (MAC-1), were expressed by mouse peritoneal mast cells (81). The finding that a targeted disruption of the aM subunit in mice resulted in reduced basal numbers of mast cells in several tissues suggested the potential relevance of MAC-1 as a homing determinant for mast cells and their progenitors, at least in rodents (81). Unlike the b_2 integrins, mast cells of both humans and mice abundantly and constitutively express several b1 integrins, many of which are counterligands for extracellular matrix proteins (82). The major fibronectin receptor of both mouse and human mast cells, a_5b_1, is highly expressed by mast cells dispersed from several human tissue sources (80,82). Dispersed mature human mast cells also express two receptors for laminin, a_3b_1 and a_6b_1 (80). A vitronectin receptor, avb_3, is absent from human mast cell progenitors derived *in vitro* from cord blood, but it is acquired as a feature of mast cell maturation *in vitro* (60), and it is expressed by mature human mast cells dispersed from tissues (80). Thus, mast cells express several receptors that likely facilitate their interactions with extracellular matrices and basement membranes *in vivo* under basal conditions, and at least one of these, avb_3, is acquired during maturation.

Along with lymphocytes, eosinophils, and basophils, mast cells express the a_4b_1 heterodimer (very late antigen-4; VLA-4) (80,82), which is especially important for the recruitment of other effector cells to sites of allergen-induced inflammation in experimental models (83). For blood-borne cells, VLA-4 serves as the major receptor for vascular cell adhesion molecule-1 (VCAM-1), which is induced on endothelial cells with priming by IL-4, IL-1, and other proinflammatory cytokines (84). The VLA-4/VCAM-1 interaction permits the rolling, firm adhesion, and transmigration of leukocytes across the endothelium. Additionally, lymphocytes and eosinophils, and likely mast cells, use VLA-4 as a receptor for fibronectin in the tissues. The functional importance of VLA-4 is supported by studies with the systemic administration of either monoclonal antibodies or small molecule inhibitors that block VLA-4/VCAM-1 and VLA-4/fibronectin interactions. These perturbations substantially attenuate allergen-induced pulmonary inflammation in rats, sheep, and guinea pigs (83,85,86,87 and 88). These studies support the role of VLA-4 not only in inflammation-based leukocyte homing but also in the activation of mast cells, because mast cell degranulation in a mouse model of passive cutaneous anaphylaxis was inhibited by antibody blockade of VLA-4 (89). Furthermore, the administration of an anti–VLA-4 antibody before allergen challenge attenuated immediate increases in pulmonary resistance, as well as several biochemical indices of early-phase mast cell activation (histamine and tryptase levels in bronchoalveolar lavage fluid, and biliary concentrations of *N*-acetyl-LTE_4) in sensitized brown Norway rats (90). Thus VLA-4 integrin may be particularly pertinent to mast cell function.

Finally, additional integrins that are lacking on mast cells constitutively may be induced by the cytokines available during inflammatory responses. For example, aLb_2, also known as lymphocyte function antigen-1 (LFA-1), is induced on the surfaces of mature cultured human mast cells by their incubation with IL-4 (91). The aE integrin is induced on mouse mast cells by transforming growth factor-b (TGF-b) (92). The induction of aLb_2 and aEb_7 on mast cell surfaces may facilitate their interaction with their counterligands on epithelial cells: intercellular adhesion molecule-1 (ICAM-1) and E-cadherin (93), respectively. Thus, the repertoire of mast cell–associated adhesion molecules is modified not only during cell maturation in tissues but also likely by microenvironmental factors encountered under inflammatory conditions.

MORPHOLOGIC AND FUNCTIONAL FEATURES OF MAST CELLS

Cytochemical and Immunochemical Heterogeneity

The unique staining characteristics of mast cells led to their identification by Ehrlich in the late 1800s (94). Mature tissue mast cells have abundant secretory granules with avidity for anionic stains, such as Alcian blue or toluidine blue, readily permitting their identification in tissue sections. Such stains elicit conversion to purple in mast cell granules, a property known as metachromasia (Fig. 24.1A). In all mouse mast cells and in most human mast cells, the granules also react with a substrate for chloroacetate esterase (a substrate for chymotryptic proteases), which results in a bright red color that is restricted to mast cells (Fig. 24.1B) and, to a lesser extent, neutrophils, which react less strongly. In humans, immunoreactivity with an antitryptase antibody (Fig. 24.1C) provides a reliable marker for mature mast cells.

The histochemical heterogeneity of mast cells was first studied by Enerback (95,96 and 97), who recognized that rat mast cells at different anatomic locations had different staining properties. The trivial terms “mucosal mast cells” (defined only for the small intestine) and “connective tissue mast cells” were adopted to designate anatomically and histochemically distinct populations in rodents. Mucosal mast cells in the gastrointestinal lamina propria and near and within the epithelial cell layer were identifiable by their metachromatic staining with Alcian blue dye, but not with safranin dye, and were rendered invisible by formalin fixation (95). Large numbers of mast cells with these mucosal features appeared in the small intestinal epithelium of mice infected experimentally with helminths and were absent in athymic mice, a finding implying T-cell dependence for their derivation as reactive mast cells (34). Connective tissue mast cells, exemplified by those in the mesentery and submucosa of the intestine and in the skin, were formalin-tolerant, safranin-positive cells (95,96 and 97), and they developed in normal numbers (constitutive mast cells) even in

athymic mice (34). The concept of mast cell heterogeneity has since encompassed histamine content, capacity for arachidonic acid metabolism in response to immunologic stimulation, and composition of neutral proteases, and it is likely to reflect several patterns of phenotypic behavior in different tissues. Indeed, as determined by neutral protease composition of their secretory granules, several tissue-related phenotypes of mast cells exist in mice (98). Although human mast cells do not counterstain with safranin, differences in the functional, ultrastructural, and biochemical features of the mast cells in different tissues support the concept of mast cell phenotypic heterogeneity in humans as well as in rodents.

The observed differences among mast cells at different locations fueled a debate on whether distinct mast cell populations arose from different progenitor cell sources. However, committed mast cell progenitors derived *in vitro* from bone marrow with IL-3 gave rise to both connective tissue and intestinal mucosal mast cells when they were infused into mast cell–deficient *W/Wv* mice (99), a finding supporting a common lineage. The concept that all mast cells arise from a single source was further supported by the observation that the injection of clonally derived peritoneal cell colonies from +/+ littermates restored the mast cells of all anatomic compartments in *W/Wv* mast cell–deficient mice with the appropriate tissue-specific mast cell phenotypes as defined by histochemical stains for the secretory granule proteoglycan class (100). Furthermore, the progeny of an immature, transformed mast cell line (V3-MC) injected into histocompatible BALB/c mice also acquired distinct tissue-specific patterns of protease expression that required both the acquisition and the deletion of proteases relative to the parent cell line (101). Finally, clonally derived, hyperplastic mast cells from patients with systemic mastocytosis exhibit tissue-specific phenotypic variations similar to the mast cells of normal individuals (102). Taken together, these observations support the conclusion that mast cells are derived *in vivo* from a single lineage of committed progenitors both in rodents and in humans, and the ultimate phenotype of the mature mast cells arising from these progenitors is the composite result of tissue-specific and regional differences in microenvironmental factors.

Ultrastructural Features

Mast cells can be readily identified in tissues on the basis of their ultrastructural features. Mast cells range between 7 and 20 µm in diameter, with 1- to 2-µm processes (microplicae) emanating from their plasma membranes (103,104 and 105) (Fig. 24.2A). The cytoplasm of mast cells is dominated by secretory granules. The secretory granules of human mast cells contain electron-dense material that is either amorphous or crystalline. The crystalline granules contain one of three structural arrangements: scrolls (Fig. 24.2B), gratings (parallel, electron-dense lines separated by lucent areas) (Fig. 24.2C), or lattices (two sets of parallel, electron-dense lines running in different directions) (Fig. 24.2D). All three patterns of crystal may be present in a single granule (104). When the cell is stimulated by IgE-dependent mechanisms, the crystalline structures become amorphous, and only amorphous granule material is discharged from the granules as they fuse with the plasma membrane (104). The mast cells in the bronchial epithelial layer of humans with asthma contain sparse, amorphous material, a finding indicating likely ongoing degranulation *in vivo* in the asthmatic state (68).

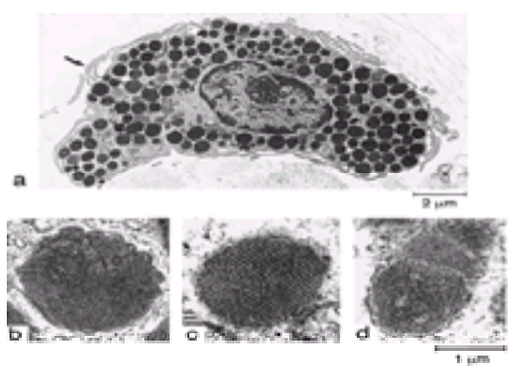

Figure 24.2. Ultrastructural features of mast cells from human skin. **A:** Transmission electron micrograph showing a mast cell containing many prominent electron-dense granules. Note 1- to 2-µm processes (microplicae) emanating from the plasma membrane *(arrow).* Higher magnification of electron-dense granule crystals shows three structural arrangements: scrolls **(B),** gratings **(C),** and lattices **(D).** (Photomicrograph courtesy of Daniel S. Friend, M.D., Harvard Medical School, Boston.)

The relative amounts of these granule structures differ among the mast cells at different tissue locations in humans. For example, the granules of mast cells in breast parenchyma, skin, axillary lymph nodes, and bowel submucosa contain relatively few scrolls (“scroll-poor” granules) but are rich in gratings and lattices (105,106 and 107), whereas mast cells in the lung alveoli and in the bowel mucosa contain many scrolls (“scroll-rich”) but relatively few gratings and lattices (103,104,106,107). The differences in ultrastructural features of human mast cell granules probably reflect corresponding differences in protease and proteoglycan content (see later). The findings that the induction of heparin proteoglycan biosynthesis in mouse IL-3–driven mast cells by 3T3 fibroblast coculture is accompanied by changes in granule ultrastructure (108,109) and that the induction of chymase expression in cultured human mast cells by recombinant IL-4 is accompanied by the appearances of grating and lattice-type granule crystals (110) support the correlation between granule content and ultrastructural appearance, as well as a flexible developmental program for both these mast cell features.

The ultrastructural flexibility of mast cell granule phenotype is illustrated by experimental *Trichinella spiralis* infection in BALB/c mice (111). The mast cells in the jejunal muscle and submucosa of infected mice exhibited spheric, electron-dense granules without crystals before infection and remained unchanged throughout the course. The reactive mast cells that appeared in the jejunal mucosa had fewer, larger granules, which were irregularly shaped, owing to their content of lattice-rich crystals. During the resolution of the intestinal mastocytosis, the numbers of intraepithelial mast cells declined, and those remaining showed fragmentation and dissolution of their crystals. Some of the mast cells in the lamina propria during resolution (but not the onset) of the mast cell hyperplasia contained fragmented crystals with a lattice structure similar to those in the intraepithelial mast cells during the peak of the hyperplasia. These alterations in granule ultrastructure are accompanied by sequential changes in the protease phenotype of the mast cells (111). That the ultrastructural and immunohistochemical phenotype of mast cell granules changes with the evolution and resolution of a T-cell–dependent intraepithelial jejunal hyperplasia highlights the plastic nature of mast cells.

Mast Cells and FceRI

FceRI is the major activating receptor on the mast cells of both humans and rodents (112), and its high-level expression is a hallmark of mast cells. When occupied by monomeric allergen-specific IgE and cross-linked by a multivalent allergen, FceRI transduces signals that result in immediate granule fusion and exocytosis, arachidonic acid metabolism, and induction of cytokine and chemokine gene transcription. IgE-mediated activation of mast cells occurs exclusively through FceRI and is thought to account for the clinical manifestations of rhinitis, conjunctivitis, urticaria, angioedema, and bronchoconstriction that immediately follow allergen challenge in susceptible hosts. Additionally, FceRI is a target of autoantibodies produced by a subset of patients with chronic urticaria (113).

The FceRI of mast cells and basophils is a tetrameric receptor composed of one Fc-binding a subunit with a single transmembrane domain, a pair of identical g subunits with short extracellular domains and long cytoplasmic tails, and a single b subunit that possesses two extracellular loops, four transmembrane domains, and two long cytoplasmic tails (Fig. 24.3) (112). The a subunit is required for binding to the Fc portion of IgE but has no intrinsic signaling properties, because deletion of its cytoplasmic tail does not abrogate FceRI-mediated cell activation (114). Conversely, the two g subunits and b subunit of FceRI have no intrinsic ligand-binding activity but associate as a functional subunit in FceRI-mediated signal transduction after receptor activation. Both the b and g subunits possess immunoreceptor tyrosine activation motifs (ITAM) in their cytoplasmic tails, which are conserved among multiple receptors involved in the initiation of diverse immune responses. The b subunit is especially important for amplification of FceRI-mediated signaling (114,115). Although FceRI can exist as an ag2 heterotrimer on human monocytes, dendritic cells, and other hematopoietic cell types (116,117,118 and 119), its signaling efficiency is markedly attenuated compared with the tetramer on mast cells and basophils. The amplifying capacity of the b subunit may be pertinent to the genetics of human atopic disease, because polymorphisms in the chromosome 11 locus that encodes this subunit are associated with an increased risk of asthma and bronchial hyperreactivity in some, but not all, human populations (120,121 and 122).

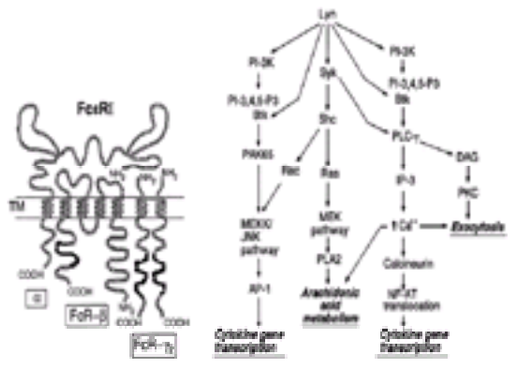

Figure 24.3. Left: Schematic representation of the tetrameric FceRI structure. The functional receptor is composed of single FceRIa (a) and FcR-b subunits and a pair of FcR-g subunits. The a and FcR-g subunits possess single transmembrane domains (TM), whereas FcR-b has four. The immunoreceptor tyrosine activation motifs of the b and g subunits are represented in bold. (From Kinet JP. The high-affinity IgE receptor (FceRI): from physiology to pathology. *Annu Rev Immunol* 1999;17:931–972. With permission from the *Annual Review of Immunology,* Vol. 17, © 1999, by Annual Reviews www.AnnualReviews.org.) **Right:** After FceRI cross-linkage, Lyn and Syk initiate a cascade of signal transduction pathways that couple to mast cell effector responses (bold type). See text for abbreviations.

The antigen-induced clustering of IgE-bound tetrameric FceRI results in several downstream signaling events that couple to mast cell effector systems (Fig. 24.3) (112). The Lyn tyrosine kinase, which is constitutively bound to the b subunit, is phosphorylated and, in turn, phosphorylates and activates the ITAMs on the b and g subunits of adjacent receptors. Two phosphorylated tyrosines on the FcRg ITAM serve as the membrane-targeting signal for the tandem SH-2 domains of Syk tyrosine kinase and bring it into proximity with Lyn. Lyn then phosphorylates Syk on tyrosine residue 518, resulting in autophosphorylation of Syk on other residues. Syk phosphorylates phospholipase C-g (PLC-g), which generates inositol 1,4,5-trisphosphate (IP_3), essential for the mobilization of calcium that is necessary for degranulation and nuclear import of calcineurin-dependent nuclear factor of activated T-cell (NFAT) transcription factors, leading, in turn, to the transcription of cytokine genes. Syk also phosphorylates Shc, leading to activation of Rac and Ras, and their downstream pathways (MEKK/JNK and MEK, respectively). Lyn activates phosphatidylinositol-3-kinase, which generates phosphatidylinositol-3,4,5-P3 in the vicinity of the signaling cluster. The phosphatidylinositol-3,4,5-P3 becomes the membrane targeting signal for the PH domains of the Tec family kinases, which includes Bruton tyrosine kinase (Btk), bringing them into proximity so that Lyn can phosphorylate them at tyrosine residue 551. Tec kinases then become further autophosphorylated and provide an additional or alternative route for the activation of PLC-g. Btk also phosphorylates and activates PAK65, leading to activation of the MEKK/JNK pathway, with activation of activator protein-1 (AP-1) transcription factors. Based on studies with mast cells derived from Btk-deficient mice, Btk is especially important for the transcription of cytokine genes after FceRI-mediated mast cell activation (123,124), and it is less important for exocytosis. More comprehensive discussions of FceRI-mediated signal transduction pathways appear in published reviews (112,125).

Although the high-level expression of FceRI on mast cells implies a potentially important physiologic role, such a function has been elusive. Mice rendered genetically deficient in FceRI by targeted disruption of the FceRIa or the Fcb subunits are developmentally and immunologically normal (126). When infected experimentally with the nematode *Schistosoma mansoni,* FceRIa-deficient mice normally eliminate worms from the intestine but develop an increased volume of hepatic granulomas and a greater degree of hepatic fibrosis than their FceRIa-sufficient littermates (126). In the FceRIa-deficient mice infected with *Schistosoma mansoni,* a splenic non-B, non-T cell population of unclear origin (possibly an innate natural killer [NK] cell–related population) did not secrete in IL-4 *in vitro* compared with the same cell population extracted from their normal similarly infected littermates. Thus, although FceRI is not required for elimination of *Schistosoma mansoni,* it does appear to modify the extent of hepatic disease that occurs in response to this infection. An additional role for FceRI has been proposed in the host response to *Haemaphysalis longicornis* ticks, because deficiencies in either mast cells or IgE markedly impair the ability of mice to eliminate the parasite (127,128).

Regulation of Mast Cell FceRI Expression and Function

The recognition that serum IgE levels correlate with the numbers of IgE binding sites on circulating human basophils prompted speculation that IgE and its receptor share a common regulatory control mechanism (129). Incubation of transformed rat basophilic leukemia (RBL) cells *in vitro* with monomeric IgE increased their surface levels of FceRI (130,131). This IgE-mediated upregulation of FceRI was attributed to stabilization of existing receptors at the cell membrane, because it was insensitive to the protein synthesis inhibitor cycloheximide. Later, mast cells from an IgE-deficient strain of mouse were found to be profoundly deficient in surface FceRI (132). This condition was corrected *in vivo* by the injection of exogenous IgE into the mice. Furthermore, FceRI expression by IL-3–driven mast cells derived from the bone marrow of these mice could be upregulated by incubation with IgE *in vitro.* Incubation of mast cells with IgE enhanced not only their FceRI expression but also signal transduction and mediator release in response to FceRI cross-linking. These observations confirmed that IgE regulates the surface levels of its own receptor, with associated incremental functional effects, and that decreased IgE concentrations *in vivo* lead to decreased levels of membrane expression of FceRI. The finding that administration of a humanized murine antihuman IgE antibody to atopic individuals markedly decreased the levels of FceRI on the surfaces of their circulating basophils (133) validates these observations in humans.

Locally derived cytokines may also serve a regulatory role in FceRI expression and signaling. Human mast cells derived *in vitro* from cord blood mononuclear cells that were cultured in the presence of SCF and IL-6 responded to the addition of exogenous IL-4 with dose- and time-dependent enhancement of their surface FceRI expression (134). Similarly, human mast cells purified from dispersed intestinal tissue responded to recombinant IL-4 *ex vivo* with upregulated FceRI expression and priming for IgE-dependent activation (66). Unlike the effect of IgE, IL-4 caused an increased steady-state level of mRNA encoding FceRIa (134). Given that they act by separate mechanisms, it is not surprising that IL-4 and IgE are synergistic for FceRI expression and FceRI-mediated mast cell activation *in vitro* (135).

Mast Cells and FcgRIII

Although IgE-dependent mast cell activation and anaphylaxis absolutely require the intact FceRI (136), anaphylaxis can also occur, at least in rodents, through IgG-dependent mechanisms. Rodent mast cells bear the low-affinity receptor for IgG, FcgRIII (137), which has not yet been identified on human mast cells. When ligated *in vitro* by antibody, FcgRIII induces mouse mast cells to degranulate and release LTC_4, similar to their responses through FceRI (137), whereas mast cell activation by FcgRIII *in vivo* can mediate anaphylaxis in mice (138,139). Furthermore, FcgRIII-mediated mast cell activation plays a dominant role in an experimentally induced Arthus reaction by facilitating the deposition of immune complexes (140). Both FceRI and FcgRIII are multimeric receptors that share the b and g subunits essential for signal transduction (136,137). FcgRIII-mediated anaphylaxis is amplified in mice that are deficient in FceRIa, a finding reflecting competition between the distinct a subunits of FceRI and FcgRIII for a limited number of shared b and g subunits (139). The common g subunit also participates in heterodimeric or homodimeric arrangements with other multimeric immune receptors, such as the T-cell antigen receptor, FcgRI, and the Fc receptor for IgA (FcaR), depending on the cellular context (141,142 and 143).

Mast Cell Activation through Non-Fc Receptors

Stimulation of mouse (144) and human (145) mast cells with recombinant soluble SCF promotes their exocytosis and arachidonic acid metabolism through c-*kit,* with kinetics that are similar to IgE-dependent activation. Mast cells release histamine in response to the anaphylactogenic complement fragments, C3a and C5a (146). These complement fragments act through pertussis toxin–sensitive, G protein–linked receptors (147). Some chemokines, whose receptors are also G protein linked, can also induce exocytosis and arachidonic acid metabolism by mast cells, as demonstrated for MIP-1a (77) and MCP-1 (148) in *in vivo* models.

Inhibitory Receptors of Mast Cells

The recognition of receptors that bear a common immunoreceptor tyrosine-liked inhibitory motif (ITIM) has led to increased understanding of the regulation of immune cell activation responses (149). Mouse mast cells possess two closely related low-affinity Fc receptors for IgG, FcgRIIb1 and FcgRIIb2 (150,151), that emanate from alternative splicing events of mRNA from the FcgRIIb gene. Both bear an ITIM motif in their cytoplasmic tails (152). When coligated with FceRI, FcgRIIb1 and FcgRIIb2 inhibit mast cell exocytosis by recruiting *src*-homology inositol phosphatase-1 (SHIP-1), which results in the inhibition of sustained calcium flux and downstream events (152). The relevance of this inhibitory function for FcgRIIb isoforms in the modulation of mast cell function is supported by the finding that mice deficient in the FcgRIIb gene exhibit an augmented experimentally induced IgE-dependent anaphylactic response *in vivo* (153). Although these findings reveal a mechanism by which mast cell activation in a mouse adaptive immune response can be counterregulated, these inhibitory Fc receptors have not yet been reported for human mast cells.

Another inhibitory receptor expressed by mouse mast cells is gp49, named for its molecular size (154). gp49b1 contains two extracellular immunoglobulin-like domains and a pair of cytoplasmic ITIM motifs. gp49b1 shares homology with a group of NK cell–related receptors with immunomodulatory functions known as killer immunoglobulin-like receptors (KIR) (155). As with FcgRIIb, experimental cocross-linkage of gp49b1 and FceRI inhibits mast cell exocytosis, as well as LTC_4

generation (154). Unlike FcgRIIb, however, the ITIM motifs of gp49b1 associate with *src*-homology-2 domain-containing phosphatase-1 (SHP-1) and SHP-2 (156), and coligation of gp49 with FceRI inhibits both transient and sustained calcium flux in mouse mast cells. Human genes that are closely related to gp49 and are localized to human chromosome 19 (termed leukocyte immunoglobulin-like receptors or LIRs) have been cloned (157,158 and 159), and their mRNA transcripts have been detected in human mast cells by reverse transcriptase–polymerase chain reaction (157).

MAST CELL–ASSOCIATED MEDIATORS AND EFFECTOR FUNCTIONS

Histamine

Histamine is present in the granules of both human and rodent mast cells in all tissue compartments. Human mast cells typically contain 1 to 2 pg of histamine per cell (160), similar to the histamine content of mast cells in rodent intestinal mucosa and about 10-fold lower than the content of mast cells in rodent connective tissue (161). Histamine has a positive charge and associates with carboxyl groups of proteoglycans in mast cell granules. After exocytosis, histamine dissociates from the mast cell proteoglycans at neutral pH (see later). Histamine acts through three classes of receptors (162) to mediate several functions, including bronchial and gastrointestinal smooth muscle contraction, vasodilatation and vasopermeability, secretion of gastric acid, and induction of cutaneous pruritus. Histamine also exerts both stimulatory and inhibitory effects on immune cells, including the following: enhancement of NK cell activation; stimulation of IL-6 synthesis by B cells; inhibition of mitogen- and antigen-mediated T-cell proliferation; inhibition of neutrophil activation; and inhibition of tumor necrosis factor (TNF)-a, IL-1, interferon-g, and IL-2 production by monocytes and T cells (163). Thus, histamine has both proinflammatory and immunomodulatory functions.

Mast Cell Proteases

Granule-associated trypsin-like (tryptases) (164) and chymotrypsin-like (chymases) (165) proteolytic enzymes are hallmarks of mast cells in all mammalian species studied. Two distinct patterns of protease composition in human mast cells were initially reported by Irani and colleagues (166). Using immunocytochemical techniques with antibodies directed against tryptases and chymase, these investigators recognized that human mast cells expressed either both tryptase and chymase or tryptase alone (designated TC and T mast cells, respectively). Mast cells with these two immunohistochemical patterns were found to have different anatomic distributions, with TC mast cells predominating in the skin and submucosa of the small intestine and T mast cells predominating in the intestinal and bronchial mucosa and in the alveoli of the lung (166). Later, this same group reported that the TC subset of human mast cells possessed two additional proteases based on immunolocalization: a carboxypeptidase A (CPA) (167) and a cathepsin G–like chymotryptic protease (168). The anatomic distributions for T and TC mast cells, combined with the observation that T mast cells were depleted at the gastrointestinal tract mucosal surface in humans with acquired T-cell immunodeficiencies (with normal numbers of submucosal TC mast cells in the same specimens) (46), raise the possibility that human intestinal T mast cells are a T-cell–dependent phenotype, analogous to the intraepithelial mast cells in the mouse jejunum, whereas the TC subset is the human equivalent of the constitutive, SCF-dependent rodent mast cells found in the connective tissues. The T versus TC mast cell dichotomy in the human, however, likely does not reflect the true diversity of the human mast cell proteases. A cluster composed of at least five distinct human tryptase genes has been identified (169). It is possible that each human tryptase gene is differentially regulated, and their respective expression patterns may depend on anatomic site, locally available growth factors, and the presence or absence of disease. If so, T mast cells of human lung may not be functionally or immunohistochemically equivalent to T mast cells of other tissues and may express different tryptases in allergic inflammation than under basal conditions. Furthermore, no data are available regarding the number and phenotype of lung mast cells in humans with T-cell immunodeficiencies; thus, the expression of tryptases or chymase could be regulated differently from that in the mast cells of the intestine.

The original studies of human mast cell protease expression used immunohistochemical probes that distinguished the known tryptases and chymase. Two highly homologous human tryptase cDNAs, tryptase a (170) and tryptase b (171), also known as tryptase II (172), encode functionally distinctive and well-characterized enzymes. Studies with specific monoclonal antibodies that distinguish between immunoreactive a and b/II tryptases revealed that a tryptase was present in human plasma and reflected the total body mast cell burden (173). In contrast, b/II tryptase is not found in plasma under normal conditions but is released by mast cells on activation (174). Thus, the measurement of these tryptases in human plasma is clinically useful in the diagnosis of systemic mastocytosis and anaphylaxis, respectively. Although a and b/II tryptases are highly homologous, structural dyshomology exists in their putative substrate-binding domains and accounts for differences in substrate specificity (175). Both a and b/II tryptase genes reside on human chromosome 16, within the previously mentioned cluster containing four additional tryptase genes that have been mapped (169,176). A cDNA encoding one of these novel human tryptases has been cloned in both human and mouse and encodes a tryptase with a novel transmembrane domain (176). Thus, each of these tryptase genes likely arose from a single ancestral gene to encode enzymes with distinct functions. To date, antibody reagents and molecular probes have not been developed or used for the full characterization of the expression patterns for the additional human tryptases.

The putative effector functions of the mast cell tryptases, as defined by *in vitro* assays or by pharmacologic studies *in vivo,* are diverse. In addition to the transmembrane tryptase (176), mouse mast cells have two homologous granule-associated tryptases, designated mouse mast cell protease-6 (mMCP-6) and mMCP-7 (177,178). mMCP-7 is released into the circulation of mice after experimentally induced anaphylaxis (179), where it potently and selectively degrades plasma fibrinogen, even in the presence of plasma protease inhibitors (180). In contrast, mMCP-6 is not detected in plasma after anaphylaxis but is retained along with the exocytosed secretory granule core in connective tissues. Unlike mMCP-7, mMCP-6 does not degrade fibrinogen; but when mMCP-6 is instilled as a recombinant protein into the peritoneal cavity of mice, it elicits a marked, selective, and sustained influx of neutrophils (181), which is attributed to mMCP-6–mediated induction of IL-8 expression by endothelial cells. This property of endothelial cell stimulation for IL-8 production *in vitro* is shared with recombinant human tryptase b/II, which also induces expression of IL-1b by endothelial cells (182). Human tryptase b/II also stimulates the formation of vascular tubes *in vitro,* a finding suggesting a role in angiogenesis (15), and it elicits fibroblast (14) and epithelial cell (183) proliferation *in vitro,* thus implying a role in remodeling and repair after neutrophil-mediated tissue injury. The activating effects of tryptases on multiple cell types of stromal derivation suggest the possibility that tryptases act through specific cell-surface receptors. Indeed, both human a and b/II tryptases activate protease-activated receptor-2 (PAR-2) (184), a 7-transmembrane–spanning receptor expressed by several stromal cell types that requires proteolytic modification of its extracellular domain for its activation (185). Finally, human tryptases have been proposed to potentiate bronchoconstriction in response to histamine (186), an effect ascribed to the enzymatic cleavage of inhibitory airway neuropeptides. The finding that pretreatment of sensitized sheep with specific inhibitors of tryptase activity attenuates the pathobiologic consequences of allergen exposure (187) may reflect inhibition of each of these actions *in vivo.*

Only one mast cell–specific chymase gene has been identified in humans (188), in sharp contrast to the mouse, in which at least nine mast cell chymase genes exist (189). Human mast cell chymase, like other members of the chymase family, cleaves angiotensin I to form angiotensin II (190,191), a function that is potentially germane to the homeostatic role of mast cells in the local regulation of vascular tone and perfusion. A dog mast cell chymase cleaves and activates a 92-kd gelatinase (192,193), a member of the matrix metalloproteases implicated both in the migration of leukocytes and in tumor metastasis. A role for mast cell chymases in the extracellular cleavage and activation of angiogenic factors has been proposed as a determinant of tumor invasiveness (194). Recombinant human chymase cleaves type 1 procollagen and initiates collagen fibril formation (195). Human mast cell chymase cleaves SCF (196), and it yields a product that retains activating and mitogenic functions (197). Moreover, human chymase stimulates secretion by bronchial mucus glands (186). Thus, chymase may be involved in wound repair, extracellular matrix turnover, angiogenesis, mucus secretion, and the local modulation of vascular caliber and tone.

As noted earlier, tryptase and chymase are distributed differently in human mast cells, with both tryptase a and tryptase b/II expressed by the mast cells of all human tissue distributions and chymase limited to the mast cells in connective tissues and the perivascular regions (167). As determined by monoclonal antibodies that recognize both tryptase a and tryptase b/II, mast cells differ in their quantities of these tryptases. For example, the mast cells dispersed from the epithelial layer of nasal polyps contained fivefold less tryptase than submucosal mast cells from the same specimens (198). Dispersed human intestinal mucosal mast cells contained threefold less tryptase than dispersed skin mast cells (160). Basophils also contain minute quantities of tryptase (199); and mRNA encoding tryptase a, but not tryptase b, is detected in basophils by reverse transcriptase–polymerase chain reaction (200). Circulating metachromatic cells that would otherwise have been characterized as basophils but stained immunocytochemically for tryptase, chymase, and CPA were found in the peripheral blood of patients with allergy, asthma, and drug reactions, respectively, but not in the blood of healthy subjects (201). The finding that several patterns of protease expression and differences in tryptase content have been identified in subsets of mast cells makes it likely that tissue and microenvironment-specific factors regulate their patterns of protease expression.

The finding that patterns of protease expression by mast cells reflect microenvironmental influences is supported by extensive studies *in vitro* with mouse mast cells. IL-3–driven bone marrow–derived mouse mast cells from BALB/c mice express both protein and steady-state levels of RNA encoding two tryptases (mMCP-6 and mMCP-7) (176,177), a chymase (mMCP-5) that is highly homologous to human chymase (202), and CPA (203). Mouse mast cells derived in the presence of SCF, rather than IL-3, express a different protease profile that includes another chymase, mMCP-4 (204). The expression of two additional chymases, mMCP-1 and mMCP-2, both of which are associated with mouse mucosal mast cells *in vivo,* was induced by exposure of IL-3–driven bone marrow–derived mast cells to IL-10 (205) or IL-9 (206) *in vitro.* Nuclear run-on analyses revealed that multiple protease genes were constitutively transcribed in these mast cells, but the steady-state levels of mRNA encoding mMCP-1 and mMCP-2 depended on mRNA stabilization mediated by IL-10 (207). Thus, multiple protease genes are transcribed simultaneously by mast cells, but only some are expressed at the steady state. This mechanism may permit mast cells to change their repertoire of bioactive proteases rapidly *in vivo* under appropriate inductive circumstances, as reflected by tissue-specific differences in protease expression in mouse mast cells *in vivo* (98,208), as well as the changes in protease expression in the mast cells at a single tissue site during the onset and resolution of helminth-induced reactive mast cell hyperplasia (111). The finding that exogenous recombinant IL-4 induced chymase immunoreactivity in a population of cord blood–derived human mast cells *in vitro* that were initially positive for tryptase alone suggests that human mast cells also alter their protease composition in response to the microenvironment (110).

Mast Cell Proteoglycans

Like all granulated hematopoietic cells, mast cells have proteoglycans in their secretory granules. All secretory granule proteoglycans contain a common peptide core (209,210 and 211), called serglycin for its repetitive serine and glycine residues that allow glycosaminoglycans to attach to every second and third serine residue. This structure also likely accounts for the resistance of proteoglycans to degradation by bound proteases that are freed from their activating peptide. The secretory granules of mast cells contain biochemically distinct species of proteoglycans that differ in sulfation of their respective glycosaminoglycan side chains. Mast cells dispersed from human tissues contain proteoglycans that are rich in heparin (heparin proteoglycans) (212,213) and proteoglycans that are rich in chondroitin sulfate E (214,215). Mouse and rat mast cells also contain both species of proteoglycans, and the mast cells of their connective tissue are especially rich in heparin proteoglycans (216). Conversely, the mast cells of the rat intestinal mucosa contain lesser amounts of heparin, and chondroitin sulfate E or di-B proteoglycans predominate (217,218). These differences in proteoglycan content account for striking histochemical differences between these two populations in rodents; the heparin-rich content of rat connective tissue mast cells permits their uptake of safranin or berberine sulfate dyes. In contrast, the relative lack of heparin in mouse intestinal mucosal mast cells and in mast cells derived *in vitro* with IL-3 renders them safranin negative.

As is the case with the mast cell proteases, the proteoglycan content of rodent mast cells is not a fixed feature of their phenotype. Mouse bone marrow–derived, IL-3–driven mast cells cocultured with a monolayer of fibroblasts convert to a heparin-positive phenotype (108,219,220). This same culture condition also sustains the viability of the mouse mast cells in the absence of exogenous growth factors (219); it markedly augments their IgE-dependent exocytosis, production of PGD_2, LTB_4, and LTC_4 (220), and it promotes the development of electron-dense granules. Rat peritoneal mast cells (221), human lung-derived mast cells (222), and human cord blood–derived mast cells (223) also survive on fibroblast monolayers and acquire augmented IgE-dependent activation responses *in vitro.* These changes in functional, biochemical, and ultrastructural phenotype of mast cells that are elicited by fibroblast coculture suggest that mast cell–fibroblast interactions, mediated by the c-*kit* receptor (224), help to determine the biochemical and functional phenotype of mast cells in connective tissues.

Intracellular functions of proteoglycans include the storage and packaging of mast cell granule constituents. The acidic (negatively charged) proteoglycans form the basic structural components of large, macromolecular complexes that are stored and released from mast cell granules during exocytosis (225,226 and 227). These complexes are composed of highly acidic proteoglycans associated with basically charged proteases, as well as histamine and b-hexosaminidase. With exocytosis and release into the extracellular environment at neutral pH, histamine and b-hexosaminidase dissociate from the complex. The relative charges of the protease and the proteoglycans determine the destiny of the proteases after exocytosis. For example, the tryptase mMCP-7 interacts with proteoglycan through histidine and diffuses away from the complex after exocytosis. Conversely, mMCP-6 binds to proteoglycan through arginine or lysine, and the positive charge of these residues prevents its diffusion from the complex and results in its retention in the tissue (179). Furthermore, the chymase mMCP-1, which is induced in the intraepithelial mast cells arising in the jejunum during helminthic infection (228), diffuses from the chondroitin sulfate E proteoglycan that predominates in these mast cells. In human mast cells, chymase and CPA (which are expressed in the same subset of mast cells) are complexed with a proteoglycan subspecies (likely containing heparin) that is different from the subspecies complexed with tryptase (likely chondroitin sulfate E) (229). The targeted disruption of the *N*-acetyl/*N*-sulfotransferase-2 (NDST-2) gene, an enzyme required for heparin biosynthesis, revealed that heparin proteoglycans are required for mast cells to express immunoreactive mMCP-4, mMCP-5, and CPA (230,231), even though the mast cells of these mice contained steady-state levels of mRNA for these proteases that were comparable to those found in the mast cells of normal congenic control mice. The expression of immunoreactive tryptases mMCP-6 and mMCP-7 were unaffected in these NDST-2 knock-out mice. It thus appears that heparin proteoglycan synthesis is absolutely required for the normal expression of chymases and CPA but not tryptases.

Arachidonic Acid Metabolism

Both rodent and human mast cells rapidly synthesize the eicosanoid inflammatory mediators, LTC_4, LTB_4, and PGD_2 from endogenous membrane arachidonic acid stores when they are stimulated by FceRI (232,233) or by c-*kit* with recombinant soluble SCF (145). In response to either stimulus, arachidonic acid is released from cell membrane phospholipids by the action of a calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$) (234) and is converted sequentially to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and then to LTA_4 by the actions of 5-lipoxygenase (5-LO). 5-LO is reversibly translocated from the cytosol to the perinuclear envelope (235), and it requires the cooperation of an integral membrane protein, 5-lipoxygenase–activating protein (FLAP) (236), for its metabolic function. LTA_4 is then converted by an LTA_4 hydrolase to LTB_4 (237), or it is conjugated to reduced glutathione by LTC_4 synthase (LTC_4S) (238), an integral membrane protein with homology to FLAP, to form LTC_4, the parent compound of the cysteinyl leukotrienes. Both LTB_4 (239) and LTC_4 (240) are exported to the extracellular space by distinct, energy-dependent steps. LTB_4 is a potent chemotactic mediator for neutrophils (241), through a 7-transmembrane–spanning receptor (242). LTC_4 is sequentially converted extracellularly to the receptor-active metabolites, LTD_4 and LTE_4 (243). These mediators, acting through another 7-transmembrane receptor, Cys-LT-1 (244), are potent bronchoconstrictors in humans (245,246) and mediators of vascular permeability (247); they also induce mucus secretion by isolated airways *in vitro* (248), and they recruit eosinophils to the airway wall when experimentally instilled into human subjects *in vivo* (249). PGD_2 is generated by the conversion of arachidonic acid through the sequential actions of prostaglandin endoperoxide synthase-1 (PGHS-1) or PGHS-2, and the hematopoietic form of PGD_2 synthase (250). PGD_2, like the cysteinyl leukotrienes, is a bronchoconstrictor (251), and its active metabolite, 9a,11b-PGF_2, is a potent constrictor of coronary arteries (252). Thus, the lipid mediators of mast cells are likely involved in several aspects of allergen-induced tissue dysfunction, including acute airflow obstruction, alterations in vascular caliber and tone, and leukocyte recruitment.

As with the profile of mast cell granule constituents, mast cell subpopulations differ in their patterns of arachidonic acid metabolism. Rat peritoneal mast cells, a prototypical constitutive mast cell population, respond to IgE-dependent activation with preferred generation of PGD_2, whereas mast cells from the rat intestinal mucosa generate LTC_4 in preference to PGD_2 (233,253). Human mast cells from different tissue sites also display heterogeneity of arachidonic metabolism. Mast cells from human intestine generated a wide range of PGD_2 (1 to 55 ng, mean = 21.3 ng) and cysteinyl leukotrienes (0.2 to 14.2 ng, mean = 4.4 ng) per 10^6 mast cells, when they were stimulated *in vitro* with anti-IgE (103). Human lung tissue mast cells generated levels of LTC_4 and PGD_2 comparable to their intestinal counterparts (254,255). In contrast, human uterine mast cells generated larger quantities of both PGD_2 (89 ng/10^6 cells) and LTC_4 (45 ng/10^6 cells) (256), whereas human skin mast cells generated PGD_2 and almost no LTC_4 (257). Thus, both rodent and human mast cells exhibit several patterns of arachidonic acid metabolism based on their tissue locations. The pattern of intertissue heterogeneity of arachidonic acid metabolism exhibited by human mast cell subsets does not parallel the heterogeneity of their corresponding protease composition, a finding indicating that these features are regulated differently.

With the derivation of mouse mast cells *in vitro,* investigators have been able to infer cytokine-mediated alterations in arachidonic acid-metabolizing phenotype and to ascribe these alterations to the regulation of the constituent arachidonic acid-metabolizing enzymes and proteins involved. For example, mouse mast cells derived *in vitro* from bone marrow with IL-3 produce LTC_4 in marked preference to PGD_2 (258) but are induced to generate PGD_2 by coculture with fibroblasts (220). The fibroblast effect on PGD_2 biosynthesis likely results, at least in part, from fibroblast-derived, membrane-bound SCF, because the capacity of mast cells derived in IL-3 for PGD_2 generation is also upregulated 5- to 7-fold by a 2-day incubation with SCF and IL-10 (250). Conversely, mouse mast cells derived with exogenous SCF instead of IL-3 produced small and equal amounts of LTC_4 and PGD_2 after IgE-dependent stimulation; but when these mast cells were cultured for 2 to 4 weeks in the additional presence of IL-3, they responded with 26-fold and 3-fold increases in the IgE-dependent production of LTC_4 and PGD_2, respectively (258). Human cord blood–derived mast cells grown in an SCF-dependent culture system generate abundant PGD_2 but minimal LTC_4. Their capacity for LTC_4 generation in response to FCeRI cross-linkage is dramatically upregulated by IL-4, which induces expression of LTC_4S (259). The requirement of SCF for mast cell development *in vivo* therefore likely reflects the finding that all tissue mast cell subpopulations constitutively generate at least some PGD_2, while additional factors, including IL-4, modulate LTC_4 generation.

Cytokines Produced by Mast Cells

EARLY-ACTING CYTOKINES

Mouse mast cells produce certain cytokines in response to IgE-dependent stimulation, including cytokines that are associated with the early phases of an inflammatory response, such as TNF-a (260), IL-1 (261), and IL-6 (262,263). Such early-acting cytokines initiate hepatic acute-phase protein production, endothelial cell adhesion molecule expression, and leukocyte recruitment (264). The immunolocalization of TNF-a to the mast cells of human skin (264) and nasal mucosa (265) confirms that TNF-a is also synthesized and stored by human mast cells. The proportions of TNF-a–positive mast cells were greater in transbronchial biopsy specimens from patients with asthma than in those obtained from nonasthmatic control subjects, a finding suggesting that local microenvironmental perturbations modulate TNF-a generation (266). IgE-dependent TNF-a release by mast cells may thus favor leukocyte recruitment to the bronchial wall in asthma. The secretion of TNF-a by mast cells also facilitates their critical protective role recognized in a mouse model of septic peritonitis (25,26). In this model, mast cell activation for TNF-a generation was mediated directly by a bacterial cell wall constituent, a finding indicating a role for mast cells and TNF-a in innate immunity. Indeed, resistance to bacterial peritonitis was enhanced in mice that were injected with SCF, an effect that was mediated partly through mast cells and TNF-a (267).

ALLERGY-ASSOCIATED CYTOKINES

Human and mouse mast cells provide several cytokines that mediate allergic inflammatory responses after IgE-dependent activation. For example, IL-4 and IL-5 are localized by immunohistochemistry to the mast cells in lung tissue of patients with asthma (266), as well as to the mast cells in the nasal mucosa of patients with allergic rhinitis (268). In addition to IL-5, a second eosinophil-active cytokine, GM-CSF, was immunolocalized to the mast cells in the bronchial mucosa of patients with asthma (269). These data are supported by the observation that dispersed human mast cells transcribe and translate several T-helper cell type 2 (Th2)-associated cytokines *de novo* through IgE-dependent activation. IL-3, IL-4, IL-5, and GM-CSF were transcribed when human lung mast cells were stimulated with anti-IgE, and immunodetectable IL-4 and IL-5 were released in response to the same stimulation (270). When cord blood–derived human mast cells were primed *in vitro* with IL-4, their IgE-dependent transcription and release of IL-13 were induced (9), and dispersed human lung mast cells generated IL-13 *in vitro* in response to IgE-dependent stimulation even without IL-4 priming (10). Both immunodetectable IL-4 and IL-5 and their corresponding mRNA species were localized to human skin mast cells after cutaneous allergen challenge (271). Human intestinal mast cells responded to IL-4 priming with augmented IgE-dependent production of IL-3, IL-5, and IL-13 but decreased release of IL-6 (272). Thus, *de novo* production of cytokines occurs downstream of IgE-dependent mast cell activation, with potential relevance to asthma and allergic inflammation. The process of Th2 cytokine generation in mast cells likely has some similarity to the same process in T cells, in which IL-4 is required for optimal production of the Th2 cytokines IL-13 and IL-5. In mouse mast cells, IgE-dependent transcription of IL-5 was linked to the activity of an inducible NFAT family member (273). IgE-dependent IL-2 production by mouse mast cells was linked to NFAT and AP-1 transcription factors, both of which were downstream of FceRI-mediated activation of Btk (123). Taken together, these studies confirm that mast cells are a source of both proinflammatory and Th2-type cytokines relevant to allergic inflammation *in vivo* (274,275 and 276), and their IgE-dependent production is a potential amplifying mechanism for allergic inflammation.

CHEMOKINES

The mast cells of both mice and humans generate several members of the CC and CXC chemokine families. Human mast cells store IL-8, a potent neutrophil-active chemokine (277,278). A closely related CXC chemokine, ENA-78, is produced by mouse mast cells *in vitro* after IgE-dependent activation (279). Human mast cells derived from cord blood *in vitro* generate MIP-1a in response to IgE-dependent activation (280), and mouse mast cells secrete both MIP-1a and MIP-1b *in vivo* during experimental contact hypersensitivity (281). Production of MCP-1 by human lung mast cells is enhanced by SCF and anti-IgE *in vitro* (282). Contact between mouse mast cells and fibroblasts induces the production of eotaxin by mast cells (283). Thus, mast cells are a potential source of several chemotactic mediators involved in inflammation-based leukocyte recruitment.

FIBROGENIC AND ANGIOGENIC GROWTH FACTORS

Mast cells generate factors that are involved in fibroblast proliferation, extracellular matrix deposition, and angiogenesis, including vascular permeability factor and vascular endothelial cell growth factor (284), TGF-b (285), and basic fibroblast growth factor (9). The last property supports the role of mast cells as modulators of tissue repair, fibrosis, and remodeling. Finally, the finding that mast cells store and secrete SCF (197,286,287) illustrates a potential autocrine capability.

IMPLICATIONS OF MAST CELLS FOR HEALTH AND IMMUNITY

In summary, mast cells possess diverse effector capabilities and are ubiquitously distributed to serve diverse physiologic functions. The role of mast cells in allergic inflammation is now firmly established, and their effector pathways are important therapeutic targets for the treatment of allergic diseases. Mounting evidence holds that mast cells are important in immunity, and the constitutive and reactive mast cell developmental pathways may reflect evolutionary pressures to serve distinct functions in host defense. Resident peritoneal cavity mast cells, a constitutive population, are critically required for innate defense against gram-negative bacteria in mouse models of experimentally induced peritonitis, partly because of their provision of TNF-a that is released by nonimmune contact between the bacterium and an as yet uncharacterized mast cell binding site (25,26). Moreover, the reactive intestinal mucosal mast cell hyperplasia that is a consistent feature of adaptive, T-cell–dependent immune responses to helminthic parasites is required for normal worm expulsion in several experimental models (43,70,288,289,290,291,292 and 293). It is therefore tempting to speculate that constitutive, non–T-cell–dependent mast cells participate in innate immunity, whereas reactive, T-cell–dependent mast cells function as effectors of adaptive immunity. The ultrastructural and immunohistochemical phenotypic alterations of mast cells that occur during helminth infections *in vivo* (111), and the changes in effector repertoire of mast cells that are induced *in vitro* by experimental cytokine perturbations (134,204,205,272), likely reflect the modification of their effector capacities in accord with the requirements of a given immune response. The observed differences among mast cell subpopulations may, in turn, reflect microenvironmental influences that are based on the needs of each tissue. The putative role of mast cells in tissue remodeling and in angiogenesis is another example of a potentially beneficial role in tissue homeostasis. The ability to augment innate immunity to gram-negative bacteria in mice by the exogenous administration of SCF (267) suggests that mast cells could be manipulated to augment their function to therapeutic advantage in immunity, revascularization, or wound healing.

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25 IMMUNE COMPLEXES

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Antibodies are the humoral products of the adaptive immune system. The physiologic activities of antibodies are to bind to foreign antigens and to promote their clearance from the body. Certain antibodies may also bind to effete and damaged cells, the cellular debris of the body, and may assist in physiologic waste disposal mechanisms. Antibodies engage their effector mechanisms only when they are bound to antigen in immune complexes. In this form, antibodies may bind and cross-link Fc receptors and may also activate the complement system. It is essential to understand the physiology and pathophysiology of immune complex formation to understand the biologic activities of the humoral adaptive immune system.

The effector activities of antibody vary according to the isotype of the antibody, as described in Chapter 13. There are three key differences among the various isotypes of antibody. The first is that different antibody isotypes predominate in particular body compartments. Thus, soluble immunoglobulin M (IgM) is confined mainly to the intravascular space and to mucosal surfaces, secretory IgA is the dominant isotype at mucosal surfaces, and IgG is distributed throughout the extracellular space. The second key difference is whether the antibody mediates its effects in the fluid phase or, as in the case of IgE, is cell bound, predominantly to the high-affinity Fc receptor for IgE (FceRI) on mast cells and eosinophils. The third is the distinction between whether a single antibody bound to multiple epitopes can mediate an effect, for example, a single molecule of IgM binding to a bacterial surface, or whether the binding of multiple antibody molecules to an antigen is required, as is the case for IgG.

In this chapter, we discuss the physiology and pathophysiology of immune complexes in five parts: (a) a consideration of the physiology of immune complexes; (b) the circumstances in which immune complexes cause tissue injury, focusing on human disease; (c) how immune complexes cause tissue injury; (d) the mechanisms for the removal of immune complexes from tissues and the circulation and abnormalities of these mechanisms; and (e) the clinical usefulness of immune complex measurements in clinical practice.

PHYSIOLOGY OF IMMUNE COMPLEXES

Removal of Antigen

It is helpful to consider two separate physiologic activities of antibodies. The first is the removal of antigen, which may be a bacterial toxin or a whole pathogen such as a bacterium or virion. Antigen is removed by the formation of immune complexes that are taken up by Fc receptor–bearing phagocytic cells. These cells engulf and destroy antigen contained in immune complexes.

Many immune complexes also activate the complement system. This activation leads to the covalent binding of C3 and C4 to the immune complex and provides further ligands for recognition by phagocytic cells. Activation of complement also leads to the generation of anaphylatoxin, which recruits inflammatory cells to the site of immune complex formation. Finally, activation of the terminal complement pathway, leading to the formation of the membrane attack complex, provides another mechanism for the killing of bacteria and cells that have been tagged by the binding of antibodies. The structure of immune complexes is modified after complement activation by the incorporation of covalently bound C4 and C3. This plays an important role in augmentation of clearance of immune complexes from tissues and from the circulation (see the discussion of clearance of immune complexes later in this chapter).

The second physiologic activity of antibody is as a feedback mechanism in the regulation of antibody responses. Under different circumstances, immune complexes may either augment antibody responses or inhibit them.

Immune Complexes and the Adaptive Immune Response

COMPLEMENT FIXATION TO SOLUBLE IMMUNE COMPLEXES AUGMENTS ANTIBODY RESPONSES

The innate immune system plays a key role not only in the efferent, or effector, pathways of the adaptive immune system, but also in the afferent pathways involved in the induction of antibody responses. Mice transiently depleted of complement with cobra venom factor had diminished IgG production in response to immunization with T-cell–dependent antigens, with reduced secondary responses and development of B-cell memory (1,2). Guinea pigs (3), dogs (4), and humans (5) deficient in complement components C2, C3, or C4 showed defects in primary and secondary IgG production in response to T-cell–dependent antigens. Similar results have been described in gene-targeted mice null for the expression of C3 and C4 (6).

These results are explained by two pathways in which immune complexes containing antigen, cognate antibody, and complement provide feedback, which augments and regulates antibody responses. First, evidence indicates that complement is crucial for the correct delivery, presentation, and retention of antigen in lymphoid organs. B cells and follicular dendritic cells express a series of complement receptors specific for the split products of C3, and these play an important role in localizing immune complexes that have bound C3 to these cell types. Follicular dendritic cells retain unprocessed antigen in the form of immune complexes (7), and they are thought to be involved in the generation of antibody responses within the germinal center and the maintenance of immunologic memory (8,9). Immune complex localization to germinal centers (10) and splenic lymphoid follicles (11) was abolished in mice depleted of complement with cobra venom factor and in rats treated with anti-C3 monoclonal antibody (12).

Second, complement, bound directly to antigen or to antigen-antibody complexes, lowers the threshold required for B-cell activation. Thus, the activation of B cells is enhanced through interaction of C3 with CD21 (13). The mechanism of this enhanced activation is mediated by the association of CD21 with CD19, a B-cell membrane protein that regulates B-cell responses, differentiation, and development (14). The threshold for specific B-cell activation *in vitro* was lowered when antigen was covalently coupled with C3d (13). Competitive inhibition or blockade of CD21-C3d interaction by soluble CD21-Ig fusion protein (15), or monoclonal antibodies with specificity for CD21 (16), abolished antibody production *in vivo.* Similar data were obtained from mice null for the expression of CD21, in which humoral immune responses to thymus-dependent antigens were reduced (17,18). Evidence that it was the absence of CD21 expression on B cells, rather than on other types of cells, came from chimeric mice in which CD21 expression was absent exclusively on B cells. In these animals, antibody responses to thymus-dependent antigens were reduced and were comparable to those in mice completely null for the expression of CD21 (19).

INTERACTION OF SOLUBLE IMMUNE COMPLEXES WITH FC RECEPTORS MAY ALSO MODIFY ANTIBODY RESPONSES

The observation that complement augments antibody responses led to the hypothesis that immunization with immune complexes may augment antibody responses. This hypothesis was shown to be the case (20), and it appears to be generally true for soluble immune complexes involving IgG. Data show that ligation of Fc receptors by the IgG component of soluble immune complexes may be as important as complement fixation in augmenting antibody responses. Fc receptors are primarily found on circulating leukocytes and cells of the fixed mononuclear phagocytic system. The three main groups are FcRI (high-affinity receptors for monomeric IgG), FcgRII, and FcgRIII. The last are lower-affinity receptors that bind mainly to IgG incorporated into immune complexes.

Experiments with Fc receptor null mice showed that the enhancing effect of specific IgG on antibody responses depended on the presence of FcgRI and illustrated, by

contrast, the suppressive effect of ligation of FcgRII on antibody responses (21). The mechanism of enhancement may be by increased binding of antigen, in the form of immune complexes, to antigen-presenting cells. A similar enhancing effect on antibody responses was also seen in the presence of specific IgE antibodies and depended on the presence of the low-affinity FceRII receptor, CD23 (22,23). The effects of ligation of B-cell coreceptors on thresholds for B-cell activation have been reviewed (24).

Evidence supports a role for genetic variants of FcgRII in modifying disease susceptibility to systemic lupus erythematosus (SLE) in both mice (25,26 and 27) and humans (28,29). This raises the interesting possibility that malfunction or depressed activity of this receptor on B cells could impair the negative feedback effect of immune complexes on these cells and could contribute to the B-cell hyperreactivity that is characteristic of SLE.

ANTIBODIES TO PARTICULATE ANTIGENS MAY SUPPRESS OR ENHANCE ANTIBODY RESPONSES

In remarkable contrast to the enhancement of antibody responses by soluble immune complexes comprising IgG and antigen is the suppression of antibody responses by immune complexes containing IgG and particulate antigens, such as erythrocytes. Indeed, an example of the deliberate formation of immune complexes as therapy in medicine is the use of IgG anti-Rhesus D antigen in the prophylaxis against Rhesus hemolytic disease in the newborn (30). This is one of the major contributions of immunology to clinical medicine. If red cells are administered at the same time as specific anti–red cell IgG, no anti–red cell antibody response results. The mechanism of this occurrence is not fully understood, and three hypotheses have been considered. The first is that the binding of IgG anti-Rhesus D to erythrocytes leads to their clearance and destruction in the spleen without evoking an antibody response. The second is that the IgG anti-Rhesus D masks epitopes on the Rhesus D antigen from binding to specific Ig receptor on B cells. The third is that the IgG bound to the red cell ligates the inhibitory FcgRII receptor on B lymphocytes and directly inhibits B-cell activation. This possibility has been virtually excluded by the observation that IgG anti–red cell antibodies still inhibit antibody responses in mice genetically deficient in FcgRII receptor expression (31).

Completely opposite to the inhibitory effect of specific IgG anti–red cell antibodies on antibody responses to red cells is the enhancing effect on antibody responses of specific IgM antibodies to red cells (and also to parasites). In the case of IgM antibodies, there is no Fc receptor with specificity for IgM, and strong evidence indicates that IgM-mediated enhancement of antibody responses is mediated by IgM-induced complement fixation to the immune complex. Indeed, an important physiologic activity of IgM may be to enhance antibody responses to low doses of antigens by forming immune complexes that activate complement (32,33).

To summarize this section, the formation of immune complexes is a mechanism that modulates antibody responses. The usual consequence is an augmentation of antibody responses, with the notable exception of IgG antibodies directed against particulate antigens such as red cells, which inhibit the development of antibody responses. For further reading on this topic, the reader is referred to the review by Heyman (34).

CIRCUMSTANCES IN WHICH IMMUNE COMPLEXES CAUSE TISSUE INJURY

Immune complexes are a major cause of human diseases. These diseases may be acute or chronic, depending on whether the antigen within the immune complex is present transiently or chronically. We first consider acute diseases caused by immune complexes, as typically represented by serum sickness, and then chronic immune complex–mediated diseases, associated with chronic infection or autoimmunity.

Serum Sickness

Serum sickness is a transient, although sometimes severe, illness that follows a large systemic load of a foreign antigen, which is effectively cleared by the immune system. During the process of clearance, large amounts of immune complexes may form, either locally in tissues or systemically. Disease normally resolves spontaneously after the clearance and disposal of the immune complex load.

In humans, serum sickness is caused mainly by the medicinal administration of large volumes of foreign antigen, as drugs or systemically injected proteins. It was originally described by von Pirquet and Schick in patients injected with horse antisera to antigens such as diphtheria toxin (35). Now, serum sickness is most commonly seen in patients treated with antilymphocyte globulin (36), streptokinase (37), and drugs such as antibiotics (38,39).

The typical clinical presentation occurs approximately 10 days after administration of the inciting antigen, with a rash, which may be vasculitic and frequently has an urticarial element, indicating the activation and degranulation of mast cells. Other symptoms include fever, arthralgia, abdominal pain, and sometimes glomerulonephritis or neuropathy.

Chronic Immune Complex–Mediated Diseases

Serum sickness should be contrasted with the chronic diseases caused by immune complexes, in which the antigen is not effectively cleared; the antibody response therefore persists, and chronic organ damage occurs. Antigen may persist in two situations. The first is when the antigen is an autoantigen and, by definition, cannot be cleared. The second is when the antigen is foreign but persists. This may result from long-term and recurrent exposure to foreign exogenous antigen, for example, in hypersensitivity pneumonitis (also known as extrinsic allergic alveolitis), or to foreign antigen produced in the body, for example, in infectious diseases in which the immune response is ineffective in controlling the infection.

AUTOIMMUNE IMMUNE COMPLEX–MEDIATED DISEASES

The autoimmune diseases mediated by immune complexes may be divided into three categories. The first is the autoimmune cytopenias, in which an autoantibody response to one of the cellular elements of the blood leads to accelerated clearance of that cell from the circulation. Disease results from deficiency of the cell type, from anemia, neutropenia, or thrombocytopenia, and, less commonly, from the consequences of its accelerated destruction, for example, pigment gallstones in hemolytic anemia. These diseases may be regarded as special cases of immune complex disease in which the antigen is particulate, and the mechanisms of clearance of erythrocytes are analogous to those of the immune clearance of bacteria from the circulation (40). The clearance mechanisms of antibody-sensitized cells from the circulation have been studied in detail, initially as models to explore the mechanisms of clearance of incompatible erythrocytes (41,42). Indeed, antibody-coated erythrocytes were the first examples of “model immune complexes” to be injected experimentally into humans as probes for the clearance of immune complexes from the blood in health and disease (43). The following discussion considers the results of these studies in our description of experimental studies of immune complex clearance.

In the case of the spontaneous autoimmune hemolytic anemias and autoimmune thrombocytopenic purpura, a summary of our understanding of the mechanism of the accelerated destruction of these cells is as follows and is reviewed elsewhere (44,45 and 46). IgG autoantibodies cause accelerated clearance of cells in hemolytic anemia and thrombocytopenic purpura, mainly in the spleen. An IgG coating of between 200 and 15,000 molecules per erythrocytes causes splenic clearance by red pulp macrophages in the spleen; at higher levels of IgG coating, some hepatic clearance of cells occurs (47). Some cells are cleared completely by uptake by phagocytes; others have small pieces of membrane “pinched” from their surface, and this reduces the ratio of surface area to volume of the cells (48). This provides the explanation for the presence of spherocytes in autoimmune hemolytic anemia. The identity of the autoantibody subclass also affects the severity of the anemia. The most severe hemolysis is associated with IgG3 autoantibodies, followed by IgG1, then IgG2; IgG4 autoantibodies cause minimal hemolysis. This hierarchy corresponds to the binding hierarchy of these antibody subclasses to Fc receptors IIA and IIIA (49,50).

In some cases, removal of the spleen results in near-complete resolution of disease, in spite of continuing autoantibody production. This finding illustrates that other organs, such as the liver, loaded with Kupffer cells, that carry Fc receptors, are less able to extract IgG-coated particles from the blood. There is also direct evidence for the involvement of Fc receptors on cells in the spleen in the cellular clearance process because anti-Fc receptor antibodies can block the clearance of IgG-coated erythrocytes and platelets (51).

IgM autoantibody-coated cells, as found, for example, in cold agglutinin disease, are processed in a different fashion by the mononuclear phagocytic system. There is no Fc receptor with specificity for IgM, but this isotype efficiently activates the complement system. The characteristic phenotype of erythrocytes in cold agglutinin is microspherocytic, with more than 10,000 C3dg molecules bound per erythrocyte. The liver is the main organ of clearance of erythrocytes in cold agglutinin disease (52,53). Many erythrocytes are delayed in transit through the liver, where portions of cell membranes are pinched from the cells. Cells that escape destruction are released back into the circulation with abnormal morphologic features and carry the final catabolic product of C3 cleavage *in vivo,* C3dg. These cells circulate for prolonged periods, an observation that shows that no clearance receptor exists for particles carrying C3dg in the circulation.

The second category of autoimmune immune complex–mediated disease is that in which the autoimmune response is restricted to a particular organ or to a limited range of organs. Examples are Goodpasture syndrome, membranous nephritis, myasthenia gravis, pemphigus, Graves disease, and chronic urticaria. These diseases illustrate several key and often overlooked concepts about the nature of inflammatory disease induced by immune complex formation. The first key principle is that the nature of an inflammatory response to immune complex formation may be fundamentally modified by the anatomy of the organ. In Goodpasture disease (54), the autoantigen is type IV collagen, which is widely distributed in basement membranes throughout the body (55). In the case of the kidney, because of the fenestrated

endothelium in glomeruli, the autoantigen is exposed to circulating autoantibody, and inflammatory glomerulonephritis is the cardinal feature of the disease. However, pulmonary disease develops only in about 40% of patients, in spite of the presence of the autoantigen throughout the alveolar basement membrane. Pulmonary hemorrhage is almost exclusively found in patients with pulmonary injury from smoking or, possibly, solvent exposure (56). This finding illustrates the importance of antigen accessibility for the development of immune complex disease. Only when alveolar basement membrane is exposed, for example, by neutrophil-mediated lung injury from smoking, can anti–glomerular basement membrane antibodies bind autoantigen in the lungs and cause potentially life-threatening injury.

The second key principle is that the nature of an inflammatory response may also be constrained by the anatomy of the target organ. This concept is illustrated by comparing Goodpasture disease with membranous nephritis. In the former disease, the autoantigen is fully exposed to the circulation, and the tissue injury is a severe inflammatory glomerulonephritis involving complement activation and cellular infiltration by neutrophils and mononuclear cells. In membranous nephritis and the animal model, Heyman nephritis, which has close similarities, the autoantigen is located beneath the glomerular basement membrane; in the case of Heyman nephritis, antigens are named megalin and receptor-associated protein on tubular epithelial cells (57). At this site, cells cannot penetrate the glomerular basement membrane, and immune complexes mediate tissue injury by activation of complement (58,59), which alters the filtration properties of glomeruli (58) and causes damaging protein to leak from plasma into the urine.

The third key principle is that the diseases mediated by autoantibodies to autoantigens restricted to particular tissues depend on the function of the particular autoantigen. Autoantibodies that bind to receptors may stimulate organ activity, as in the case of Graves disease in which antibodies to the thyroid-stimulating hormone receptor stimulate thyroxine production and cause hyperthyroidism, or in chronic urticaria, in which the autoantibodies are directed to the high-affinity FceRI on mast cells (60). Alternatively, the autoantibodies may lead to inflammatory destruction of organs, as occurs in myasthenia gravis, in which antiacetylcholine receptor antibodies are implicated in the destruction of motor neuron end plates on muscles.

The third category of immune complex disease resulting from an autoantibody response comprises a group of systemic diseases. These are caused either by production of an autoantibody to an autoantigen with a systemic distribution, such as essential mixed cryoglobulinemia, in that the autoantigen is IgG, or by the production of different autoantibodies that react with antigens in many tissues. Examples of this category of disease are SLE, rheumatoid arthritis, and Sjögren syndrome. Each of these diseases is considered elsewhere in this volume. Here we consider the evidence that immune complexes play a role in their pathogenesis and emphasize the heterogeneity of the effects of the autoimmune response, depending on the identity of the autoantigen.

Systemic Lupus Erythematosus and Other Systemic Autoimmune Diseases

Diseases such as SLE and rheumatoid arthritis, in which immune complexes are thought to be important in pathogenesis, are associated with prolonged autoantibody production driven by the presence of persistent autoantigen. In these diseases, immune complexes are formed in large antibody excess and tend to be large, complement-activating aggregates. The immobilization of these immune complexes in tissues, either from *in situ* formation or from deposition from the circulation, causes inflammation and organ injury. Complement is often portrayed as a villain in this situation, activated by immune complexes and contributing to inflammatory tissue injury. However, data suggest that an intact complement system may play an important role in limiting tissue injury in autoimmune disease by inhibiting the formation of large immune complexes and by promoting their disposal, together with the disposal of cellular debris such as apoptotic and necrotic cells, by the mononuclear phagocytic system (61). This protective role of complement may explain the association of inherited deficiency of classical pathway proteins of complement with the development of SLE. However, it does not exclude a proinflammatory role for complement in SLE, in circumstances in which immune complexes overwhelm the physiologic mechanisms for their clearance from tissues. Then, the proinflammatory effects of the anaphylatoxins and of the membrane attack complex are probably the main effector pathways of complement-mediated tissue injury (62,63).

Immune complexes are implicated in the pathogenesis of both renal and extrarenal injury in SLE. In animal models, anti-DNA antibodies localize under certain conditions in glomeruli (64). However, it is difficult experimentally to induce the formation of subepithelial deposits containing exogenous antigens by the infusion of immune complexes. Highly charged cationic, preformed immune complexes can produce this picture, possibly as a result of the attraction of cationic antigens or antibodies to oppositely charged anionic components of glomeruli, such as heparan sulfate and collagen type IV. The subepithelial localization of immune deposits may result from filtration forces, whereas granularity may be a consequence of the condensation of immune complexes containing polyvalent antigens into larger deposits, which may be detectable by electron microscopy (65).

In SLE, the main candidate antigens for involvement in immune complex formation in kidneys are double-stranded (ds)DNA and histones, especially when complexed as nucleosomes. Nucleosomes bind to glomerular basement membrane by charge interactions between their cationic histones and anionic components of glomerular basement membrane. Immune complexes containing nucleosomes may be deposited from the circulation, or immune complexes may form *in situ* by the binding of autoantibodies to nucleosomes trapped by the basement membrane (66).

CHRONIC IMMUNE COMPLEX DISEASES CAUSED BY FOREIGN ANTIGENS

Disease Caused by Extrinsic Foreign Antigens

Hypersensitivity pneumonitis (also known as extrinsic allergic alveolitis and in some clinical settings as farmer's lung) results in some patients from long-term exposure by inhalation to foreign antigens, and, if it is not recognized early enough, it may prove fatal. Important antigens include the spores of thermophilic actinomycetes, the usual cause of farmer's lung, and avian serum proteins, which cause bird fancier's lung. High levels of precipitating antibodies to the relevant antigen may be detected in serum of patients with pneumonitis, although similar precipitins may be detected in some persons in the absence of respiratory disease.

The typical pulmonary lesion in this syndrome is a granulomatous inflammatory response located around peripheral bronchioles. It has been modeled experimentally by inhalation of antigen or antibody into mice and other species that have been infused systemically with either antibody or antigen (67). However, this is an acute model of immune complex–mediated pulmonary injury and may not be a good surrogate for the chronic inflammatory process of extrinsic allergic alveolitis, in which evidence suggests that T-cell–mediated effects may be more important than the inflammatory consequences of immune complex formation (68).

Disease Caused by Infections

Some persistent bacterial and viral infections induce a host immune response, including an antibody response, that is not effective in eliminating the microorganism. The immune complexes that result from an antibody response in the context of the persistence of microbial antigens may play an important part in the pathogenesis of these infections. Bacterial infections at anatomic sites that are inaccessible to the host immune response are commonly associated with immune complex disease. An example is infective endocarditis resulting from chronic bacterial colonization of cardiac valvular structures, most commonly by streptococci (69,70 and 71). The clinical manifestations associated with the presence of the chronic immune response and immune complex production (72,73 and 74) include glomerulonephritis (75), rash, and splenomegaly. Infected ventriculoatrial shunts (76,77), Dacron grafts (78), and intravascular catheters and other access devices may cause a similar clinical syndrome. The antibody response to deep-seated bacterial infections resulting from *Staphylococcus aureus* and chronic respiratory infections in cystic fibrosis may be linked to chronic immune complex production, which may be associated occasionally with the development of arthritis and vascular inflammation (79).

A second category of infection that may cause chronic immune complex–mediated disease is persistent virus infections, in which infection persists in the presence of a strong antibody response. The chronic hepatitides, hepatitis B (80) and hepatitis C (81,82), and human immunodeficiency virus (83) are examples of such viral infections in which a specific antibody response coupled with persistent generation of antigen may cause immune complex disease, typically manifest as cutaneous vasculitis or glomerulonephritis. There are several models of chronic viral disease in animals, such as lymphocytic choriomeningitis virus, that, under certain circumstances, cause a prolonged disease mediated by immune complexes (84,85).

An important manifestation of hepatitis C infection is cryoglobulinemia, which may be associated with cutaneous vasculitis, peripheral neuropathy, or glomerulonephritis (86). The cryoglobulin may contain specific anti–hepatitis C virus antibodies, viral RNA, and typically IgM rheumatoid factor, which may be oligoclonal or monoclonal (87).

In addition to the development of chronic immune complex disease, the formation of immune complexes may also enhance some infectious diseases by targeting and enhancing their uptake by host cells carrying Fc and complement receptors. This is believed to be the explanation for the finding that dengue hemorrhagic fever and dengue shock syndrome occur most commonly after a second infection with a dengue virus that has a serotype different from that of the first. The presence of a subneutralizing antibody, which can not destroy the virus, leads instead to immune complex formation and uptake of the virus in the form of an immune complex through Fc receptors on macrophages, the main target cell for growth of the virus (88,89). Human immunodeficiency virus infection may similarly be enhanced by binding to Fc and complement receptors on macrophages (90).

HOW IMMUNE COMPLEXES CAUSE TISSUE INJURY

Studies of experimental models of disease mediated by immune complexes have elucidated many of the pathways of inflammatory injury. Two types of model system

have been studied. The first is a localized model of injury by immune complexes, known as the Arthus reaction. The second encompasses models of systemic immune complex disease: induced models of disease, typified by serum sickness, and spontaneous models, such as murine SLE.

Arthus Reaction

The most commonly used experimental system for studying the inflammatory effects of immune complex formation in tissues is the Arthus reaction. The original reaction described by Maurice Arthus was induced by the repeated injection of horse serum into rabbits (91). Initial injections of horse serum induced no reaction, but later injections induced an inflammatory reaction after several hours that was characterized by the presence of edema, hemorrhage, and neutrophil infiltration, which frequently progressed to tissue necrosis. The development of the reaction depended on precipitin formation in the blood, and the reaction could be blocked by systemic depletion of the precipitin by injection of large quantities of antigen.

Most investigators now use passive models of the Arthus reaction in which either antibody is infused systemically and antigen is given locally (passive Arthus reaction) or antigen is infused systemically and antibody is injected locally (reverse passive Arthus reaction). The passive Arthus reaction typically develops much faster than Arthus reactions produced by repeated antigen injection. A detailed account citing key references of early studies of the Arthus reaction is given in the classic textbook of Kabat and Mayer (92).

The traditional view of the mechanism of the Arthus reaction is of an inflammatory reaction caused by the formation of perivascular immune complexes, which activate complement. The consequences of the activation of complement are binding of C3 and C4 to the immune complexes, the production of anaphylatoxins by cleavage of C3 and C5, and generation of the membrane attack complex. The anaphylatoxins recruit and activate neutrophils and other phagocytes.

The location of the inflammatory response adjacent to small blood vessels results in activation of endothelium, which changes phenotype to allow the adhesion and emigration of leukocytes from the blood to the tissues. Activation of platelets and endothelial cells promotes local thrombosis. The Arthus reaction resolves by uptake and clearance of immune complexes by phagocytic cells through ligation of Fc and complement receptors. This picture of the Arthus reaction developed in studies of the reaction in experimental animals in which the inflammatory response was modified by depletion of cells or complement (93,94), as well as in animals with natural hereditary deficiencies of proteins such as C4 (95).

The development of gene-targeted mice with selective deficiencies in many proteins implicated in inflammatory mechanisms has led to a reevaluation of the mechanisms of the Arthus reaction, and also, to a tendency to reinvent the wheel by rediscovery of mechanisms that were well established with traditional techniques. The major shift in our understanding of the mechanisms of immune complex–induced injury has come from the use of mice with engineered deficiencies of selected Fc receptors or complement proteins. These studies have shown that the dominant mechanism of induction of inflammation by immune complexes in the Arthus reaction is by ligation of Fc rather than complement receptors (96), with FcgRIII and, to a lesser extent, FcgRI playing the major role. However, in some situations, complement may also play an important role in mediation of inflammatory injury in the Arthus reaction, as illustrated by experiments in mice with deficiency of the C5a receptor (97). New pathways of inflammation mediated by immune complexes have also been discovered, including a role for the substance P receptor (98).

The popularity of the Arthus reaction as a model for immune complex–mediated inflammation has also illustrated the extraordinary complexity of the inflammatory response to immune complexes (67). Most investigators use the reverse passive Arthus reaction, in which antigen is infused systemically and antibody (the reagent usually in shorter supply) is given locally into the tissues. This reaction can be induced in the lungs by intratracheal administration of antibody, in the skin by intradermal antibody injection, and in the peritoneum by intraperitoneal injection of antibody. Different mechanisms may be dominant at each of these sites. Moreover, differences exist in the dominant mechanisms of the Arthus reaction among species and, indeed, among various inbred strains of mice. For example, the Arthus reaction appears to be much more dependent on the complement system in rats and guinea pigs than in mice (99). Complement has a more important role in inflammation mediated by complement in CBA mice compared with C57BL/6 mice (100).

With respect to the role of different Fc receptor types, FcgRI was found to be critical in the mediation of an inflammatory response in immune complex–induced peritonitis (100). In contrast, another series of experiments showed that the expression of FcgRIII on mast cells was critical for the expression of the Arthus reaction (96,101,102 and 103).

Experimental Models of Systemic Immune Complex Disease

The Arthus reaction provides an important model to study local tissue injury by immune complexes developing in the vicinity of blood vessel walls. Many models have been developed to study systemic disease mediated by immune complexes.

Serum sickness may be induced in rabbits by the injection of a large dose of foreign antigen (104,105 and 106); the effects are similar to those seen in serum sickness in humans. With this model, it was possible to demonstrate immune complexes deposited in the glomeruli and to show that the site of immune complex deposition in the kidneys (mesangial, subendothelial, or subepithelial) varied according to the strength of the antibody response and according to whether repeated injections of antigen were given. In the presence of chronic antigen excess, the immune deposits were located in the subepithelium. These early studies were interpreted on the basis that immune complexes were passively trapped in the kidney, with the site within the glomerulus determined by the size and composition of the complex. However, it subsequently proved remarkably difficult to induce disease by the injection of immune complexes systemically into experimental animals; although it was possible to identify complexes in the mesangium and beneath the endothelium, deposits were not seen beneath the epithelium. Experiments with antisera raised against renal antigens that caused Heyman nephritis and nephrotoxic nephritis showed that it was possible to obtain subepithelial immune deposits when antibody was targeted to antigens within glomeruli (107). This led to the hypothesis that subepithelial immune complexes formed *in situ,* rather than being passively deposited from the circulation, and this hypothesis has been supported by studies (107).

Spontaneous models of murine lupus have provided additional important information about the mechanisms of tissue injury mediated by immune complexes. In particular, studies with mice null for the expression of Fc receptors and complement proteins have, in general, shown similar results to studies of the Arthus reaction. Thus, New Zealand B/W mice lacking Fc receptors I and III had antibody deposition with complement activation in the kidneys but no inflammation (108). Parallel to these results, mice lacking complement proteins C1q, C2, and factor B developed inflammatory glomerulonephritis with antibody deposits in the glomeruli in the absence of glomerular complement deposition (109).

CLEARANCE OF IMMUNE COMPLEXES

The physiologic clearance of immune complexes is facilitated both by their interaction with Fc receptors on cells of the fixed macrophage system and as a result of activation of the complement system. The mechanisms of clearance of soluble and particulate immune complexes are similar, although they differ in some important respects. Because of these differences, we consider the mechanisms of clearance separately.

Clearance of Soluble Immune Complexes

The interaction of soluble immune complexes with the complement system has two main consequences. First, the binding of complement proteins and their incorporation into the complex lattice result in physicochemical modification of the complex, thereby influencing size and solubility. Second, opsonization of immune complexes by complement, particularly C3, facilitates the ligation of specific complement receptors on a range of cells, either in the circulation or in the organs of the mononuclear phagocytic system.

COMPLEMENT MODIFIES THE STRUCTURE OF IMMUNE COMPLEXES

In the 1940s, Heidelberger discovered that immune precipitates contained more nitrogen when they formed in normal serum than in heat-treated serum (110). This was the first demonstration that complement proteins may be incorporated into immune complexes. He then showed that the “particulation” or precipitation of immune complexes from serum was inhibited by complement activation. This phenomenon was rediscovered many years later by investigators developing radioimmunoassays, who observed that complement interfered with precipitation reactions (111,112). This interaction of nascent immune complexes with complement, described as “inhibition of immune precipitation,” is primarily mediated by activation of the classical complement pathway (113,114).

Activation of complement by immune complexes not only inhibits the formation of immune precipitates but also has the capacity to solubilize immune precipitates that have already formed (115). The solubilization of immune precipitates is mediated by the alternative pathway, in contrast to inhibition of immune precipitation, which requires classical pathway activation. The covalent binding of fragments of C4 and C3 is necessary to maintain immune complex solubility. This covalent binding involves the formation of amide bonds between C4A and IgG (116,117 and 118), and ester bonds between C3B and the IgG heavy chain (119,120 and 121).

Two complementary explanations have been suggested for the mechanism by which complement proteins modify immune complex solubility. The first is that the incorporation of complement into the lattice reduces the valency of antibody for antigen by occupying sites of interaction between antibody and antigen (122). The

second explanation is that complement incorporation interferes with noncovalent Fc-Fc interactions that promote the rapid aggregation of immune complexes (123).

Only antibody isotypes that activate the classical pathway can induce complement-mediated inhibition of immune precipitation. Thus, inhibition of immune precipitation occurs with immune complexes containing IgG and IgM, but not with those containing IgA alone (124). However, the capacity of an immune complex to activate the classical complement pathway does not parallel precisely its ability to incorporate C3b into the lattice. This is of some clinical relevance. For example, complexes of monoclonal IgM rheumatoid factor and IgG, found in patients with type II (mixed essential) cryoglobulinemia, deplete complement rapidly but do not incorporate C3 effectively into the complex (125).

The solubilization of immune precipitates is associated with covalent binding of C3b to precipitated immune complexes and is inefficient, requiring a considerable amount of complement activation. Investigators have estimated that solubilization of an immune complex requires approximately one molecule of C3b to bind per antibody molecule (126). Less than 10% of activated C3 binds covalently to an immune aggregate, and it follows therefore that considerable quantities of complement are consumed during solubilization. This process may result in formation and deposition of the membrane attack complex and generation and release of anaphylatoxins into the tissues. Alternative pathway amplification is required to generate the degree of complement activation needed for solubilization. Insoluble immune aggregates act as “protected” surfaces (127) on which factor H has reduced access to bound C3b in comparison with factor B, which favors the amplification of complement activation by the amplification pathway. All the proteins of the alternative pathway, including properdin, are required. Activation of the classical pathway alone is neither necessary nor sufficient to solubilize immune precipitates, although partial solubilization by classical pathway activation has been demonstrated *in vitro* with immune precipitates (128,129).

In summary, there are two important differences between the processes of solubilization of precipitates and inhibition of immune complex precipitation. First, the capacity of the complement system to inhibit immune precipitation is tenfold greater than its capacity for solubilization, a finding that probably reflects the ease of prevention of Fc-Fc interactions and lattice enlargement, compared with disruption of a lattice that is already formed. Second, differences exist in the proinflammatory potential of the two reactions, because inhibition of immune precipitation generates smaller amounts of the anaphylatoxins and of the membrane attack complex than does solubilization (130).

IMMUNE COMPLEXES ARE USUALLY TRANSPORTED THROUGH THE CIRCULATION ATTACHED TO CELLULAR RECEPTORS

Immune complexes that are formed within the circulation, or that enter it from tissues, have to travel some distance around the body before reaching one of the organs of the fixed mononuclear phagocytic system. In many species, immune complexes travel through the circulation attached to receptors on the surface of circulating cells, rather than free in the fluid phase in plasma. On arrival in the microcirculation of the fixed mononuclear phagocytic system, immune complexes are transferred from the carrier cell to fixed macrophages (e.g., Kupffer cells in the liver). The process of the binding of immune complexes to receptors on carrier cells was first described for complement-coated microorganisms in 1915 (131). Shortly, thereafter, investigators found that trypanosomes bound to platelets in immune rats and to erythrocytes in humans and other primates (132,133). The adhesion reaction was found to be mediated by complement (134,135 and 136).

These early observations were overlooked between the late 1930s and early 1950s, and no mention is made of complement-dependent adherence reactions in two major reviews of the complement system published during this period (137,138). The field of study was restarted in 1953, when Nelson rediscovered immune adherence reactions between human erythrocytes and opsonized treponemes and pneumococci (139). The complement-dependent nature of these reactions was confirmed, and the term “immune adherence” was first suggested. A major advance in understanding the molecular basis of these reactions resulted from the isolation, by Fearon (140), of the receptor responsible for the adherence reactions of human erythrocytes, the C3b/iC3b receptor (complement receptor type 1, CR1, CD35).

CR1 binds large immune complexes and plays a role in the transport of soluble immune complexes *in vivo* in primates (141). In humans and other primates, most of the CR1 in the circulation is located in clusters on erythrocytes (142). This spatial organization of CR1 on erythrocytes facilitates high-avidity interactions with ligand, in comparison with other cell types on which the receptor is expressed as cell-surface monomers. The absolute number of CR1 molecules per red cell is low, varying between 50 and approximately 1,000 receptors per erythrocyte in humans (143,144). In contrast, there are 5,000 to 50,000 receptors per neutrophil, depending on the state of cellular activation (145). However, erythrocytes vastly outnumber other cell types in the circulation. Therefore, the red cells, in primates including humans, play a key role in the binding and transport of C3b- and iC3b-coated immune complexes and particles through the circulation.

CR1 displays an inherited polymorphism of both structure and expression levels. Because of the role of this molecule in transporting immune complexes, the hypothesis has been tested that this inherited variation of CR1 may influence susceptibility to diseases mediated by immune complexes.

Two types of genetic polymorphism of CR1 exist, each of which causes differences in the transport of immune complexes. First is a structural polymorphism. Four alleles of CR1 have been characterized, with molecular weights of approximately 210 kd (F' or C allotype), approximately 250 kd (F or A allotype), approximately 290 kd (S or B allotype) and approximately 330 kd. This variation of approximately 40 kd between allelic variants is caused by variable internal repetition of the long homologous repeats that form the structural core of the receptor. The approximately 210-kd (F' or C) allotype has reduced binding affinity for C3b dimers, corresponding to absence of one long homologous repeat containing a C3b binding site (146). This rare variant appears to have an increased prevalence among patients with SLE (147), although it is uncommon even in this population.

CR1 also exhibits an inherited numeric polymorphism of expression on red cells in the normal population, with numbers varying between 50 and 500 CR1 per erythrocyte (136,148,149 and 150) . The molecular mechanism of this numeric polymorphism has not been established, but a restriction fragment length polymorphism within the CR1 gene is correlated with high or low expression of CR1 on red cells (149).

The discovery that patients with SLE expressed fewer receptors per cell than healthy persons raised the possibility that low expression of CR1 could constitute a disease susceptibility gene for the development of SLE. However, studies have demonstrated that low expression of CR1 in SLE and in certain other conditions associated with complement activation on erythrocytes or in the fluid phase results from acquired mechanisms (151). For example, the infusion of erythrocytes bearing “normal” numbers of CR1 into patients with SLE, hypocomplementemia, and low red cell CR1, during the course of therapeutic blood transfusion, resulted in a fall in CR1 numbers on the transfused cells (152).

IMMUNE COMPLEXES INTERACT WITH OTHER CIRCULATING CELLS AS WELL AS ERYTHROCYTES

As noted earlier, neutrophils have both Fc and complement receptors. The kinetics of the interaction between neutrophil CR1 and immune complexes is different from the kinetics of the interaction between erythrocyte CR1 and immune complexes (153). CR1-dependent binding of immune complexes to circulating leukocytes is not of major importance in immune complex processing in primates. Indeed, one of the primary roles of erythrocyte CR1 may be to prevent potentially harmful interactions among immune complexes, leukocytes, and vascular endothelium by maintaining the complexes attached to red cells within the central stream of the vessel. This hypothesis is supported by *in vitro* observations that erythrocyte CR1 can protect cultured human umbilical vascular endothelial cells from immune complex–mediated and neutrophil-mediated injury (154).

SOLUBLE IMMUNE COMPLEX PROCESSING: IN VIVO *STUDIES IN ANIMALS*

The cells of the fixed mononuclear phagocytic system clear the majority of immune complexes from the circulation. In most species, including primates, rodents, and lagomorphs, the tissue macrophages of the liver and spleen are the primary sites in the circulation for clearance of foreign substances (155) and immune complexes (156). Pulmonary intravascular macrophages are also found in pigs, cows, sheep, goats, and cats, and they are important in the clearance of particles (157) and soluble immune complexes (158).

Some important findings have come from experimental studies of immune complex processing *in vivo.* First, the sites of processing of immune complexes have been delineated. Second, the receptors on circulating cells that act as transport receptors for immune complexes have been characterized, as have the receptors on the macrophages of the mononuclear phagocytic system that are responsible for the removal of immune complexes from the circulation. Third, variables in immune complex size and composition have been shown to influence clearance pathways and kinetics. In particular, the type of antigen in the immune complex may profoundly alter immune complex clearance pathways. Fourth, immune complex uptake by the mononuclear phagocytic system may, in some circumstances, be saturable. Fifth, abnormalities of the processing of immune complexes have been identified in animal models of immune complex disease, particularly models of SLE. We briefly review some of these findings and note circumstances in which variation among different species has been characterized.

In mice, guinea pigs, and rabbits, soluble immune complexes, injected intravenously, were predominantly removed in the liver and spleen (159,160 and 161), as in humans (see later; however, the complexes were not transported in the circulation bound to erythrocytes, which, in these species, do not bear CR1). Platelets in these species carry C3b receptors, and in one study, rapid *in vivo* binding of immune complexes to platelets was observed after intravenous injection (159).

The physiologic importance in primates of erythrocyte CR1 in immune complex clearance was first shown in studies with baboons (162). In decomplemented animals, the immune complexes did not bind to red cells and were cleared more rapidly, depositing in organs other than the liver, including the kidney (163). The importance of

the complement system in immune complex clearance was confirmed in this model by the observations that IgA-containing complexes, which fix complement poorly, failed to bind to baboon erythrocyte CR1, were cleared rapidly, and were localized in organs other than the liver (164).

Direct evidence indicates that the antigen component and the character of the antibody have roles in determining the mode and kinetics of clearance of immune complexes in animals. In murine studies of the clearance of immune complexes containing as antigen either orosomucoid or ceruloplasmin or their desialylated derivatives (165), the asialoorosomucoid-containing complexes were cleared 20-fold more rapidly than those containing the sialylated molecule. Blocking studies showed that the rapid clearance phase was mediated by a hepatocyte carbohydrate receptor, now known to be a member of the scavenger receptor family.

The concept of saturation of the mononuclear phagocytic system originated in studies of the clearance of carbon particles. Intravenous injection of large amounts of particles saturated the capacity for their clearance in the liver and resulted in their deposition in other organs (155). Investigators hypothesized that a similar phenomenon may exist with respect to the clearance of immune complexes, and, as discussed later, the hypothesis developed that saturation or abnormality in the clearance mechanisms for immune complexes in humans could underlie susceptibility to autoimmune disease. In experimental models, investigators showed that the capacity of the fixed macrophage system to process complexes was saturable, after the injection of escalating doses of soluble immune complexes. After “saturation” of hepatic uptake, spillover of immune complexes into other organs was observed (161). After these studies, it was found that immune complex clearance in murine models of SLE was abnormal (166), with a particular defect in the ability of macrophages to take up and retain immune complexes effectively (167). However, no evidence of saturation of immune complex uptake in murine SLE was noted (168).

SOLUBLE IMMUNE COMPLEX PROCESSING: IN VIVO *STUDIES IN HUMANS AND ABNORMALITIES IN SYSTEMIC LUPUS ERYTHEMATOSUS AND COMPLEMENT DEFICIENCY*

A range of radiolabeled model immune complexes has been used to explore the processing *in vivo* of preformed, soluble immune complexes in humans. Three model immune complexes have been employed: heat-aggregated IgG (169), tetanus toxoid (TT)/anti-TT (170,71), and hepatitis B surface Ag (HBsAg)/anti-HBsAg immune complexes (172).

Broadly comparable results have been obtained with each of these model immune complexes. The immune complexes were cleared from the circulation after fixation of complement, binding to CR1 on erythrocytes, and clearance in the liver and spleen. In SLE and other hypocomplementemic states, the immune complex clearance followed a different pattern, with reduced binding to CR1 on erythrocytes (169,171), accelerated clearance from the blood by the liver, and reduced splenic uptake (173).

In a C2-deficient patient studied before and after therapy with fresh frozen plasma to reconstitute complement activity, there was no red cell CR1 binding and no uptake of immune complexes in the spleen before therapy. Both CR1 binding and splenic uptake of immune complexes reverted to normal after correction of classical pathway complement activity (173). This result illustrates the importance of the spleen in the clearance of particulate immune complexes. It appears that the spleen clears immune complexes only if they are delivered attached to red cells. By contrast, the liver is most efficient at clearance of soluble immune complexes and shows accelerated uptake of immune complexes not bound to erythrocyte CR1.

No evidence supports the concept that the mononuclear phagocytic system in patients with SLE is saturated. However, as was found in mice with SLE (167), hepatic retention of immune complexes was abnormal in patients with SLE (173), and the mechanism is not understood.

One criticism that may be leveled legitimately at these studies of immune complex processing is that they involve the exogenous administration of large immune complexes prepared *in vitro* in the absence of complement, which may not be representative of potentially pathogenic immune complexes forming *in vivo.* However, similar results have been obtained from studies of immune complexes formed *in vivo,* both in an animal model (160) and in human patients receiving radioimmunotherapy (174). In these studies, immune complexes that formed *in vivo* also bound to erythrocyte CR1, with hepatic and splenic clearance.

Clearance of Particulate Immune Complexes

CLEARANCE OF A POTENTIAL AUTOANTIGEN: ERYTHROCYTES

We previously discussed the mechanisms of clearance of erythrocytes from the circulation in patients with autoimmune hemolytic anemias. Erythrocytes coated with IgG or IgM autoantibodies have been used as experimental probes of the function of the mononuclear phagocytic system in patients with SLE and other diseases in which immune complexes play an important role in pathogenesis. Erythrocytes coated with IgG anti-Rhesus D show impaired clearance in the spleen of patients with SLE (43,175). A similar defect has been seen in patients with other diseases associated with the presence of high levels of immune complexes, such as Sjögren syndrome (176), essential mixed cryoglobulinemia (177), and acquired immunodeficiency syndrome (178).

This impaired splenic clearance of immune complexes was initially interpreted as being a consequence of saturation of the mononuclear phagocytic system by immune complexes, and one study showed reversal of the defect in patients with SLE after treatment by plasma exchange (179). However, other studies with soluble immune complexes described earlier failed to support the hypothesis of saturation of the mononuclear phagocytic system in autoimmune disease in mice or humans. Instead, it seems more likely that there may be a defect, either primary or secondary, in Fc-receptor–mediated clearance activity associated with SLE. This is seen in the spleen in the context of the clearance of IgG-coated erythrocytes and in the liver in the context of the impaired uptake and retention of soluble immune complexes.

MEASUREMENT AND CHARACTERIZATION OF IMMUNE COMPLEXES IN THE CIRCULATION

Many different assay systems have been described for the detection of immune complexes in the circulation (180). Some were based on the ability of immune complexes to bind the first component of complement, C1q (181,182 and 183). The detection of complement proteins associated with antibodies as a marker of immune complexes has been the basis for a family of assays for immune complexes. Raji cells are a CR2-positive lymphoblastoid cell line that has been used to detect immune complexes bearing iC3b and C3dg (184). Similarly, conglutinin, which binds iC3b (185), and monoclonal antibodies binding to neoantigens of C3 (186) have been used to capture the complement component of antibody-complement immune complexes. A different approach has been to take advantage of the macromolecular nature of immune complexes to identify immune complexes by precipitation by polyethylene glycol (187).

The most common clinical indication for the measurement of circulating immune complexes has been as an aid in the diagnosis and monitoring of SLE. Many groups have reported an increase in the prevalence of circulating immune complexes in SLE (181,182,188,189,190 and 191), and some evidence also indicates that higher immune complex levels correlate with the severity of disease. However, the difficulty in the interpretation of these assays is illustrated by studies that have shown little correlation between the results with different assays and the levels of immune complexes and disease activity (182,192).

A serious problem exists with the interpretation of results from studies that measured immune complexes with C1q used as a solid-phase ligand to “capture” immune complexes. This is an assay for anti-C1q autoantibodies and not for immune complexes at all. A significant proportion of the IgG binding to C1q was shown to be monomeric in size (193,194). Purified F(ab')2 fragments of the immunoglobulin containing the C1q-binding material had the capacity to bind to C1q, and investigators also demonstrated that this material bound not to the globular heads of the molecule (which bind to immune complexes), but to the collagen-like region of C1q. The conclusion from these observations is that the C1q-binding IgG, which cosediments with normal IgG, consists of autoantibodies to C1q. This conclusion has been confirmed by affinity chromatography (195).

One can rarely identify either specific antibodies or specific antigens in immune complexes recovered from patients' samples. Mixed essential cryoglobulinemia, in which IgM rheumatoid factor (frequently monoclonal) is found, represents an exception to this rule. In SLE, much effort has been devoted to the determination of the composition and size of immune complexes. In the serum, a range of autoantibodies is easily detected, but there is little firm evidence for the participation of these autoantibodies in fluid-phase immune complexes. DNA (both single-stranded and double-stranded) and chromatin are among the most important autoantigens in SLE, but the identification of circulating immune complexes comprising anti-DNA antibodies and DNA is extremely difficult (196,197,198 and 199).

Even in immune complex disease caused by the administration of an exogenous antigen, such as drug-induced serum sickness, it is exceptional to identify immune complexes containing the relevant antigen and antibody. However, there has been occasional success in patients with bacterial endocarditis or in patients treated with large doses of streptokinase (37).

In summary, the clinical use of immune complex assays for the diagnosis and monitoring of disease is not often useful, with the exception of essential mixed cryoglobulinemia. A major underlying difficulty may be that clinically important immune complexes have only a short transit time in the circulation before clearance by the mononuclear phagocytic system or trapping in tissues. This was illustrated by a study of the formation of immune complexes *in vivo* in human patients that showed the transient presence of immune complexes in blood (174).

SUMMARY

Immune complexes are of great physiologic importance. The effects of the antibody response are mediated by the formation of immune complexes. Most of the time, the formation of immune complexes leads to the removal of antigen and the augmentation of immune responses. However, immune complexes are also an important cause of disease, and their long-term formation in the contexts of infection and autoimmunity has major consequences. Much has been learned about how immune complexes cause tissue injury from the study of patients and experimental models of disease. The availability of many strains of mice with gene-targeted deletions of key proteins of the immune and inflammatory systems has altered our perception of some of the mechanisms of tissue injury mediated by immune complexes. In particular, strong evidence is now emerging that tissue injury is predominantly mediated by ligation of activating Fc receptors, whereas complement may be friend or foe, depending on the circumstances in which immune complexes form. Some intriguing abnormalities of Fc-mediated clearance of immune complexes in patients with SLE are not understood. Much remains to be discovered about the immunobiology and pathology of immune complexes in health and disease.

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26 COMPLEMENT SYSTEM

Michael M. Frank, M.D., and John P. Atkinson, M.D.

Complement is a collective term designating a group of plasma and membrane proteins that play a key role in host defense against infection. These proteins are expressed in all vertebrates, and the origins of this system predate the appearance of chordates in the animal kingdom. The complement system participates in both innate and adaptive immunity.

The complement proteins and their activation cascades serve two major functions (Fig. 26.1). First, they modify the membranes of microorganisms to promote the process of host defense. This is accomplished through opsonization and direct lysis of the organism. Opsonization refers to the coating of a target with large component fragments that are recognized by receptors on peripheral blood cells, tissue macrophages, and follicular dendritic cells (FDCs). This interaction leads to adherence and thereby immobilization of the opsonized material that facilitates phagocytosis and antigen (Ag) processing. In some situations, organisms may be directly lysed by the complement proteins. The *second* function is to promote the inflammatory response by liberating small peptide fragments from complement proteins. These peptides bind to their respective receptors to produce cellular activation phenomena such as mast cell degranulation, directed migration of motile cells (chemotaxis), and smooth muscle contraction. Thus, after complement activation, the leukocyte is directed to an inflammatory site and finds a readily ingestible target. These same events, if excessively engaged or improperly directed, such as by auto-antibodies (auto-Abs), mediate undesirable inflammation and tissue damage in autoimmunity. Some excellent books and monographs provide an in-depth discussion of the complement system (1,2,3,4,5,6,7 and 8).

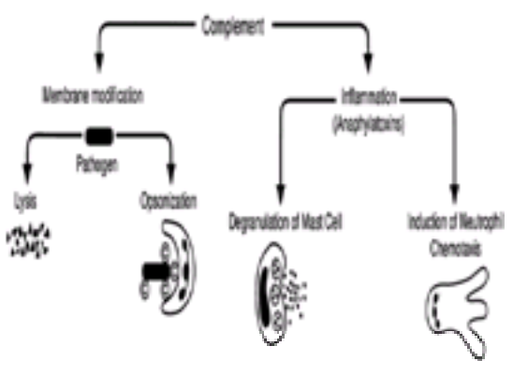

Figure 26.1. Function of the complement system. The most important function of complement is to alter the membrane of a pathogen by coating its surface with clusters of complement components (the phenomenon of opsonization). These components, in turn, facilitate interactions with complement receptors and lead to phagocytosis. In some cases, such as with certain gram-negative bacteria and viruses, the acting components induce lysis. The second function of complement is to promote the inflammatory response. The complement fragments C3a and C5a activate many cell types such as mast cells to release their granule contents and phagocytic cells to migrate to an inflammatory site (chemotaxis). (Modified from Lizsewski MK, Atkinson JP: *Primer on the rheumatic diseases,* 2nd ed. Atlanta: Arthritis Foundation, 1997, with permission.)

HISTORICAL ASPECTS

Before the discovery of antibiotics, bacterial infection was the major cause of death and disability. By the latter part of the nineteenth century, the role of specific bacteria in the pathogenesis of infectious disease was understood, and vaccination was becoming an established procedure. It seemed reasonable that a vaccination program for each bacterial pathogen would provide long-term protection and could become a general solution to the problem of infectious diseases. Analysis of the events responsible for the protective effect of vaccination against bacteria led to the discovery of the complement system (1,5,8,9).

In the 1880s, experimental pathologists demonstrated that the cell-free portion of blood lysed certain bacteria *in vitro,* and this killing property of serum was heat labile, destroyed at 56°C. This lytic factor was named *alexin.* In a classic experiment, a heat-labile factor in fresh serum of vibro immunized guinea pigs was capable of killing *Vibrio metschnikovii,* whereas the serum of control animals was not. Other investigators extended these studies to the peritoneal fluid of immunized versus unimmunized animals. Further, heat-inactivated cholera immune serum could afford protection when it was transferred to normal animals. Next, it was recognized that a heat-stable organism-specific factor (Ab) existed in the serum of immunized animals. When this was added to fresh normal serum (the source of the bactericidal or complement activity), it was able to kill the organism. The heat-labile bactericidal activity was nonspecific in that it was effective with a variety of organisms and was present in everyone. Thus, a heat-labile bactericidal factor was necessary to “complement” the heat-stable organism specific or acquired factor. In these early experiments, investigators correctly suggested that Ab combined with a specific site on a bacterial cell surface. Moreover, the Ab was correctly visualized as having a

specific Ag-recognition site as well as a distinct domain that was a binding site for complement.

At about the same time, blood transfusion specialists made a similar series of observations (10). Because the concept of blood groups had not yet been developed, some patients who received blood transfusions did well, whereas others had massive transfusion reactions. Several weeks later, when blood from a patient who had recovered from a transfusion reaction was examined, a heat-stable, specific material that recognized the transfused cells was found. In the presence of fresh serum, this factor produced lysis of erythrocytes. Thus, from these two sets of observations, the general concept of complement was born: *a heat-labile, innate, serum factor that allowed the heat-stable, acquired factor to manifest its lytic activity.* From the foregoing, it is easy to understand how the study of complement and Ab dominated immunology for the next 60 years. Moreover, the first autoimmune disease to be recognized as such in 1895 was a hemolytic anemia (paroxysmal cold hemoglobinuria) in which this same combination of Ab and complement was shown to mediate erythrocyte lysis (9).

Next, other investigations established that complement was not a single substance. Serum could be treated in a variety of ways that separated complement activity into two or more fractions. Mixing the two fractions restored activity. By the first half of the twentieth century, four distinct components of complement were recognized. During the 1960s and 1970s, techniques of modern protein chemistry allowed resolution of what was termed the *third component of complement* into six proteins acting in an ordered sequence (11). Today, we know that the lysis of Ab-sensitized erythrocytes or bacteria by the classical pathway (CP) of complement activation requires the ordered action of nine distinct components. In addition, two other pathways of complement activation and multiple inhibitors and receptors have been defined (Fig. 26.2 and Table 26.1).

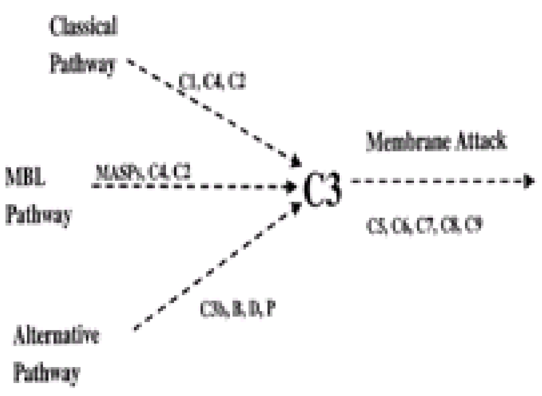

Figure 26.2. Pathways of complement activation. The three pathways of complement activation are shown. Each may lead to the activation of C3 and formation of the membrane attack complex. The classical pathway is triggered by antibody interacting with antigen, and the lectin pathway is activated by a lectin binding to a sugar. The alternative pathway is continuously turning over and becomes engaged only in the setting of a foreign material. Not shown are the anaphylatoxins, C3a, C4a, and C5a, which are liberated into the surrounding milieu. (Modified from Cooper NR. *Inflammation: basic principles and clinical correlates,* 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 1999, with permission.)

Native State	Activation Sequences	Regulatory	Receptors
Plasma	C1q, MBL	C1-INH	
	C1r, C1s, MASPs	C4bp, factor H	
	C2, B	C3a/C5a INA	
	D	S protein	
	C3, C4, C5	Properdin	
	C6, C7, C8, C9	Factor I	
Membrane[b]		DAF, MCP, MAC INH	C1qR C3aR, C5aR CR1, CR2 CR3, CR4

[a] [illegible]
[b] [illegible]
[illegible]

TABLE 26.1. Proteins of the Complement System[a]

After the initial discovery of complement, standardized methods were needed to allow investigators to study the system. First, a choice had to be made about which test particle to examine for lysis, because this was the only function of complement then recognized. The lysis of bacteria was technically difficult to study because the bacteria had to be grown to log phase and killing determined by colony counting. In contrast, lysis of erythrocytes was easy to study. The surviving cells could be sedimented by centrifugation, and the degree of erythrocyte lysis could be determined by measuring released hemoglobin in the supernatant with a spectrophotometer. Because it was believed that the lysis of erythrocytes and the lysis of bacteria were equivalent, the decision was made to study the lysis of erythrocytes. In a similar fashion, sheep erythrocytes were chosen because, unlike the erythrocytes of most other species, they are exquisitely sensitive to the lytic action of Ab and complement. The Ab used recognized the Forssman Ag on sheep erythrocytes. This lipopolysaccharide is widely distributed in nature and is expressed by bacteria, plants, and animals. The rabbit, however, is a Forssman-negative species and responds to this Ag by developing high-titer Ab. Therefore, an assay system evolved that employed two readily prepared reagents: rabbit Ab to the Forssman Ag and sheep erythrocytes.

As the source of complement, fresh serum from various species was screened. Because guinea pig serum had the most active lytic complement, guinea pig serum became the standard for complement tests and studies. Unfortunately, important functions of complement were missed because the model system (rabbit Ab, sheep erythrocytes, and guinea pig serum) is highly artificial, and studies were limited to defining the factors that were responsible for the lysis in this system. Thus, the opsonizing role and the finding that bacteria could also be lysed by complement through activation of a series of *distinct initiating* proteins (the alternative pathway; AP) were not appreciated until relatively recently.

TERMINOLOGY

The nine proteins of the CP are designated by the uppercase letter C followed by a number (Table 26.2). These numbered components generally act in numeric order, except C4, which acts before C2 and C3. Components of the AP are designated by capital letters (e.g., factor B). Regulatory proteins are designated by a descriptive title (e.g., C4 binding protein) or, in the case of the AP, a letter (e.g., factor H). Single components or multimeric complexes that have enzymatic activity are designated by a bar (e.g., $C1\bar{s}$). The loss of hemolytic activity by a component is usually designated by a lower case prefix (e.g., iC3b). Fragments generated during complement activation are designated by a lowercase letter suffix (e.g., C3a, C3b). Except for the fragments of C2, the a fragment is smaller and is liberated into the surrounding milieu, whereas the larger b fragment becomes cell bound and continues the cascade. Receptors were named in order of their discovery, but they also are commonly noted relative to their ligand specificity or CD number (4) (Table 26.3).

Component	Molecular Weight (kd)	Serum Concentration[a] (μg/mL)
Classical pathway		
C1	750	80
C1q	410 (6 × A, B, C)[b]	
C1r	85	
C1s	85	
C4	210	200–600
C2	110	20
Lectin pathway		
MBL	32	150 (wide range)
MASP-1	83	6
MASP-2	80	6
Alternative pathway		
Factor B	93	150–250
Factor D	25	1–2
Properdin	220	25
Central protein		
C3	196	550–1,200
Terminal pathway		
C5	190	70
C6	128	60
C7	121	60
C8	150	60
α-γ	64, 64	
β	22	
C9	79	60

[a] [illegible]
[b] [illegible]
[illegible]

TABLE 26.2. Components of the Cascades

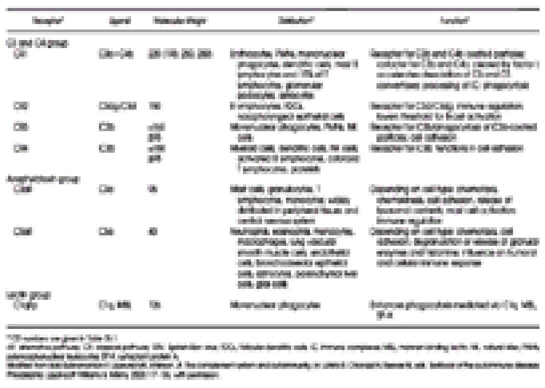

TABLE 26.3. Complement Receptors

CLASSICAL COMPLEMENT PATHWAY

Binding, Activation, and Control of C1

The *classical complement pathway,* so named because it has been a major focus of study since the beginning of the twentieth century, begins with the binding and activation of the first component of complement, C1 (Fig. 26.2 and Fig. 26.3). In this pathway, the Fc portion of Ab is responsible for C1 binding and activation, although other means (e.g., urate crystals, certain bacteria and viruses) to activate C1 in the absence of Ab have been described. The isotypes of human Ab that bind C1 are immunoglobulin M (IgM) and IgG, subclasses 1, 2, and 3. IgG subclass 4, IgD, IgE, and IgA do not bind C1. The mechanism of C1 binding and activation, after interaction of Ab with Ag, is unknown. In the case of binding, there may be a distortion in the Ab Fab region such that an interaction with Ag allows the C1q subcomponent access to the CH2 domain (12,13). Another hypothesis is that aggregation of Ig leads to the apposition of multiple C1 binding domains and resultant C1 binding (12,13).

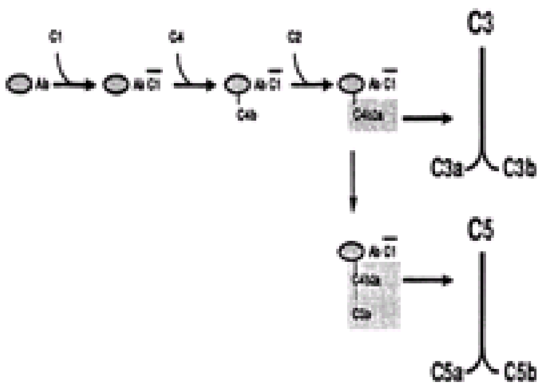

Figure 26.3. Assembly of complement convertases in the classical pathway of complement activation. Ab, antibody of immunoglobulin G (IgG) or IgM class; *circles,* antigenic target. The bimolecular C3 convertase and the trimolecular C5 convertase are *shaded.* These same C3 and C5 convertases are formed by the lectin and classical pathways. C5b continues the cascade by forming the membrane attack complex with C6, C7, C8, and multiples C9s (see Fig. 26.11). (Modified from Volanakis JE. XXXX. Volanakis JE, Frank MM, eds. *The human complement system in health and disease.* New York: Marcel Dekker, 1998, with permission.) Overview of the complement system, pp. 9–32.

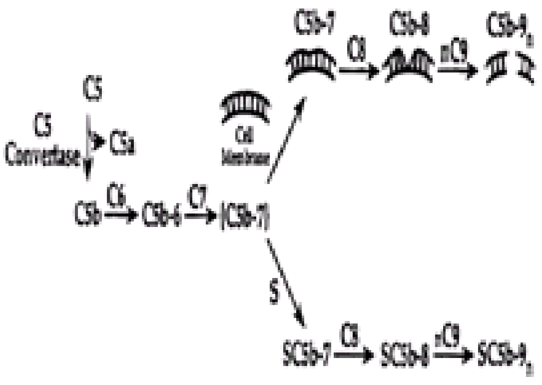

Figure 26.11. Assembly of the membrane attack complex. After the addition of C7 to the C5b-6 complex, a stable interaction with the membrane can be formed. The end result is the formation of lytically active C5b-9 (membrane attack complex) on target cell membranes or the inactive, soluble SC5b-9 complex. N, multiple (5 to 10) C9s join the complex; S, soluble (fluid-phase) complex. (Modified from Plumb M, Sodetz J. Proteins of the membrane attack complex, pp. 119–148. In: Volanakis JE, Frank MM, eds. *The human complement system in health and disease.* New York: Marcel Dekker, 1998, with permission.)

Two molecules of IgG, side by side (a so-called Ab doublet), act cooperatively to bind one molecule of C1. In contrast, a single molecule of IgM, bound to multiple Ag sites, is capable of binding and activating C1. These data point to conformational changes in the Ab molecules as well as cooperative binding of multiple Fc domains needed to form a C1 binding and activating site. A critical angle may be required among the Fab, Fc, and arms of C1 for successful activation to proceed.

The finding that two molecules of IgG side by side are required to bind one molecule of C1 has medical implications (Chapter 57) (14). Thus, sufficient Ag site density on a surface is necessary to allow two molecules of IgG to cluster sufficiently close to bind C1. On many surfaces, thousands of molecules must be deposited before an effective C1 fixing site is formed. The reason that IgG Abs to Rhesus (Rh) Ags, commonly detected in warm Ab (IgG)–mediated hemolytic anemia, do not activate complement efficiently is likely a consequence of the distribution of the Rh Ag on erythrocyte surfaces, rather than an unusual property of the Ab or complement. Alternatively, non–complement-binding Ig may intercalate between complement-binding IgG, thereby preventing effective doublet formation. This situation may occur in the meningococcal Ab response in which IgA can inhibit complement activation by IgG (14).

C1 is a complex macromolecule containing three individual protein subunits held together by calcium ion (Ca^{++}) (12,13). The largest of these subunits, C1q, binds to the Ag-Ab complex. C1q, an 18-chain molecule with six copies each of three A, B, and C chains, has a central core with six radiating, fibrillar arms (Fig. 26.4). The amino-terminal end of each arm is a triple-helical structure similar to collagen, and the carboxy-terminal end forms a globular or podlike domain. These structures form an ionic bond with the CH2 domain of IgG or IgM. By binding to multiple IgGs, C1q cross-links IgG bound to Ag and thereby stabilizes the Ag-Ab complex.

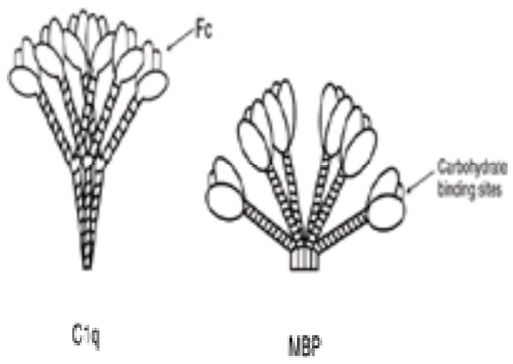

Figure 26.4. Molecular models of C1q and mannose-binding protein (MBP). The tuliplike structure protein of C1q consists of six major chains. The collagenlike sequences (shown by *cross-hatching*) adopt a triple-helical structure. Three highly homologous chains make up each of the six arms and give rise to three parts of each head. The C1$\bar{r}$ and C1$\bar{s}$ binding sites are located in the collagen regions, whereas the binding sites for the Fc regions of IgG and IgM are in the globular heads. A side view of the hexameric form of human serum MBP is shown in the panel on the **right.** In contrast to C1q, the three chains in each monomeric subunit are identical. Each head of MBP contains three C-type lectin domains. (Modified from Reid K. In: Volanakis JE, Frank MM, eds. *The human complement system in health and disease.* New York: Marcel Dekker, 1998, with permission.) Clay and mannose; binding lectin, pp. 33–48.

Associated with C1q in the presence of Ca^{++} are two chains of C1r and two chains of C1s, bound as a tetramer to the central core of the C1q. In unactivated C1, C1r and C1s are proenzyme serine esterases. Binding of C1q to the Ag-Ab presumably leads to distortion of the structure such that, in an unknown way, C1r is activated, and this, in turn, activates C1s. During activation, the two 85,000-d C1r and C1s chains undergo cleavage to a 28,000-d light chain and a 57,000-d heavy chain. The chains remain attached because of disulfide bonds. The enzymatic site on the C1$\bar{s}$ light chains cleaves the next two proteins in the complement cascade, C4 and C2.

Certain materials bind and activate C1 without the requirement for Ab binding to Ag (12). For example, monosodium urate crystals activate C1 in the absence of Ab and likely account for some of the inflammatory response in gout. Similarly, certain viral envelope proteins and bacterial surfaces bind and activate C1 in the absence of Ab. Moreover, complexes of polyions such as heparin and protamine can also activate C1. C-reactive protein (CRP), an acute-phase reactant, rises strikingly at times of inflammation (15). CRP, a lectin that binds to certain carbohydrates and phospholipids, attaches to the capsule of pneumococci and initiates the CP attack on the organism's surface. This protein, like many of the CP-initiating proteins, is a multimer, in this case featuring five identical subunits in cyclic symmetry. On one face of each subunit is a Ca^{++} containing the phosphocholine binding site and on the opposite face a cleft that contains the C1q binding site. CRP binds through multiple low-affinity interactions and then interacts with C1q to initiate the CP. Lectin-mediated activation is considered further in discussion of the mannan-binding lectin pathway.

C1 INHIBITION

C1$\bar{r}$ and C1$\bar{s}$ are regulated by the C1 inhibitor (C1-INH) (Table 26.4) (16,17 and 18). This protein binds stoichiometrically to the activated protease on each of the light chains of C1r and C1s and destroys their enzymatic activity. On binding of the inhibitor to C1, C1$\bar{r}$ and C1$\bar{s}$ are released into the fluid phase in the form of two C1r-C1s:$(C1\text{-}INH)_2$ complexes, whereas C1q remains attached to the Ab. This protease-antiprotease complex is highly stable and may be measured in plasma as a guide to C1 activation.

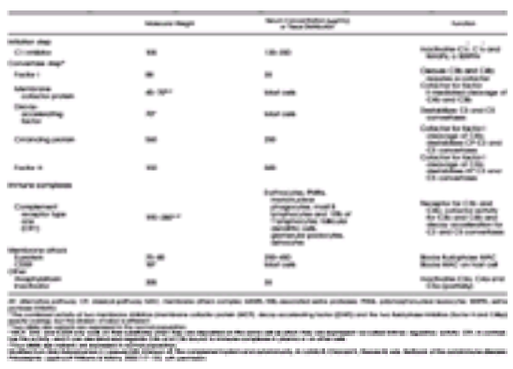

TABLE 26.4. Complement-Regulatory Proteins

The C1-INH is a member of the family of plasma inhibitory proteins called *serine protease inhibitors* (serpins) (16). Serpins provide a bait sequence that mimics the active site of substrate. When C1$\bar{r}$ or C1$\bar{s}$ cleaves the protease inhibitor at the bait sequence, a new functional site is revealed on the protease inhibitor that binds covalently to and thereby destroys the protease. In patients with hereditary angioedema, one of the two structural genes encoding for the C1-INH produces a nonfunctioning protein or no product. In this disease, patients are unable to control the activated C1 complex adequately (Chapter 30). The presence of the C1-INH has *no* influence on the activation of the CP by immune complexes. C1-INH is responsible for preventing fluid-phase activation and for degrading C1 after complement activation has occurred.

Binding and Activation of C4

The C1$\bar{s}$ subcomponent of C1 cleaves the next protein in the reaction sequence, C4. C4 is composed of three disulfide-linked chains (a, b, g) with molecular weights of 93,000, 75,000, and 33,000 d, respectively. On interaction with C1$\bar{s}$, C4 is cleaved with release of a 9,000-d C4a fragment from the amino-terminal of the a-chain. The larger C4b fragment couples covalently through an ester or amide linkage to a constituent of the cell membrane and then continues the cascade (Fig. 26.5). This remarkable ability to transfer from the fluid phase to a surface is the consequence of the action of a labile thioester bond and is essentially a nonreversible reaction. In association with C4 cleavage to C4a and C4b, C4b undergoes a major change in shape, and the internal thioester bond is broken. The reactive carbonyl species thus is immediately attached to a hydroxyl or amino group on the target of attack or becomes hydrolyzed.

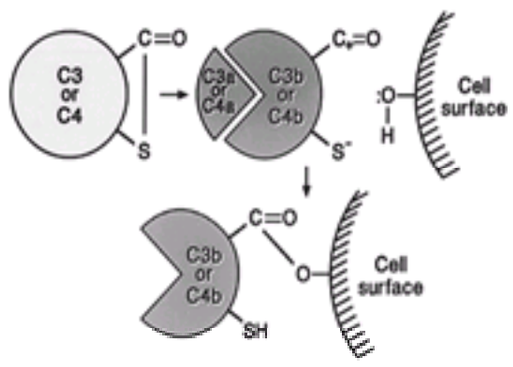

Figure 26.5. The thioester bond of C4 and C3. The activation of the thioester bond in C3 and C4 and its condensation with a hydroxyl group (formation of an ester bond). Amino groups on the target can also be engaged producing an amide linkage. (Modified from Hughes-Jones NE. The Classical Pathway, pp. 21–44. In: Ross GD, ed. *Immunobiology of the complement system: an introduction for research and clinical medicine.* Orlando, FL: Academic Press, 1986, with permission.)

Two C4A and C4B alleles code for C4 and give rise to four C4 genes in most persons (17). The proteins differ by as few as five amino acids in the a-chain. C4A tends to form amide bonds with targets, whereas C4B tends to form ester bonds. These two C4 alleles are associated with two human E blood group Ags, Chido and Rogers. The Chido and Rogers Ags are located in the C4d (thioester bond site) and are derived from plasma C4 (18,19).

Multiple C4 molecules are cleaved by C1, yet approximately 10% actually couple (the remainder are lost into the fluid phase) in a cluster about the Ag (where Ab is bound) site. Nevertheless, in this amplification process, each C1 fixed by Ab is amplified many times. For example, 10 to 20 C4s were bound per C1 in one well-studied example (20).

C4b deposited on a target is an opsonin, recognized by complement receptor type 1 (CR1), and thus is able to bind to a specific complement receptor, CR1, a process termed immune adherence. Further, certain viruses are neutralized by the deposition of multiple C4bs on their surface, and this prevents their binding to suitable host cells. The liberated C4a fragment is known as an anaphylatoxin and has weak proinflammatory activity. It is inactivated by the anaphylatoxin inhibitor (Table 26.4). C4b is inactivated in the fluid phase by the plasma regulator known as C4 binding protein (C4bp) and on self-tissue by membrane cofactor protein (MCP, CD46) (see later).

Binding and Activation of C2

C2 follows C4 in the CP cascade. This 105,000-d protein binds to C4b in the presence of magnesium ion (Mg^{++}). Once bound to C4b, C2 is cleaved efficiently by the C1s̄ subcomponent of C1, into two fragments, a small fragment C2b (34,000 d) and a large fragment C2a (74,000 d) (Fig. 26.3). The latter remains bound to C4b to form a new enzyme complex, the C3 convertase or C3 splitting enzyme. The enzymatic (catalytic domain) site resides on the C2a portion (a serine protease), and the C4b portion anchors the enzyme complex to the target. This bimolecular enzyme complex is unstable and decays spontaneously, with release of the C2a in an inactive form into the fluid phase surrounding the cell. The C4b is covalently bound to the cell surface and can accept another C2, which, in the presence of active bound C1, is then cleaved to reform the C3 convertase.

Regulation of the Classical Pathway C3 Convertase

The ability of C4b to bind C2 and to form a C3 cleaving enzyme is under strict regulatory control. C4b in the fluid phase is rapidly bound by the C4 binding protein (C4bp), which is a cofactor for a plasma serine protease factor I to cleave C4b (Table 26.4). After cleavage by factor I, the 45,000-d C4d and 170,000-d C4c fragments are rapidly cleared from the circulation. Further, C4bp binds C4b in the C3 convertase and, in so doing, displaces C2a (decay-accelerating activity), thereby destroying convertase activity. Like C1-INH, however, C4bp has no influence on CP activation on a suitable target.

Decay-accelerating factor (DAF or CD55) is a widely expressed GPI-linked membrane protein. DAF accelerates decay of the C4b2a enzyme (just like C4bp) and prevents this complex from forming (Fig. 26.6). DAF monitors host cells to dissociate convertases that form on the same cell in which DAF is expressed. DAF is one member of a group of GPI-linked proteins that are absent from erythrocyte membranes in patients with paroxysmal nocturnal hemoglobinuria (21,22). MCP is a widely expressed regulator that has cofactor activity for C4b bound to *self-tissue.* It patrols host cells and tissues and inactivates C4b. MCP acts intrinsically in that it only interacts with C4b attached to the same surface on which it is expressed. MCP serves as a cofactor (like C4bp) for factor I, and the cleavage fragments produced are as noted earlier. The C4d fragment remains covalently bound to the host cell (an immunologic scar). Thus, most cells of the body express DAF and MCP that function synergistically to prevent formation of the CP C3 convertase on host tissue (23).

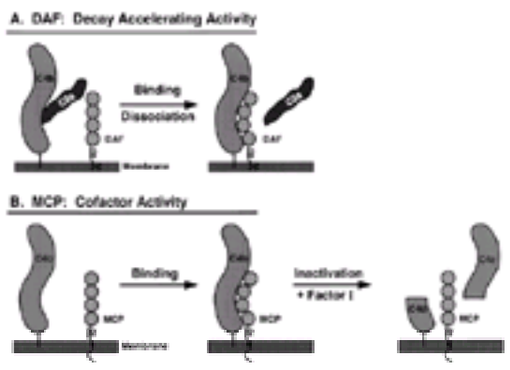

Figure 26.6. The classical pathway C3 convertase is shown. Decay-accelerating factor (DAF) displaces the protease, C2a, from C4b, and this C2a cannot rebind. To prevent C4b from interacting with a newly formed C2a, C4b is cleaved by membrane cofactor protein (MCP). The residual C4d has no known biologic activity. Classical pathway C5 convertase can be similarly inactivated. Moreover, AP C3 and C5 convertases are also disassembled in an identical fashion by DAF and MCP, except C3b is cleaved to iC3b rather than to C3dg.

CR1, a membrane-bound protein receptor for C4b and C3b, has both decay-accelerating activity for CP C3 convertase and cofactor activity for C4b (Table 26.4). This receptor is primarily expressed on peripheral blood cells including granulocytes, monocytes, B lymphocytes, approximately 15% of T lymphocytes, and erythrocytes of primates (see later). These activities likely serve to dampen complement activation by C4b- and C3b-bearing immune complexes and prepare them for transfer to immunocompetent cells in the spleen and lymph nodes.

MANNOSE OR MANNAN-BINDING LECTIN PATHWAY

A series of plasma, complement-activating, protein lectins (also known as collectins) have been defined that play a major role in innate immunity (24,25,26 and 27). The two best studied are CRP (discussed earlier) and mannan-binding lectin (MBL). Like C1q, MBL has a triple-helical “collagenlike” arms; however, instead of the arms terminating in an Ig Fc binding region, a lectin region binds to repeating polysaccharides on surfaces such as occur on microorganisms (Fig. 26.4). The polypeptide chains of MBL form oligomers, from dimers to hexamers. MBL attaches to the terminus of polymeric carbohydrate chains (mannose>GlcNAc>fucose>glucose). MBL, on interaction with an appropriate polysaccharide, binds to and activates one of three serine proteases with which it circulates in plasma, termed MBL-associated serine proteases 1, 2, 3 or MASP 1, MASP 2 and MASP 3. These serine proteases have many structural and functional similarities to C1r and C1s including Ca^{++} dependency and overall size and domain organization. On activation, MASP 2 cleaves C4 and C2, initiating the CP, just as C1 cleaves C4 and C2 (Fig. 26.2 and Fig. 26.3). The MBL pathway can be looked at as another means of activating the CP, but with its own initial proteases. MASP 1 and MASP 2, like C1r and C1s, are inactivated by the C1-INH.

The relationship of MASP 1 with complement activation is less clear. Evidence indicates that MASP 1 bypasses the early steps of the CP to cleave and activate C3 directly. Other studies suggest that MASP 1 activates MASP 2. MASP 3 has been described, and its function is still being investigated. This pathway was discovered in the 1990s, and much remains to be learned about the activation and regulation of its proteases.

C3 AND THE ALTERNATIVE PATHWAY

As discussed previously, CP C3 convertase (C4b2a) is responsible for binding and cleavage of the next component in the cascade, C3 (Fig. 26.3 and Fig. 26.7). C3, present in serum in the highest concentration of any component (approximately 1.2 mg/mL), is central to the function of the system (28). The major goal of the complement system is to deposit large quantities of clustered C3b on a target. For this reason, pathways converge at the step of C3 activation, and the steps up to C3 activation are characterized by an amplification process. Because the steps leading to the formation of AP C3 convertase mimic closely those of the CP, we now consider the AP in some detail.

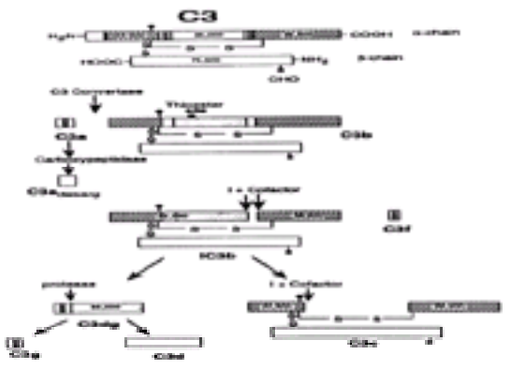

Figure 26.7. Activation and degradation of C3. The cleavage sites are indicated by *arrows.* Only disulfide bridges relevant to the degradation pattern are shown. The cleavages of C3b to iC3b by factor I require a cofactor. CR1, factor H, or membrane cofactor protein can mediate this function. Further cleavage of iC3b to C3dg by factor I is mediated by CR1. Several plasma proteases such as kininogen, trypsinlike enzymes, and others can cleave C3dg to C3d and C3g. The fragment bearing the thioester (C3b, iC3b, C3dg, or C3d) remains attached to the target. (Modified from Lambris J, and Sahu A. The Chemistry and Biology of C3, C4, and C5, pp. 83–118 (Chap. 5). In: Volanakis JE, Frank MM, eds. *The human complement system in health and disease.* New York: Marcel Dekker, 1998, with permission.)

The existence of a second pathway of complement activation was first suggested in 1954 by reports by Pillemer (29), who was interested in defense mechanisms in the unimmunized host (innate immunity). They reasoned that the components of the CP would be useful for protecting the host against infection once Ab had formed, but they thought that there must be a mechanism for defense against microbial invasion that was active before the host had time to form Ab. To explore this question, these investigators examined, in a remarkable series of experiments, the interaction with serum of an insoluble yeast particulate polysaccharide called zymosan. They discovered a factor that did not appear to be Ab but could activate complement. They named this protein properdin (from *perdere,* Latin for to destroy). Various cofactors were required for zymosan to remove this protein from serum, including a heat-labile factor and an ammonia-sensitive factor. Moreover, properdin-depleted serum had altered biologic activity in general. For example, it no longer sustained the lysis of many types of microbes *(in the absence of Ab),* or lysed the erythrocytes of patients with paroxysmal nocturnal hemoglobinuria, or neutralized certain viruses.

During the next two decades, the concepts of properdin and an AP of complement activation fell into disrepute. It was assumed that the experiments performed by Pillemer and associates reflected the depletion from serum of IgM (so-called natural Ab) antizymosan Ab that was required for complement activation. Restoration of this Ab to depleted serum would restore its complement-activating properties. Thus, properdin was thought to be a special type of Ab that was present in low titer in normal serum. The only remaining question was why absorbed serum failed to lyse a *variety* of microorganisms. If the antiyeast (zymosan) Ab cross-reacted with multiple types of microbes and cells, the failure of the absorbed Ab to lyse the microbes and cells would be explained. Later work proved conclusively that this line of reasoning was incorrect, and, during the late 1960s and 1970s, the theories of Pillemer (29), Muller-Eberhard & Schreiber (30) and colleagues were resurrected and reexamined (30). Series of proteins, similar to the early proteins of the CP, were rediscovered that constituted the AP.

Factor D

Factor D is a 24,000-d single-chain serine protease that cleaves factor B of the AP, analogous to $C1\bar{s}$ cleaving of C2 (Fig. 26.8). No evidence of activation exists for this protein, and it appears to function as a serine protease in its native state. However, it can only cleave factor B if factor B is bound to C3b. This protein is identical to a control protein in fat cells, adipsin (31).

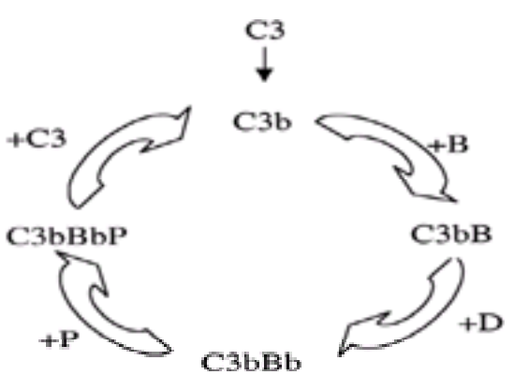

Figure 26.8. Feedback or amplification loop of the alternative pathway. If C3b is deposited on a target by the classical pathway or the lectin pathway, it then can engage the alternative pathway components shown here to generate more C3b. C3bBbP is the physiologic C3 convertase of the alternative pathway. The reaction cascade is anchored to the target by C3b. Factor D (a serine protease) cleaves factor B but only if it is bound to C3b. Properdin stabilizes the enzyme complex. The liberated Ba fragment is not shown.

Factor B

Factor B and C2 are approximately 30% homologous serine proteases, having arisen by gene duplication. As with C2, the catalytic activity is on the larger fragment of factor B (Bb) and requires Mg^{++}, like its C2 equivalent in the CP. Factor B is cleaved by factor D when it is bound to C3b, the C4b equivalent of the AP (discussed in the next section).

C3b: C4b Equivalent Protein of the Alternative Pathway

Having found a protein analogous to C1 (factor D) and one analogous to C2 (factor B), investigators believed that an AP protein analogous to C4 would also be discovered. In fact, such a protein was described during the initial studies of Pillemer (29). He defined a protein that, like C4, was sensitive to ammonia, did not require metal ions to act, and was necessary for complement activation to proceed. After a prolonged and difficult search, the protein analogous to C4 in the AP was shown actually to be C3. However, native C3 does not function in this capacity, and an understanding of the structure and function of C3 is required before continuing the discussion of the AP.

STRUCTURE AND FUNCTION OF C3

C3 is a two-chain glycoprotein with an a-chain of 110,000 d and a b-chain of 75,000 d (Fig. 26.7) (28). Within the a-chain of C3, as in the a-chain of C4, is an internal thioester bond formed between a cysteinyl and a glutamyl, just three amino acids apart (Fig. 26.5). The thioester bond between these two amino acids is highly reactive chemically and is easily attacked by nucleophiles. In the native protein, this linkage is buried within a hydrophobic pocket. If the thioester is hydrolyzed, C3 undergoes a conformational change. It then has about 60 microseconds for the reactive carbonyl group to participate in a transacylation reaction. If not, it interacts with H_2O to form $C3(H_2O)$. As such, it is no longer cleavable by AP or CP C3 convertase or able to bind covalently to cellular targets and thus has no hemolytic activity. However, the conformationally altered C3 [iC3 or $C3(H_2O)$] can interact with factor B in the presence of Mg^{++} to form a complex; in other words, it acts like cleaved C3 (i.e., C3b) (Fig. 26.8). Factor B bound to C3b or $C3(H_2O)$ can be cleaved by D to form the large fragment Bb. The complex of altered C3 [$C3(H_2O)$] or C3b with Bb, as with the complex of C4b with C2a, is an enzyme capable of cleaving native C3. This is AP C3 convertase.

Additional molecules of native C3 (with an intact thioester) are cleaved into two fragments by either CP or AP C3 convertase. The smaller 8,900-d fragment is called C3a, and the larger 185,000-d fragment is termed C3b. When C3 is so cleaved, the thioester bond is exposed. It can interact directly with an acceptor to form an ester or amide bond. The particle-bound or fluid-phase C3b, with its open thioester loop, can bind fresh factor B. Thus, two forms of C3, $C3(H_2O)$ and C3b, can act like C4b of the CP. Both bind factor B in the presence of Mg^{++} and, in the presence of a suitable factor B cleaving enzyme (factor D), the factor B is cleaved to form a C3 cleaving enzyme just as C4b binds C2 in the presence of Mg^{++} and the C2 is cleaved by C1 to form a C3 cleaving enzyme. Both $C3(H_2O)$ and C3b, which have bound

factor B, participate in AP convertase. The convertase formed interacts with native C3 (feedback loop) to lead to further deposition of C3b on the activating surface (Fig. 26.8).

As mentioned, the C3 internal thioester loop undergoes slow hydrolysis (30). The hydrolyzed molecule can bind factor B to initiate the AP. The generation of altered C3 is slow but constant. This slow hydrolytic step is likely responsible for the effects originally observed by Pillemer and colleagues. When an AP-activating substance such as zymosan is added to plasma in the absence of Ab, it is exposed to native C3. The internal thioester of the native C3 undergoes slow, spontaneous scission to yield altered C3 that can interact with B and D to form a C3 cleaving enzyme. A portion of the C3b generated by this enzymatic activity may bind to the activating surface, thus initiating and propagating complement binding to the target. The requirement for spontaneous hydrolysis of native C3 for initiation of the AP explains why this pathway requires high concentrations of serum and is relatively slow to activate. In these characteristics, it differs from the CP, which is efficiently and rapidly activated by Ab, even in dilute serum.

Properdin

To complete this discussion of the AP, we must discuss Pillemer's original protein, properdin. Properdin contains two to four 53,000-d subunits, held together by noncovalent interactions. Its function is to stabilize the AP C3 convertase by decreasing the rate of decay of $C3(H_2O)Bb$ or C3bBb. By stabilizing this enzyme, it allows continued activation of the AP (Fig. 26.8). Therefore, the key protein originally described by Pillemer and associates, properdin, is not central to AP activation scheme *per se,* but it is critical to “successful” activation by preventing rapid decay of AP convertase. In fact, without properdin, rarely, if ever, is the AP capable of depositing clusters of C3b on a target.

C3 Nephritic Factor

Under certain pathologic circumstances, an Ab is formed that recognizes C3bBb (32). This Ab, termed C3 nephritic factor (C3NeF), occurs at times in patients with systemic lupus erythematosus or partial lipodystrophy and more frequently in patients with membranoproliferative glomerulonephritis. The behavior of C3NeF is similar to that of properdin in that it stabilizes AP convertase. Such stabilization permits the convertase to remain (excessively so) in its active state, thereby allowing continued C3 cleavage. Thus, patients with C3NeF have markedly low serum C3 levels and a depleted AP system.

Function of Antibody in Alternative Pathway Activation

As is apparent from the reaction scheme, specific Ab to an Ag is not a requirement for AP activation. Nevertheless, Abs of many classes (IgA, IgG, IgM, IgE) facilitate AP activation (33). F(ab)'2 fragments of Ab trigger AP activation, in contrast to the CP, in which the Fc fragment is required. The mechanism of this effect is not clear. C3b bound to IgG resists degradation by factors H and I (34,35), so stabilization of C3b by IgG may explain part of its ability to augment AP activation. Other possibilities include alteration of physical or chemical properties of Ags by exposing or covering various chemical groups that make the Ags better acceptors for C3b. The heterodimer containing one or two molecules of C3b and one molecule of IgG has potent biologic effects, acting as a superlysin and a superopsonin (35). IgG is a preferred acceptor for C3b, so IgG-C3b complexes readily form at sites of CP activation.

The binding of C3b to a particle after C3 cleavage by either CP or AP convertase is inefficient (20). Many molecules of C3 are cleaved to C3b by each convertase, but only up to 10% of the C3bs successfully bind covalently to the particle surface. Those C3b molecules that do not bind are inactivated in the fluid phase by control proteins, namely, factor H in concert with factor I (as shown for another cofactor protein in Fig. 26.6 and Fig. 26.7).

Biologic Activity Associated with C3 Activation

As mentioned, the C3 convertases cleave native C3 into C3a and C3b (Fig. 26.7). The smaller C3a fragment is an 8,900-d peptide that is cleaved from the amino-terminal of the a-chain. C3a is a potent anaphylatoxin and binds to its receptor to produce cell activation phenomena such as histamine release from mast cells. The larger 185,000-d cleavage fragment, C3b, has many important biologic activities after it combines covalently with Ag surfaces. In addition to its ability to activate the AP and to form part of the C5 convertases (see later), C3b and its principal decay fragments comprise one of the two major opsonins (IgG being the other) of serum. Microbes or other surfaces coated with opsonins are more easily ingested by phagocytic cells bearing receptors for these ligands. Because this is a vital host defense activity and the most important function of the complement cascade, we next discuss the complement receptors that interact with C3 fragments.

COMPLEMENT RECEPTORS

The receptors and regulators of C4 and C3 are a family of closely related proteins (36,37 and 38) (Table 26.3 and Table 26.4). The regulator of complement activation (RCA) gene cluster is located on chromosome 1q 3.2 and consists of six proteins with related structural features (Fig. 26.9). C4bp and factor H are two plasma regulators, whereas DAF and MCP are two membrane regulators (Fig. 26.6). They have the same profile of regulatory activity, although the division of labor is slightly different. The other two members of this tight gene cluster are receptors and are discussed later.

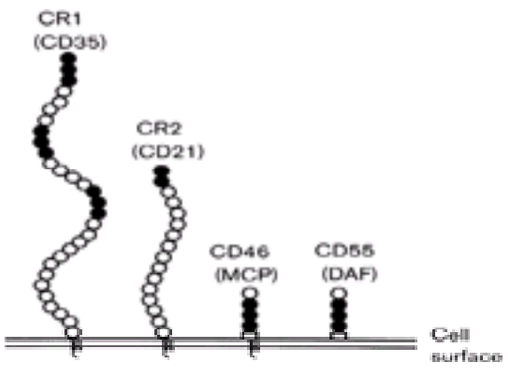

Figure 26.9. The structure of human complement membrane regulators and receptors in the regulator of complement activation family. The *circles* represent the approximately 60 amino acid repeating modules that are a prominent feature of the extramembranous portion of these proteins and contains the ligand-binding domains *(filled circles). Filled circles* indicate domains required for complement regulation and/or for binding of complement components. The *open boxes* at the juxtamembranous segment of CD46 and CD55 represent serine/threonine–rich regions that are extensively *O*-glycosylated. DAF, decay-accelerating factor; MCP, membrane cofactor protein. DAF is linked by a glycophospholipid, whereas CR1, CR2, and MCP are type 1 transmembrane proteins. (Modified from Lindahl G, Sjobring U, Johnsson E, et al. Human complement regulators: a major target for pathogenic organisms. *Curr Opin Immunol* 2000;12:44–51, with permission.)

CR1

CR1 binds C4b/C3b-bearing immune complexes (37,39,40 and 41). CR1 is a rodlike, 220,000-d (most common allelic form) protein whose extracellular domain is composed of 30 linked repeating modules known as short consensus repeats (SCRs) or complement control protein (CCP) repeats (Fig. 26.9). Twenty-eight of the 30 CCPs are grouped into 4 long homologous repeat (LHR) structures, each containing 7 repeating modules, that arose by duplication of a basic 7 CCP unit. CR1 has 3 binding sites for C4b and 2 binding sites for C3b. In addition to immune adherence (binding of C3b- and C4b-bearing immune complexes), CR1 also possesses cofactor activity for C4b and C3b and decay-accelerating activity for the C3 and C5 convertases. Cleavage of C3b to C3bi and then to C3dg or C3d is important for the proper handling and disposal of Ags (Fig. 26.7).

To facilitate further processing, these two C3b-derived products (i.e., iC3b and C3dg) can interact with other receptors (CR2, CR3, CR4) (see later). CR1, by interacting with C4b and C3b, also promotes the disassociation of CP and AP C3 and C5 convertases by displacing the C2a and factor Bb catalytic domains from the convertase. By this mechanism, CR1 may prevent unnecessary and undesirable complement activation by immune complexes.

The biologic activity of CR1 has been examined on phagocytes (39,40). CR1 is expressed on the cell membrane, but more is located on the membrane of endocytic vesicles. On cell activation, cytoplasmic CR1 is transported to the cell surface, thus leading to a severalfold increase in CR1 expression. Binding of C3b-coated particulate Ags, such as a C3b-coated erythrocyte, promotes adhesion efficiently but ingestion poorly. A second signal is required to initiate the phagocytic process. This second signal can be provided by IgG binding to the phagocyte Fc receptor, by a carbohydrate such as occurs on a bacterial surface binding to its lectin receptor, or by exposure of the phagocytic cell to the appropriate cytokines (Chapter 10). If a C3-coated Ag is bound to CR1 and then is endocytosed, it is sorted from the

endocytic vacuole and is transported into a phagocytic vacuole, where it is digested.

Erythrocytes express 100 to 1,000 CR1 per cell, the average number (approximately 300) being an inherited characteristic (41). If immune complexes form in the circulation, they may activate complement and may lead to deposition of C4b and C3b. The complexes bearing sufficient C4b or C3b attach to cells expressing CR1. Numerically, the largest pool in the blood of CR1-expressing cells is erythrocytes. Complexes bound to erythrocytes are effectively removed from the circulation (presumably to prevent deposition in tissue sites such as the renal glomerulus). Erythrocytes bearing immune complexes traverse the sinusoids of the liver and spleen, where they come into close contact with fixed phagocytes (41,42,43 and 44). Phagocytes, which express CR1, CR3, and Fcg receptors, effect a transfer of the immune complexes to their surface. The erythrocytes then leave the liver and spleen freed of their complexes and ready for another round of immune complex binding and transfer. This transfer may be simply an increased affinity for the multiple receptors on macrophages, but there is also evidence for proteolytic cleavage of CR1 near its transmembrane domain. In addition to preventing deposition of immune complexes in undesirable locations, Ags in the immune complex are delivered to the lymphoid system for processing and an immune response.

CR1 of B lymphocytes and FDCs functions in the localization and processing of Ag that is bound by Ab and complement. Triggering CR1 on B lymphocytes induces lymphokine release (45). C3b receptors are also expressed on the surface of the epithelial cells of the human glomerulus (46). In patients with glomerulonephritis who have deposition of C3 fragments in the kidney, the C3b receptors are either occupied or partially destroyed and lose their activity. How this occupation of C3b receptors on glomerular cells relates to the development of glomerulonephritis and whether the receptors ordinarily play a protective role in the kidney are still unclear.

As discussed in Chapter 57, the function of complement receptors and the concepts of first and second signals controlling phagocytosis are important for understanding the pathophysiology of diseases such as immune-mediated hemolytic anemias. In summary, in both *in vitro* and *in vivo* studies, particles coated with C3b are more easily ingested by phagocytes, but C3b is usually not a sufficient stimulus to initiate the phagocytic process. The phagocyte commonly requires one of several additional signals to proceed to ingestion of the membrane-bound particle.

CR2

The second complement receptor to be described, CR2, binds the complement degradation fragments iC3b and especially C3dg and C3d (47,48,49 and 50). This receptor is present on most B lymphocytes and FDCs but is not present on granulocytes, monocytes, or macrophages. Coligation of CR2 with the B-cell Ag receptor decreases the threshold for B-cell activation by approximately 100-fold (51,52). Further, the presence of C3 fragments such as C3d on an Ag increases its antigenicity many times (perhaps up to 10,000-fold) (52). It accomplishes this first by facilitating Ag localization and then by serving as a coreceptor for B-cell signaling through the Ag receptor. CR2 is also the receptor for the Epstein-Barr virus on B cells. The virus codes for a 350,000-d protein with a short amino acid sequence that mimics C3d and mediates binding to the CR2-expressing cell (53).

Factors H and I

Factor H is a 150,000-d plasma protein that consists entirely of 20 CCP repeating modules common to other members of the RCA class (54). It binds to C3b and possesses decay-accelerating activity for C3b-bearing convertases and cofactor activity for C3b. Factor I is a 90,000-d glycoprotein that consists of two disulfide-linked chains of 50,000 and 38,000 d. It is a serine protease that converts C3b to iC3b (Fig. 26.7). Factor I requires a cofactor protein such as factor H, MCP, or CR1. These cofactors are thought to induce a conformational change in C3b that exposes a cryptic site and thereby allows it to be cleaved by factor I. After cleavage by factor I, the cofactor is released from the complex. The cleaved C3b, known as iC3b, cannot bind factor B and therefore does not participate in the AP activation cascade. The catalytic domain of factor I is on the 38,000-d chain. The enzyme is an unusual serine protease because it is not inhibited by diisopropylfluorophosphate, soybean trypsin inhibitor, or other common inhibitors of this class of proteases.

In the common situation, C3b, attached to the surface of a foreign AP activator, binds factor B, which is then cleaved by factor D to initiate the AP (Fig. 26.8). Conversely, if C3b is bound to non-AP activator, factor H may preferentially bind and may thereby inhibit further AP function. When the AP cannot be efficiently activated on a foreign target, the surface has usually been modified to inhibit complement activation, and one mechanism for this is that it promotes factor H binding (54,55).

The chemical nature of the interaction that confers protected site status to Ag-bound C3b is unknown (54,55). In some cases, sialic acid, a charged sugar on many cell surfaces, is of key importance to this interaction. For example, sheep erythrocytes are not activators of the human AP. C3b deposited on these cells is rapidly inactivated by factors H and I. If the cells are treated with neuraminidase, which releases sialic acid, deposited C3b no longer rapidly interacts with factors H and I. Such cells, even if not sensitized with Ab, are rapidly lysed by AP activation. The strains of *Escherichia coli* and group B streptococci, which are dangerous pathogens especially for the newborn, contain sialic acid in their capsules and are poor activators of the AP. Similarly, *Treponema pallidum* has a sialic acid–rich outer coat. In other situations, microbes may escape complement activation by synthesizing their own complement regulators or by coating themselves with the host's own inhibitors (Table 26.5). To summarize, the most critical factor in allowing the AP to activate on a target is the absence of membrane regulatory proteins (in contrast to self-tissue); however, microbes have evolved mechanisms to inhibit AP by modifying their surfaces.

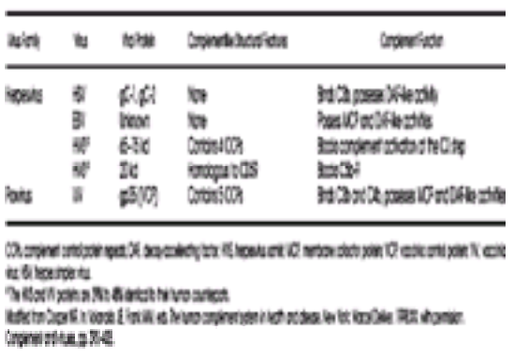

TABLE 26.5. Viral Gene Products Possessing Complement-Regulatory Functions

CR3 and CR4

Although unable to interact with the proteins of the AP, iC3b is of critical importance to host defense. Two receptors for iC3b (CR3 and CR4) are expressed on phagocytic cells. The receptors are two-chain proteins with a-chains of 165,000 and 150,000 d, respectively; both have an identical b-chain of 95,000 d. CR3 and CR4 are members of the b integrin protein family (56). The a-chain confers receptor specificity, whereas the b-chain directs the receptor into the proper configuration on the cell membrane. CR3 is expressed by phagocytes and there also exists an intracellular pool. Polymorphonuclear leukocyte activation induces its upregulation by inducing fusion of the CR3-containing vesicles with the outer cell membrane. A second signal for antigens coated with iC3b efficiently triggers ingestion. After complement activation, Ags become rapidly coated with clusters of C3b, many of which are then converted to iC3b and C3dg (see later). In this way, multiple receptors can be engaged to facilitate microbial destruction and Ag processing.

C3DG AND C3D

iC3b is converted by CR1 and factor I (cofactor activity) to C3dg (Fig. 26.7). Certain proteases, such as neutrophil elastase, can then cleave C3dg to C3d. Both C3dg and C3d interact with CR2 on FDCs and B lymphocytes. On B lymphocytes, CR2 serves as a coreceptor for the B-cell Ag receptor (47,48). On FDCs, CR2 facilitates Ag localization (49,50).

In summary, C3 is a central component of the complement system. The presence of C3b is required for AP activation and engagement of the feedback loop. C3 degradation fragments and IgG are the two major opsonins in blood and play an important role in immune adherence, Ag processing, and phagocytosis. Not surprisingly, the activation and degradation of C3 are under exquisite control. Limited cleavage to iC3b and C3dg is important in mediating phagocytosis and Ag clearance. C3b is also the entry to membrane attack complex (MAC), as part of the enzyme complex that cleaves C5. The components before C3 in the CP, lectin pathway, and AP function primarily as an amplification mechanism to produce clusters of C3b on a target.

Cobra Venom Factor

Since the beginning of the twentieth century, investigators have known that a protein in the venom of Egyptian and Asiatic cobras is capable of activating the complement system. Most animals including humans can be depleted of complement by injecting cobra venom factor (CVF). CVF is a cobra C3b homolog produced by glandular tissue in the venom site that functions like mammalian C3b (57). It differs, however, from mammalian C3b in that it is not efficiently *inactivated* by human regulatory proteins such as factors H and I; therefore, it continues to interact with AP components until the system is exhausted. Thus, CVF produces an AP convertase that cleaves C3 but cannot be properly regulated.

C5 CONVERTASES AND ANAPHYLATOXINS

The attachment of C3b to either CP or AP C3 convertase converts this enzyme to a trimeric complex capable of binding and cleaving C5 (Fig. 26.3 and Fig. 26.10). In the case of CP convertase, considerable evidence points to a preferred acceptor site on C4b for this C3b. C5 is cleaved by the C5 convertases into an 11,000-d fragment (C5a) and a large 190,000-d fragment (C5b) (see next section). C5a has anaphylatoxic activity and causes mast cells to degranulate and, further, is a potent chemotactic factor for phagocytic cells (58,59). C5a, on binding to its receptor on phagocytic cells, causes upregulation of CR1 and CR2, aggregation of neutrophils, and adherence of neutrophils to the surface of endothelial cells. Patients undergoing renal hemodialysis or cardiac bypass procedures may experience profound pulmonary dysfunction because the membranes activate the AP and thereby induce the generation of C5a (60). C5a causes neutrophil aggregation, and the neutrophil aggregates embolize to the lung.

Convertase	Classical or Lectin Pathway	Alternative Pathway
C3	C4bC2a	C3bBbP
C5	C3b / C4bC2a	C3b / C3bBbP

Figure 26.10. C3 and C5 convertases. C4b or C3b anchors the enzyme to the target. The catalytic domains are the serine proteases C2a and Bb. The C3 convertase becomes a C5 convertase when a C3b attaches to a preferred acceptor site on C4b or C3b. Properdin (P) stabilizes the alternative pathway convertases.

Another important biologic consequence of C5a generation is the directed migration of cells, particularly neutrophils, monocytes, and macrophages. When C5a interacts with these cells, it not only alters their membranes to induce adherence but also profoundly alters their migratory properties. The cells migrate along a concentration gradient to the site of complement activation. C5a plays a major role in the local response at sites of bacterial invasion and tissue injury (see later).

C5a is the most potent of the three complement-derived anaphylatoxins that cause mast cell degranulation (58). As with the anaphylatoxins mentioned earlier, C4a and C3a, the activity of C5a is regulated by carboxypeptidase N or Y, which cleaves an essential terminal arginine. Although these des-arg anaphylatoxins lose much of their anaphylatoxin activity, C5a des-arg, unlike the inactivated C3a, retains considerable chemotactic and neutrophil-activating activity. Evidence suggests that part of the effect of C5a des-arg results from its ability to bind to a cofactor, probably vitamin D binding protein in plasma, and, in this form, to mediate chemotaxis (61).

MEMBRANE ATTACK COMPLEX: FORMATION AND CONTROL

C5b is the initiating component of the MAC (Fig. 26.11 and Fig. 26.12). C5b is unstable and rapidly loses activity. Stability is conferred by the presence of C6, a C5b binding component that follows C5 in the cascade. In the presence of the next component in the sequence, C7, a C5b67 complex is formed. This complex is highly hydrophobic, and, if it is formed near the surface of a cell or bacterium with a lipid bilayer, it tends to associate with and insert into the lipid bilayer. The association is not covalent like C4b and C3b binding, but the proteins firmly attach to form a stable complex in the lipid bilayer through hydrophobic interactions. C8 and C9 interact with the membrane-bound C5b67, perturb the membrane, and produce lysis of some pathogens. If formed in the plasma at a distance from a lipid bilayer, C5b67 interacts with a plasma molecule termed S protein and is inactivated. The C5b67S-protein complex can interact with C8 and C9 but is unable to induce cell damage.

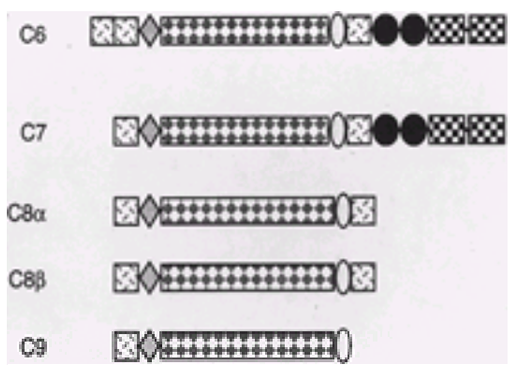

Figure 26.12. Modular structure of terminal pathway components. The structures become smaller from C6 to C9. From amino-terminal to carboxy-terminal, the proteins consist of one or two thrombospondin repeats, a single low-density lipoprotein receptor class A repeat, membrane attack complex (MAC) proteins or perforinlike segment, one epidermal growth factor–like repeat, another thrombospondin repeat, two complement control protein repeats, and last, in C6 and C7, two factor I/MAC modules. (From Morley BJ, Walport MJ. *The complement facts book.* San Diego: Academic Press, 2000, with permission.)

The molecular basis for the lytic activity of C5b-C9 continues to be intensely studied (62,63). C5b, C6, C7, C8, and multiple C9s form a macromolecular complex that resembles a cylinder, as visualized by electron microscopy. The complex has a hydrophobic outer surface and a hydrophilic central core. It tends to orient perpendicular to and within the lipid bilayer of the cell. The complex allows the free passage of water and small ions through the central hydrophilic channel, thus placing the inside and outside of the cell in communication. The cell cannot maintain its osmotic equilibrium, and it swells and lyses. In most cases, binding of C5b, C6, C7, and C8 is sufficient to cause a slow lysis of targets, including erythrocytes and some bacteria. C9 increases the rate of hemolysis. Purified C9 polymerizes when it is incubated in free solution to form a cylindric complex. It is believed that a small channel is formed in the cell membrane when C8 binds to the C5b67 complex. Multiple C9 molecules bind to the C5b678 complex. The function of C9 appears to be to bind and polymerize, thereby enlarging the pore and rapidly causing cell lysis. Although lysis of cells is emphasized in the foregoing comments, MAC insertion also produces cellular responses in many cell types in which lysis does not occur (64,65 and 66).

Even at the level of transmembrane lesion formation, the system is under considerable regulatory control. Investigators have long known that complement activation is less efficient in damaging host cells than in damaging cells from most other species. This capability, called homologous restriction, results from the presence of MAC inhibitor, CD59 (36,37 and 38). This GPI-linked membrane protein binds C8 and C9 and thereby inhibits their proper insertion into the lipid protein bilayer. Homologous restriction reflects the finding that CD59 is optimally designed to inhibit its own C8 and C9, not that of distantly related species.

COMPLEMENT IN THE INNATE AND ADAPTIVE IMMUNE RESPONSE

In 1974, Pepys reported that injection of CVF into mice to destroy normal plasma complement activity attenuated the animal's immune response (67). Klaus observed that CVF-treated mice did not generate memory B cells (68). In multiple studies, C4- or C2-deficient animals and humans did not respond efficiently to intravenous

administration of a phage Ag (51,69,70 and 71). Although producing IgM Ab, they did *not* show the normal class switch from IgM to IgG Ab or demonstrate immunologic memory on reexposure to the Ag. CR2 fused to the Fc region of IgG by molecular engineering techniques competes with cellular CR2 for C3 binding (71). By this interaction, it suppresses the Ab response to T-dependent Ags and inhibits normal isotype switching. Attachment of C3d to an Ag increases its immunogenicity by several logs and appears to serve as nature's adjuvant (52). By this mechanism, complement instructs the adaptive immune system to respond to certain Ags (Fig. 26.13). To summarize, C3d on the Ag surface instructs adaptive immunity. It links innate to adaptive immunity, amplifies the immune response, and facilitates the switch from an IgM to an IgG Ab, especially to T-cell–dependent Ags.

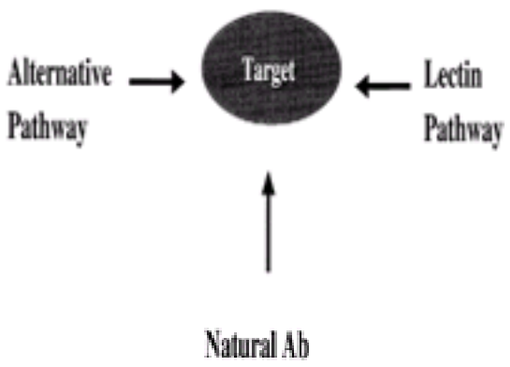

Figure 26.13. Activation of the complement system in innate immunity.

HOST DEFENSE FUNCTIONS OF COMPLEMENT IN CONTROL OF INFECTIOUS AGENTS

As discussed earlier, complement plays a key role in innate immunity. Whether by the AP, lectin pathway, or natural Ab and the CP, complement fragments are deposited on membranes of infectious agents, and the liberated fragments generate an inflammatory response at the site of contact. These events, in addition, provide instructions to the adaptive immune response, and once a specific Ab response is developed, complement is a major effector arm of Ab-mediated events.

Membrane Modification

DIRECT LYSIS

Complement forms membrane pores in susceptible bacteria and enveloped viruses (62,63). Although the exact mechanism of microbial killing is not known, in the case of susceptible gram-negative bacteria some evidence indicates that the formation of a lytic lesion triggers metabolic processes that, in turn, cause death. For complement to kill a bacterium, the organism must be in log-phase growth and not in stationary phase. Bacteria have developed mechanisms of avoiding complement-induced death. Gram-positive bacteria contain a thick peptidoglycan layer in the periplasmic space that precludes penetration by C5b-9 to the cytoplasmic membrane. Many pathogenic gram-negative organisms avoid complement lysis, an important prerequisite of spread in the circulation. For example, long-chain lipopolysaccharide appears to facilitate binding of active complement components far from the bacterial surface to avoid lysis. Numerous bacterial surface proteins also inactivate complement proteins or block their binding to preclude effective lesion formation. *Neisseria* species particularly require direct lysis for control because patients with late complement and AP deficiencies are at risk of disseminated meningococcal and gonococcal infections.

OPSONIZATION

Specific complement fragments, namely, C4b and C3b, deposit on an organism's surface, where they facilitate phagocytosis by interacting with specific receptors to immobilize the organism at the phagocyte surface. These fragments and their limited degradation products are critical for the host to control microbes. C3-deficient children develop a clinical syndrome of recurrent pyogenic infections, analogous to children with hypogammaglobulinemia and children lacking b integrins on their phagocytes (leukocyte adhesion defect), who also have frequent infections (Chapter 30).

Inflammation

Receptors for proinflammatory complement-derived peptides, C3a and C5a, signal the cell to induce movement (chemotaxis) or other cell activation phenomena. At one time, investigators believed that C3a and C5a receptors were largely restricted to granulocytes, monocytes, and mast cells (58,59). We now know that these receptors are much more widely expressed, including on endothelial cells and many types of epithelial cells (72,73). The cloning of C3a and C5a receptors and the preparation of gene-targeted mice lacking these receptors will allow a more definitive dissection of cell activation phenomenon mediated by C3a and C5a and of the role of these anaphylatoxins in health and disease.

EVASION OF COMPLEMENT BY MICROORGANISMS

In addition to the preceding examples, some microorganisms (including bacteria, viruses, fungi, and parasites) synthesize or acquire proteins that mimic the action of complement regulatory proteins (74,75 and 76) (Table 26.5). These virulence factors facilitate dissociation and degradation of active complement enzymes deposited on the surface of the organisms or bind components so they are not available to cause damage. Particularly noteworthy examples are the vaccinia CCP (77) and related proteins of other poxviruses and gC1 and gC2 of herpes simplex viruses (78). They possess cofactor and decay-accelerating activity, analogous to that of the host's own regulatory proteins. In some cases, the organism uses complement components or receptors to enter a host cell. Tuberculous and leprosy bacilli, *Leishmania,* and *Legionella* use limited complement deposited on their surface to attach themselves to phagocyte complement receptors and thereby to gain entrance (79) (Table 26.6). Mycobacteria have even been reported to synthesize a lipid that substitutes for C2a in the C3 convertase to facilitate further complement activation on their surface (80).

RCA Protein	Microorganism	Ligand in Microorganism
Membrane RCA protein		
CR2 (CD21)	EBV	gp350/220
MCP (CD46)	MV	Hemagglutinin
	Streptococcus pyogenes	M5 protein
	Neisseria gonorrhoeae	Pili
	Neisseria meningitidis	Pili
	Herpesvirus 6	Unknown
DAF (CD55)	*E. coli*	O-like antigens
	Picornavirus: Echovirus 7	Capsid
	Coxsackievirus A21	Capsid
Fluid-phase RCA protein		
C4BP	*Streptococcus pyogenes*	Some M proteins
	Bordetella pertussis	Unknown
Factor H	*Neisseria gonorrhoeae*	Porin
	Streptococcus pyogenes	Some M proteins
	Neisseria gonorrhoeae	LOS or porin
	Streptococcus pneumoniae	Unknown
	Human immunodeficiency virus type 1	gp41, gp120

DAF, decay-accelerating factor; EBV, Epstein-Barr virus; LOS, lipooligosaccharide; MCP, membrane cofactor protein; MV, measles virus.
[a] The I (Ig) -like binding domains of these proteins are composed of complement control protein (CCP) repeats and there is overlap (CR2 and EBV, CD46 and measles, and DAF and *Escherichia coli* or echovirus) between the physiologic ligands and pathogen binding sites.
Modified from Lindahl G, Stalhammar-Carlemalm M, Areschoug T: Surface protein virulence factors of Gram-positive pathogens: interaction and subversion of the human immune system. In Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, eds: Gram-Positive Pathogens, 2nd ed. Washington, DC: ASM Press, 2006, pp 478–496.

TABLE 26.6. Interactions Between RCA Proteins and Microorganisms[a]

BIOSYNTHESIS OF COMPLEMENT PROTEINS

Studies of the synthesis of complement proteins have proceeded largely *in vitro* in cell cultures and in established cell lines and, to a much lesser extent, *in vivo* in animals and humans. However, analysis of the polymorphic variants of complement plasma proteins after orthotopic liver transplantation indicates that they became largely those of the donor. Except for properdin (leukocytes) and C7 (granulocytes), components of the activation cascades and plasma regulators are synthesized in the liver (4). Nevertheless, peripheral blood monocytes, endothelial cells, tissue macrophages, fibroblasts, keratinocytes, and many types of epithelial cell types synthesize many different complement components and regulators. Synthesis at local sites is likely important for initiating an inflammatory response. Complement components are present in body fluids, in concentrations ranging from a few to 50% of that in serum. Especially low levels of complement proteins are found in

cerebrospinal fluid and bronchial alveolar lavage (BAL). Presumably, an extensive inflammatory reaction may be detrimental to normal organ function. On breakdown of the blood–brain or capillary–alveolar barrier, plasma proteins flood in and promote inflammation.

A major feature of the regulation of expression of many complement proteins in plasma relates to their being acute-phase proteins. Thus, under the direction of cytokines such as interleukin-1, interleukin-6, tumor necrosis factor-a, and others, complement protein synthesis may increase up to severalfold. These and other aspects relative to transcriptional control of complement proteins have been reviewed (81).

COMPLEMENT IN OTHER SPECIES

This chapter is specifically written to describe the human complement system. In general, the activating system is nearly identical for primates and other mammals as well as for birds, reptiles, and bony fishes. However, there seems to be more variation among receptors and regulators, probably because, as noted earlier, they have become the target of pathogens. Among mammals, components, inhibitors, and receptors can usually be substituted, one for the other (e.g., guinea pig for human and vice versa), but one-way or two-way incompatibilities have been noted. In general, the more closely related are the species, the more likely it is that the components will be compatible.

Some interesting differences between human and other species have been characterized, as follows:

1. Mouse complement is less stable (for unknown reasons) and less lytic; the lytic aspect is in part related to the inability to form an efficient C5 convertase except through the AP (82).
2. In the mouse, sex-limited protein (Slp) is a C4 homolog (approximately 90% identical) that has some interesting regulatory features and likely has functional activity (83).
3. There is tissue-specific expression of smaller size variants of CR1 on erythrocytes of many primates other than humans; this may relate to susceptibility to malarial infections (74).
4. In the mouse, CR1 and CR2 are expressed by a single gene with only the amino-terminal one-fourth of CR1 attached to the full-length CR2 gene (84).
5. In the rat and mouse, MCP is expressed predominantly in the testis (on the inner acrosomal membrane of spermatozoa), whereas in other tissues, a protein known as Crry, which is more closely related to CR1, is widely expressed and possesses the regulatory activity of MCP and DAF (84,85).
6. In New World primates, the first CCP repeat of MCP is spliced out, possibly related to the finding that repeats 1 and 2 are required for measles virus binding; in addition, MCP is expressed on most primate erythrocytes but not on human erythrocytes (74).
7. In the sand bass, a regulatory protein is expressed with structural and functional features of both C4bp and factor H, a finding suggesting that it is a precursor of the mammalian forms (86).

C FIXATION REACTIONS

Shortly after the discovery of complement, investigators recognized that the interaction of Ag and Ab was detectable by its effect of lowering complement titers. Thus, when Ag-Ab complexes are formed in the presence of fresh serum, the complement titer of the fresh serum falls, and the complement is said to have been fixed by the Ag-Ab complex. This test has been valuable, particularly in the study of infectious diseases, because it is sensitive, and neither the Ag nor the Ab needs to be available in purified form. Clearly, however, anything that lowers the complement titer of fresh serum is detected as an Ag-Ab complex. In most cases, this test has been replaced by enzyme-linked immunosorbent assay, flow cytometry, Western blotting, and related tests that often employ purified Ags and monoclonal antibodies to create greater specificity.

CLEARANCE OF DEAD, DAMAGED, AND APOPTOTIC CELLS AND OTHER CELLULAR DEBRIS

The complement system likely plays an important role in facilitating the removal of damaged cells (87,88). Natural antibodies, lectins, and the AP recognize altered surface characteristics of injured cells and, through opsonization, promote their efficient and safe removal (Fig. 26.13). Such a system may have evolved to promote clearance of nonviable or poorly viable tissue, especially traumatized skin and apoptotic cells. If this sytem fails, such as could happen in C1q or C4 deficiency, the individual may be predisposed to develop autoantibodies (87). Ischemia-reperfusion injury is likely to be a related situation (88). In this case, complement activation has been clearly shown to mediate damage of viable (but at risk) tissue surrounding a myocardial infarction.

IMMUNE COMPLEX HANDLING AND COMPLEMENT

The complement system is important for the processing and clearance of immune complexes (41,42,43 and 44). As immune complexes form, activation of the CP leads to C3b deposition that, in turn, prevents the immune complex from precipitating in a vessel wall or tissue site (so-called maintenance of immune complex solubility). The deposition of C3b on the Ab and Ag may reduce the ability of Ab to cross-link and therefore precipitate the immune complex. Even preformed immune complexes can be solubilized by exposure to fresh serum. The soluble immune complex, bearing clusters of C3b, then becomes bound to peripheral blood cells, especially erythrocytes. This phenomenon is called immune adherence. Erythrocytes possess more than 80% of the CR1 in blood and serve as a taxi or shuttle, taking the immune complexes to liver and spleen, where they are dissociated from the erythrocytes. Most immune complexes are destroyed in liver or spleen Ags. This transfer of immune complex from the erythrocyte to tissue macrophages may be mediated by simple affinity differences (multiple types of receptors with higher affinity for C3 fragments and IgG than CR1 on erythrocytes) and also by a proteolytic cleavage event that occurs near the stalk of CR1-bearing immune complexes. The erythrocytes then return to the circulation, minus a few of their receptors, for another round of immune adherence and immune clearance. This handling system for immune complexes evolved to keep the complexes from depositing in undesirable locations such as the kidney. Based on the foregoing discussion, it is apparent how this immune complex clearance mechanism could fail: (a) CP deficiency up to and including C3, (b) complement receptor deficiency, (c) synthesis of a non-CP fixing Ab such as IgG subclass 4 or IgA, and (d) hepatic dysfunction or after splenectomy.

COMPLEMENT IN THE IMMUNE RESPONSE

Investigators have known since the 1970s that complement depletion by cobra venom protein (see section on *in vivo* models) inhibits the immune response to a variety of T-dependent Ags (51,68,89,90). This is, perhaps, most strikingly demonstrated with the fX-174 bacteriophage in which animals deficient in C4, C2, or C3 have a weak IgM response, limited or no isotype switching, and poor immunologic memory (91). Further, Ab to CR2 decreases immune responses to T-dependent and T-independent Ags. B cells express CR1 and CR2 as well as B-cell Ag receptors. Complement binding to an Ag leads to C3dg on the Ag surface that can interact with CR2. An Ag with multiple C3dg peptides bound to its surface has a far higher affinity for the B-cell receptor because it binds the cells at multiple receptor sites (B-cell receptor or Ag receptor plus the possibility of multiple complement receptors). Thus, investigators have shown that C3d bound to Ag in model systems can increase antigenicity 100- to 10,000-fold (51,52,92). Moreover, Ag retention in germinal centers by FDCs is far more efficient when C3 fragments are bound to the Ag (93). This likely leads to continuous Ag presentation, driving the formation of Ag-specific B cells and memory cells. CR2 tends to associate on the B-cell surface with CD19, CD81, and Leu-13 (92). Aggregation of CD19 augments B-cell triggering and Ag with multiple CR2s may aggregate these accessory molecules to promote activation of B cells (47,48). Triggering of complement receptors may also induce the surface expression of cofactor molecules that facilitate the immune response. Finally, Ag with bound complement may promote B-cell and FDC interactions.

Animals with a targeted gene deletion of complement receptor CR1/CR2 have a decreased response to Ag and a failure to show normal isotype switching (49,50). Mice with the B cells lacking CR1/CR2, but with these same receptors present on FDCs, have poor initial responses to Ag and a failure to class switch (93). However, if the situation is reversed (FDCs lack CR1/CR2 but B cells are normal), then poor long-term responses and a lack of immunologic memory are noted (93).

The data, therefore, are overwhelming that complement plays a major role in augmenting the immune response and in instructing adaptive immunity. Further, C4-deficient mice and guinea pigs tend to make autoantibodies, and C4 deficiency on a predisposing genetic background makes the autoimmune disease more severe (91,94,95). Investigators have shown that C4-deficient mice, but not C3-deficient or CR1/CR2 knock-out mice, fail to induce normal tolerance to certain model Ags (94). Thus, the early steps in the CP may be important in allowing tolerance, but how this works and why C3 does not appear to be a factor are still unknown. That persons who are deficient in C1q and C4 have a strong tendency to develop autoimmune disease goes along with a failure to develop tolerance (96,97). Perhaps intimately related are reports that C1q binds to apoptotic cells to facilitate their ingestion and destruction (97). If this proves important, C1q deficiency could therefore lead to failure to handle apoptotic cells properly and could allow self-Ags to be presented to Ag-presenting cells, with a consequent autoimmune response.

ANIMAL MODELS OF DISEASE

Many *in vivo* models have been established to examine the consequences of complement activation (95). In general, these models have explored the effect of complement activation (a) in mediating tissue damage in diseases featuring immune effector mechanisms, (b) in producing inflammation in more general terms, or (c) in enhancing the afferent limb of the immune response.

Deficient and Normal Animal Models

Colonies of mice deficient in C4, C3, and factor B or C receptors and regulators have been developed using the technology of homologous recombination (70). Even before these knock-out mice were available, guinea pigs deficient in C4 or C2 or with extremely low levels of C3, rats and rabbits deficient in C6, dogs deficient in C3, and pigs deficient in factor H had been characterized (95). Further, investigators discovered in the 1960s that many strains of inbred mice were deficient in C5. Experiments with all these species clearly indicated the double-edged aspects of the complement system. Thus, compared with sufficient mice, C5-deficient animals were approximately 100-fold *more* susceptible to being killed by pneumococci but approximately 100-fold less sensitive to auto-Ab–mediated tissue injury (95).

Normal animals have been compared with animals treated with cobra venom protein that had low to undetectable AP and C5-C9 values in many studies. These studies have been informative and have been corroborated by the gene-targeted animals. CVF is a strong immunogen in most species, and, after 3 to 6 days, an immune response on the part of the recipient animals usually leads to neutralization and elimination of the protein. Thus, in contrast to experiments with gene-targeted animals, these were short-term studies.

Results of research emphasize that it is essential to separate the effects of IgG Ab working through the myriad Fc receptors on phagocytes and B cells from those induced by the various complement proteins (98,99,100 and 101). Thus, for example, it has generally been believed that the Arthus reaction, an immunopathologic inflammatory reaction caused by the formation of Ag-Ab complexes in tissue, is mediated to a great extent by local complement activation. The immigration of neutrophils in this process is thought to be caused primarily by the local formation of C5a. This concept was challenged when it became possible to generate mice by homologous recombination with C3 deficiency as well as with FcgRI and FcgRIII deficiency. Investigators reported that IgG FcgRIII was required for Arthus reaction, but not complement (98). Careful studies have shown that this is model specific, with FcgRIII playing a central role in the Arthus reaction in the skin of mice (98), but complement being of critical importance in immune complex–mediated peritonitis in mice (99) and in immune complex–mediated lung disease in rats (100). Complement is also of major importance in the immune complex–mediated skin disease of guinea pigs and rats (101). Thus, the species, experimental conditions, and genetic backgrounds are of critical importance.

Investigators have generally believed that complement activation occurring during the formation of immune complexes in serum sickness is an important factor of renal damage and vasculitis. Data obtained in rabbit models and studies of humans support that conclusion, although the evidence is indirect (95,101,102). Serum sickness is generally considered to be an excellent model of systemic lupus erythematosus.

A useful model of complement-dependent cell and tissue lysis is that of Forssman shock (95,103). Forssman Ag is a lipopolysaccharide widely distributed in nature. Animals that are Forssman negative treat this Ag as highly foreign and develop a vigorous immune response. Sheep are a Forssman-positive species, and sheep erythrocytes are coated with Forssman Ag. The rabbit is a Forssman-negative species and responds to the injection of sheep erythrocytes with high-titer anti-Forssman Ab. On injection intravenously of this rabbit Ab into guinea pigs, a Forssman-positive species, the Ab travels from the vein into the lung, where it causes massive damage that is CP dependent, with alveolar capillary fluid leakage and hemorrhage. Phagocytes are not important in this cataclysmic reaction in which death occurs within a few minutes, but the lytic activities of complement appear to be essential. This model has been used, for example, to test the effect of complement inhibitors agents in preventing tissue damage (103).

Pigs with factor H deficiency die rapidly of progressive membranous glomerulonephritis resulting from unopposed activation and abnormal regulation of the AP (104,105). Large quantities of C3 fragments are deposited in the glomerulus. A deficiency in factor H has been implicated in hemolytic-anemia syndrome, especially in the familial forms in newborn humans (105).

Animal models have demonstrated alternative methods of complement activation (106,107,108 and 109). Thus, there clearly exists a mechanism for Ag-Ab complexes to activate C1 and to bypass either C4 or C2 of the CP to recruit components of the AP and to cause damage. The mechanism that bypasses C4 is clearly less efficient than that bypassing C2. Under ordinary circumstances, it would appear that these bypass mechanisms are not used. Investigators believe that these animal models, particularly those in mice in which sophisticated genetic manipulations of the immune system and the complement system are now possible, will provide many additional answers to the function of complement in immunopathologic states.

Gene-Targeted Mice

In the 1990s, C1q, factor B, factor D, factor B/C2, C3, C4, CR1/CR2, C5aR, DAF, and Crry were all deleted by targeted insertional mutagenesis (70). Except for Crry mice (see the section on reproduction later), which is an embryonic lethal phenotype, the deficient mice developed normally. C1q, C4, C2, C3, and CR1/CR2 mice demonstrated substantial defects in T-cell–dependent immune responses and had the expected reduction in immune complex clearance. The C1q-deficient mice (depending on the background strain) had enhanced humoral immunity and glomerulonephritis (lupuslike picture) with problems in clearance of apoptotic bodies (96,97). For many of the strains with defects in the CP or AP, defective clearance of bacteria and viruses was shown on challenge. The C5aR-deficient mice had a decrease in inflammatory cell infiltrates. These mice started to be developed in the mid-1990s, and over the next decade they will be used to clarify the role of complement in host defense against infection, in the immune response, and in autoimmunity.

RESISTANCE TO LYSIS

Many cell types are relatively resistant to lysis by homologous complement. This “resistance” is, in part, attributable to regulatory proteins, especially CD59. In addition, active metabolic processes have been identified that eliminate the MAC from the cell membrane. For example, MAC protein complexes may accumulate in clathrin-coated pits, may be endocytosed, and eventually may shed through vesicle formation (64,65). In the last process, a temperature of 37°C, Ca^{++} flux, and activation of kinases, phospholipases, and adenylate cyclases have been implicated. Several variations on these intracellular activation pathways and on the shedding process have been described, depending on the cell type investigated.

In several model systems, the first complement-mediated attack on a cell confers resistance to a second. The responses to MAC presumably protect the cells' integrity and alter the cells' physiology to better it to meet a stress situation more effectively. Some of these responses include the synthesis of arachidonic acid and oxygen metabolites (in phagocytes), mediator secretion, and expression of procoagulant properties through secretion of von Willebrand factor, tissue factor, and adherence promoters. In general, these responses can be viewed as self-protective, occurring in response to a danger signal, that is, MAC assault on membrane integrity. Finally, experimental evidence implicates MAC-mediated disease in conditions featuring complement activation such as rheumatoid arthritis, lupus, and several other immune-mediated glomeruli processes, and even in demyelinating syndromes. The availability of specific inhibitors such as the monoclonal Ab to C5 should facilitate our understanding of the role of C3b deposition as opposed to MAC in causing tissue damage in conditions featuring complement activation.

REPRODUCTION

Complement components, especially C3, are prominent in the secretions of reproductive organs, and, in some cases such as the rat, they represent the dominant secreted proteins of the uterus (110,111). Rat uterus is also responsive to estrogen. MCP and DAF are located on the inner acrosomal membrane of spermatozoa. DAF, CD59, and MCP are also present in seminal fluid, in which a substantial fraction is attached to prostasomes (112). Probably, in most locations, these membrane regulatory proteins in the reproductive tract are performing the same check on complement activation as they do elsewhere. Conversely, the inner acrosomal membrane location is also consistent with a more specific role such as in gamete binding through C3 and its receptors (113). DAF, MCP, and CD59 are abundantly expressed in the placenta and trophoblast cells early in fetal development. The importance of these regulatory proteins in placental protection was most dramatically shown by knocking out the DAF/MCP equivalent protein, Crry, in the mouse (85). This phenotype was lethal with embryonic death before day 10. Examination of the developing placental tissue demonstrated an inflammatory lesion with prominent C3 deposition. The phenotype was rescued by crossing these Crry-deficient mice with C3 knock-out mice.

CLOSING COMMENTS

By the 1970s, the attitude of the general immunologic community was that there just was not much left to be learned about the complement system. In part, this was because, as a result of advances in protein chemistry, the 20 or so proteins of the CP and AP had been purified, and their place in the activation cascade had been firmly established. It was also buttressed by a perception that complement did not play much of a role in the immune response, because it was primarily an effector system. However, the identification and characterization in the 1980s of at least 10 novel receptor and regulatory proteins began a renaissance of interest in the field that continues to the present. In particular, complement is now recognized to be an important identifier of foreignness and to play an instructive role in the adaptive immune response. Cellular, reproductive, and transplantation immunologists, biotechnology companies, and clinicians have come back into the field as they begin to recognize this expanded role for the complement system. Research on the complement system has been reawakened, there is much more to learn, and the future is bright for “a simple little proteolytic cascade” (114).

Chapter References

The initial eight references are comprehensive discussions of the complement system and are excellent initial sources for further information. The other references are primarily selected to provide a highly focused review or represent more recent developments.

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27 ARACHIDONIC ACID PATHWAYS

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Arachidonic acid pathways produce the eicosanoids, bioactive lipid derivatives that include prostanoids, leukotrienes, and lipoxins. Whereas other 20-carbon fatty acids may act as substrate for the eicosanoids, arachidonic acid is the most common fatty acid found in the sn-2 position of mammalian cell membrane phospholipids and is thus the major precursor of eicosanoids. Two major enzyme pathways catalyze different oxygenation functions of arachidonic acid to form the major classes of eicosanoids. Endoperoxide synthases (cyclooxygenases) form the intermediates for prostanoid biosynthesis, and lipoxygenases provide the intermediates for the formation of leukotrienes and lipoxins. Although all these lipid families are generally described as mediators of inflammation, their physiologic actions are directed to functions of both an autocrine and a paracrine nature.

This chapter reviews the current knowledge of the effects of the prostaglandins and leukotrienes in innate (inflammatory) and adaptive immunity. The characteristics of the genes, cDNA, and encoded proteins involved in eicosanoid biosynthesis, metabolism, and receptors are presented, and the phenotypic changes incurred as a result of gene manipulation of these molecules are addressed.

PREDOMINANT BIOLOGIC EFFECTS OF EICOSANOIDS

Eicosanoid functions are wide ranging and are frequently counterregulatory; however, pharmacologic analyses and antagonist studies in pathobiologic states have provided insight into some specific roles. At the cellular level, eicosanoid actions pertinent to physiologic and pathobiologic functions include ion channel activation (1,2 and 3), neural transmission, hormone release (4,5,6,7 and 8), cellular activation (9), adhesion (10), and structural remodeling (11,12 and 13). At the tissue and organ levels, homeostatic functions of the eicosanoids include regulation of sleep cycles, maintenance of gastric mucosa, vascular tone, blood clotting, ovulation and parturition, and immune responses (14,15,16,17,18,19 and 20) (Table 27.1). Studies with gene-manipulated mice have attempted to elucidate eicosanoid effects in the animal, but some concerns exist. First, in transgenic mice, protein expression leading to mediator generation at various sites may not represent a normal distribution. Second, in targeted gene disruption, a technique that seems particularly elegant, the absence of recognized phenotypes requires the imposition of pathobiologic stimuli to uncover a consequence of the defect, which is then rationalized in terms of some physiologic function. Clearly, the *in vitro* regulation and actions of lipid mediators cannot necessarily be extrapolated to *in vivo* situations. Hence, only the dominant inflammatory effects of eicosanoids and their presence in inflammatory conditions as determined by pharmacologic administration and gene-manipulation studies are briefly considered.

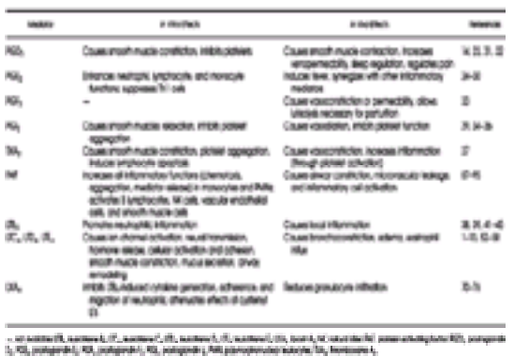

TABLE 27.1. *in Vitro* and *in Vivo* Effects of Eicosanoids

Prostaglandin D_2 (PGD_2), an important mediator of allergic responses, is synthesized by antigen-presenting cells and T-helper lymphocytes of the Th2 phenotype (21), and it is the major constitutive and inducible cyclooxygenase product of hematopoietic mast cells (22). PGD_2 constricts smooth muscle and thereby increases vascular permeability and reduces airway flow (23), regulates nociception, inhibits platelet aggregation, and induces sleep (14). PGD_2 is converted to 9a,11b-PGF_2 or to the J series of prostanoids, such as PGJ_2, d12-PGJ_2, and 15-deoxy-d12,14-PGJ_2. PGE_2 is notable for its biologic effects, which can be proinflammatory or antiinflammatory *in vitro.* Direct effects of PGE_2 on neutrophils *in vitro* are antiinflammatory, diminishing superoxide generation and leukotriene generation (24). In monocytes, PGE_2 impairs cytokine production, chemotactic migration, and adherence (25,26). PGE_2 also affects T lymphocytes; it suppresses Th1 cells relative to Th2 cells by lowering the production of interleukin-2 (IL-2) and interferon-g (27). Based on *ex vivo* culture systems, PGE_2 appears to contribute to bony erosions and periarticular osteopenia by stimulating osteoclastic activity and bone resorption and by inhibiting monocyte-induced proliferation of human bone cells (28). *In vivo,* however, there is considerable direct pharmacologic evidence for its proinflammatory potential, depending on the target cell. In the central nervous system, PGE_2 induces fever, and in the peripheral tissues it acts synergistically with other mediators such as bradykinin, C5a, histamine, and leukotriene B_4 (LTB_4) to cause typical features of early inflammation, including venopermeability and hyperalgesia (29,30) (Table 27.1).

PGF_2 functions as a potent bronchoconstricting agent in human airways; however, it appears to act minimally as a vascular agent in human skin (31,32). PGF_2 also appears to have a critical role in luteolysis, and failure of parturition is a dominant feature in mice lacking the PGF_2 receptor, as described later (33) (Table 27.1). PGI_2 induces smooth muscle vasodilatation and has an antiaggregatory effect on platelets, an effect counterregulated by the potent vasoconstrictor effects of PGF_2 and thromboxane A_2 (TXA_2) (34,35 and 36). The role of TXA_2 in cardiovascular physiology at the levels of both vascular endothelial cells and platelets balances the vasodilating and platelet-inhibiting effects of PGI_2. In addition to the hemostatic effect of TXA_2, there is enhancement of local inflammation because aggregating platelets release a variety of mediators. TXA_2 may also play a role in normal immune function in that it induces apoptosis of immature thymocytes *in vitro* (37) (Table 27.1).

The most notable effects of the dihydroxy leukotriene, LTB_4, based on *in vitro* and *in vivo* pharmacologic assays, are proinflammatory, including adhesion and directed migration of human neutrophils (38). On promoting chemotaxis, LTB_4 autoamplifies neutrophilic inflammation by mediating adhesion, migration, and accumulation of additional activated neutrophils and by stimulating degranulation (38,39). LTB_4 and the cysteinyl leukotriene, LTC_4, stimulate the development of bone marrow cells into both myeloid and erythroid progenitors (40). *In vitro* effects of LTB_4 on T cells include upregulation of the expression of the IL-2 receptor-b and cytotoxic activity (41,42). In monocytes, LTB_4 stimulates production of bioactive and immunoreactive IL-6 preferentially over IL-1 (43,44). B lymphocytes respond to LTB_4 with enhanced activation and immunoglobulin production (45). However, 5-lipoxygenase (5-LO), 5-LO–activating protein (FLAP), and LTA_4 hydrolase gene disruptions have no resting phenotypes, whereas each exhibits some attenuation in one or more models of inflammation. Leukotrienes have been implicated, on the basis of their presence and known effects, in inflammatory bowel disease (46), as well as in the skin lesions of psoriasis (47); they are also present in the synovial fluid of patients with gout, rheumatoid arthritis, and osteoarthritis (48,49,50 and 51).

The most dominant known effects of cysteinyl leukotrienes occur in bronchial asthma and relate to constriction of microvascular and airway smooth muscle that

increases venular permeability and attenuates pulmonary function (52,53). Constriction of bronchial smooth muscle in response to cysteinyl leukotriene inhalation, as in asthma, is dose dependent, temporally distinct, and more severe than that caused by histamine and methacholine (53,54). Additional features of asthma mediated by cysteinyl leukotrienes include submucosal edema from increased venular permeability (55), infiltration of eosinophils and lymphocytes (56,57), and changes of remodeling (58). Many of these features caused by leukotrienes are also present in patients with adult respiratory distress syndrome, chronic obstructive pulmonary disease, and neonatal pulmonary hypertension (59,60 and 61). The proinflammatory effects of cysteinyl leukotrienes are abundantly supported by the short-term and long-term clinical efficacy of antileukotriene drugs (62,63,64,65,66,67,68 and 69). Indeed, the relief of airway obstruction experienced by about two thirds of patients with asthma in the first hour after ingestion of these agents reveals a long-term overproduction of these mediators, because the antileukotriene drugs are not intrinsically bronchodilatory (66) (Table 27.1).

Lipoxygenase interaction products or *lipoxins* are formed by the transcellular metabolism of the respective 5- and 12-lipoxygenase-generated lipids. Lipoxins A_4 and B_4 (LXA_4 and LXB_4) function as endogenously produced chalones or "stop" signals that attenuate the effects of proinflammatory leukotrienes. They inhibit both LTB_4 and cytokine generation and the effects of LTB_4 such as adherence and migration of neutrophils and its attendant venopermeability (70,71,72,73,74 and 75). In a rat model of allergen-induced responses, LXA_4 reduces eosinophil trafficking (76) (Table 27.1).

BIOSYNTHESIS OF EICOSANOIDS

Release of Arachidonic Acid from Membrane Phospholipids

The generation of both prostaglandins and leukotrienes begins with an appropriate cell-activation signal that results in the release of arachidonic acid from cell membrane phospholipids through the action of phospholipase A_2 (PLA_2) (Fig. 27.1). Within the large family of PLA_2 isoenzymes, type IV has been established as essential to both the prostanoid and the leukotriene pathways, whereas types IIA and V have been implicated in amplification steps.

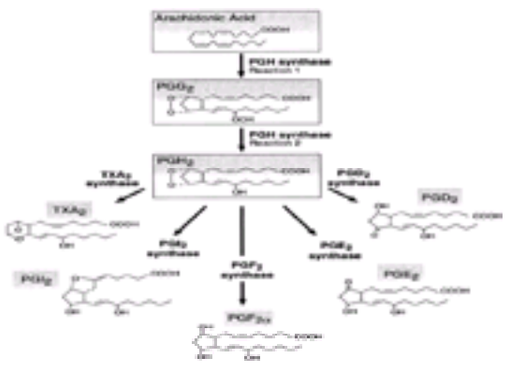

Figure 27.1. Chemical structures and biosynthetic pathways of prostanoids.

At least 16 PLA_2 isozymes exist, differing in molecular weight, subcellular localization, biochemical requirements, and preference for substrate (77). The PLA_2 isozymes have been categorized into 5 families on the basis of homology and structure. The structurally related approximately 14-kd low-molecular-weight enzymes, groups I, II, V, and X, are also referred to as secretory PLA_2 enzymes. These isozymes are characterized by conservation of 12 to 16 cysteine residues and highly homologous catalytic and calcium-binding domains (77). Within this family, group IIA PLA_2 is present in granules of mast cells and platelets, a finding suggesting a downstream extracellular function, compatible with its prominence in inflammatory exudates (78,79,80 and 81). The binding of PLA_2 group IIA and related family members to M-type approximately 180-kd transmembrane PLA_2 receptors implicates a signal transduction function that is not dependent on the catalytic domain (82,83). Bone marrow–derived mast cells from mice with a natural disruption of group IIA PLA_2 can generate cysteinyl leukotrienes and PGD_2 (77), and the extracellular role of group IIA PLA_2 is attributed to receptor-mediated group IV PLA_2-dependent eicosanoid production. Group V PLA_2 is localized to the endoplasmic reticulum and related organelles (84), and it is believed to work in concert with group IV PLA_2 to amplify prostanoid biosynthesis in macrophages and mast cells, possibly by induction of prostaglandin endoperoxide synthase-2 (PGHS-2) (77,83,84 and 85).

Group IV PLA_2, referred to as cytosolic PLA_2 ($cPLA_2$), is relatively specific for arachidonic acid hydrolysis from phospholipids and has no amino acid homology to the group I or group II PLA_2 enzymes. Group IV PLA_2 is located in the cytoplasm of cells in its mature 85-kd form. Cell activation results in micromolar increases of calcium and causes translocation of group IV PLA_2 to the perinuclear membrane and augmented function through membrane-associated protein kinase–dependent serine phosphorylation (86,87). The full-length human cDNA and the deduced amino acid sequence reveal motifs for a calcium (Ca^{++})-dependent lipid-binding domain and a plextrin homology domain that may be involved in the association of $cPLA_2$ with the cell membrane. The gene of group IV $cPLA_2$ is on chromosome 1q25, and the 3.4-kb transcript is widely expressed constitutively but is further inducible with cytokines such as IL-1, tumor necrosis factor, and IL-3 (88).

The SN-1 ether-linked lysophospholipid moiety with the general structure alkylacetyl-glyceryletherphosphorylcholine that remains after the release of the arachidonic acid from cell membrane phospholipids is termed lyso-platelet-activating factor (lyso-PAF). This moiety acts as both a precursor and a metabolite of PAF, a mediator with intrinsic bioactivity, controlled by inactivation through a specific acetylhydrolase (89,90). PAF effects, which include increased chemotaxis, aggregation, superoxide generation, cytokine production, and cytotoxic function in monocytes and also increased leukotriene generation in granulocytes, are transduced through a G protein–coupled serpentine PAF receptor to activate $cPLA_2$ (91,92,93 and 94). Other cells that exhibit increased bioactivity in response to PAF include B lymphocytes, natural killer cells, vascular endothelial cells, and smooth muscle cells (91,92,93,94,95 and 96). At the tissue level, PAF also causes airway constriction, microvascular leakage, and inflammatory cell activation; however, these effects are at least partially mediated through the release of secondary mediators, specifically cysteinyl leukotrienes (97,98 and 99).

The effects of the PAF receptor have been explored in the mouse with both transgenic overexpression and gene disruption. PAF receptor transgenic mice showed a bronchial hyperreactivity to methacholine challenge, which was inhibited by pretreatment with either a thromboxane synthesis inhibitor or a cysteinyl leukotriene antagonist; this finding suggests that both TXA_2 and cysteinyl leukotrienes are involved in the bronchial responses to PAF or cholinergic stimuli (100). Disruption of the PAF receptor gene allowed mice to develop normally and to remain sensitive to bacterial endotoxin. However, systemic anaphylactic symptoms were markedly reduced, a finding clearly showing that PAF plays a dominant role in eliciting anaphylaxis but is not essential for reproduction or endotoxic shock (101).

Prostanoid Generation

The prostaglandins are fatty acid derivatives of 20-carbons in which a five-membered prostane ring separates the olefinic bonds; thromboxanes contain a six-membered ring. The numeric subscript represents the number of carbon-carbon double bonds in the side chains. Most naturally occurring prostaglandins have two aliphatic side-chain bonds, named the a and w chains, known as the "2" series. In diets rich in fish oils, eicosapentaenoic acid gives rise to the "3" series, which, in the case of thromboxane, creates a product with attenuated function relative to the "2" series as assessed by platelet aggregation. The corresponding PGI_3, which is the only other eicosanoid produced in significant amounts, is essentially equally active in platelet aggregation (102). Modifications in the cyclopentane ring result in prostaglandins that are classified from A to I; however, A, B, and C are produced only artificially during procedures of extraction. Prostaglandins G and H share a similar ring structure but differ with the presence at C-15 of either a hydroperoxyl group or a hydroxyl group, respectively (Fig. 27.1).

PROSTAGLANDIN H SYNTHASE-1 AND PROSTAGLANDIN H SYNTHASE-2

The prostaglandin H synthase isozymes, PGHS-1 and PGHS-2, known also as cyclooxygenase-1 and -2, catalyze the two initial reactions in the biosynthesis of the prostanoids (Fig. 27.1, Table 27.2). Arachidonic acid is dioxygenated at positions C-11 and C-15 to form the bicyclic peroxide PGG_2, which, on migration to the peroxidase active site of the PGHS enzyme, is converted to PGH_2. Cell- and tissue-specific terminal enzymes then convert PGH_2 to other cyclic endoperoxides, such as TXA_2 or PGI_2, PGD_2, PGE_2, and PGF_2; the individual effects of these mediators are noted in the preceding section. Prostanoids responsible for many normal homeostatic functions in tissues such as the stomach and kidney are formed through the action of the constitutive PGHS-1 isozyme. In these organs, the cytoprotective effects of prostaglandins provided by PGHS-1 are implicated, and susceptibility to toxic effects from nonselective PGHS inhibitors occurs (103). In contrast, the

generation of prostanoids such as prostacyclin (PGI_2) and PGE_2 in inflammatory responses (104) is attributed to the PGHS-2 isozyme, which is prominently expressed in inflammatory cells such as monocytes, macrophages, synoviocytes, and fibroblasts. PGHS-1 and PGHS-2 are isozymes with 61% overall amino acid identity, each with distinct substrate specificities and binding characteristics, mechanisms of regulation, and tissue expression (Table 27.2). Both isozymes are integral membrane proteins of approximately 68-kd molecular weight, with a subcellular localization in the endoplasmic reticulum and nuclear envelope, although some data suggest that they use distinct lipid pools (105).

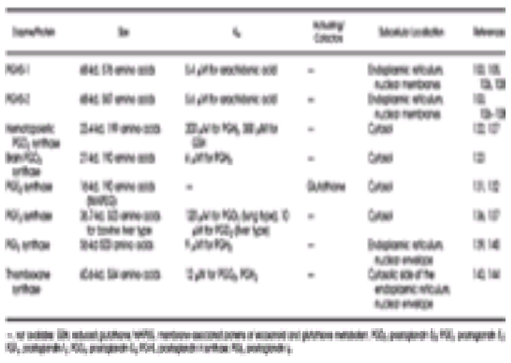

TABLE 27.2. Molecular and Biochemical Characteristics of Prostanoid Biosynthetic Enzymes

The biochemical and structural features of these isozymes are critical for the development of selective drugs to inhibit only the PGHS-2 isozyme, in an effort to avoid toxicity (106,107 and 108). Valeryl salicylate selectively inhibits PGHS-1, whereas compounds such as NS-398, celecoxib, and rofecoxib act selectively on PGHS-2 (109). Crystallographic structure analysis of both isozymes of PGHS reveals Val (instead of Ile) at position 523 in the active site of PGHS-2, as well as a slightly larger active site channel, thereby allowing isozyme discrimination with selective inhibitors (110,111).

The human transcript for PGHS-1 is detected as either 2.8 or 5.2 kb on RNA blot analysis, presumably because of the presence of a canonical polyadenylylation site 0.75 kb downstream from the translation termination codon, a finding suggesting alternative polyadenylylation of the PGHS-1 gene (112). The sequence of the 3'-untranslated region of the PGHS-1 transcripts is highly divergent from that of the human PGHS-2 transcript isoforms, a finding suggesting a distinct function in the regulation of expression at the posttranscriptional and translational levels. PGHS-1 has generally been described as constitutive in all tissues, with only modest increases of inducibility.

The 4.5-kb human PGHS-2 cDNA encodes a polypeptide of 604 amino acids, although various levels of glycosylation affect its approximate 68-kd size, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both PGHS-1 and -2 mRNAs are expressed in human endothelial cells, vascular smooth muscle cells, monocytes, and fibroblasts. However, a high-level induction of the PGHS-2 transcripts by phorbol 12-myristate 13-acetate, lipopolysaccharide, mitogens, inflammatory cytokines, and growth factors suggests a role for PGHS-2 in the inflammatory response; this induction is downregulated by glucocorticoids (113,114,115,116 and 117). Only the PGHS-2 isoform is decreased by glucocorticoids, an effect caused by reduced transcription and mRNA stability (116), characteristic of the family of glucocorticoid-sensitive inflammatory response genes. The PGHS-1 gene is a TATA-less, 22-kb, 11-exon gene on chromosome 9 (118,119), and the PGHS-2 gene is a TATA-containing, 8-kb, 10-exon gene on chromosome 1 (120,121) (Table 27.3).

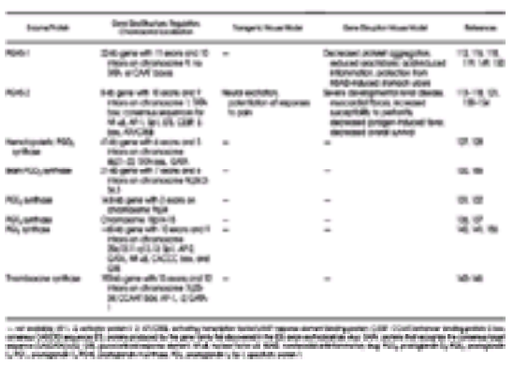

TABLE 27.3. Characteristics of CDNAs/Genes of the Prostanoid Biosynthetic Enzymes

The physiologic effects of the downstream prostanoids from PGH_2 affect all cells, tissues, and organ systems. The presence of biosynthetic enzymes and receptors for the prostanoids is regulated in both positive and negative directions by conditions in a given microenvironment.

PROSTAGLANDIN D_2 SYNTHASE

PGD_2 formation is catalyzed by at least two isozymes of cytosolic PGD_2 synthases, hematopoietic and brain forms, which are clearly distinguished by amino acid composition, catalytic properties, and antigenicity (122,123) (Fig. 27.1, Table 27.2). Hematopoietic PGD_2 is the key enzyme for production of the D and J series of prostanoids in the immune system. Biologically active PGD_2 metabolites can be dehydrated to form d12-PGJ_2, or they can be enzymatically processed by 11-keto-reductase to produce a second prostaglandin, PGF_{2a} (124,125 and 126).

The hematopoietic PGD_2 synthase, a glutathione-dependent, cytosolic isozyme, is found in spleen, mast cells, and antigen-presenting cells, such as dendritic cells, and in Kupffer cells and histiocytes. An 811-bp cDNA encodes this 23.4-kd protein, which has a Michaelis constant (K_m) of 200 µM for PGH_2 and of 300 µM for reduced glutathione (GSH), with a pH optimum of 7.5 (122,127). Crystallization reveals that the enzyme is a s class GSH *S*-transferase (GST) with a unique cleft as the active site that distinguishes it from other members of the GST family and appears to confer specificity for the isomerization from PGH_2 to PGD_2 (127). The 41-kb gene for the hematopoietic isoform of this enzyme is TATA-less, it contains six exons and five introns, and it is localized on chromosome 4q21-22 (128). Although PGD_2 is the dominant prostanoid product in mast cells, it is also produced by monocytes and macrophages (129), and by polarized T cells (21). The hematopoietic form of PGD_2 synthase is inducible in immature mouse mast cells treated with c-*kit* ligand, as demonstrated by enzymatic function and immunoreactive protein (80).

The brain form is a GSH-independent cytosolic member of the lipocalin family (123), with a K_m for PGH_2 of 6 µM at a pH optimum of 9.0. This enzyme is considered to be a dual-function protein; it acts as a PGD_2-producing enzyme and also as a lipophilic ligand-binding protein, with high affinities for retinoids and bile pigments. The 21-kb gene for this isozyme is composed of 7 exons and 6 introns, and it is found on chromosome 9q34.2-34.3 (130) (Table 27.3).

PROSTAGLANDIN E_2 SYNTHASE

PGE_2 formation is catalyzed by PGE_2 synthase. This inducible 16-kd microsomal enzyme is in the newly described family of membrane-associated proteins of eicosanoid and glutathione metabolism (MAPEG) (131) (Fig. 27.1, Table 27.3). PGE_2 synthase possesses a high level of specific activity (250 nmol/min/mg). Expression of PGE_2 synthase mRNA was detected in human placenta, prostate, testis, mammary gland, and bladder; and protein expression is induced severalfold by IL-1b (131). The 14.8-kb gene for human GSH-dependent PGE_2 synthase contains 3 exons and is localized on chromosome 9q34 (132) (Table 27.3). Reporter constructs containing the 5'-flanking region of this gene revealed that the transcript increases transiently in response to IL-1b. This pattern is similar to the effects of IL-1b on PGHS-2 expression, and it implies a transcriptional regulatory basis for the observed coregulation of these enzymes.

The formation of PGE_2 also appears to be catalyzed by 2 isomerases identified as anionic forms of cytosolic GST, which have respective K_m values for arachidonic acid of 147 and 308 µM and maximum velocity (V_{max}) values of 380 and 720 nmol/mg per minute (133,134). PGE_2 is metabolized by oxidation at the C-15 position by 15-hydroxy-prostaglandin dehydrogenase and reduction of the double bond at the C-13 position by d13-reductase. The resultant 13,14-dihydro product has substantially less biologic activity than the parent PGE_2 (135).

PROSTAGLANDIN F_2 SYNTHASE

PGF_2 synthase is a soluble dual-function enzyme that catalyzes the reduction of PGD_2 and PGH_2 and the oxidation of 9a,11b-PGF_2 and PGD_2 (Fig. 27.1, Table 27.2). Although an isozyme of a-hydroxysteroid/dihydrodiol dehydrogenase catalyzes this reaction with a K_m for PGD_2 of 57 µM, at least two specific PGF_2 synthases in the lung and the liver have K_m values of 120 µM and 10 µM for PGD_2, respectively (136,137). A cDNA for PGF_2 synthase cloned from human lung encoded a 36.8-kd protein of 324 amino acids. Its gene is localized on chromosome 10p14-15, and its 1133-bp transcript is expressed in peripheral blood lymphocytes and lung tissue. A bovine cDNA for the liver-type enzyme consists of a 969-bp encoding a 323-amino acid polypeptide with a molecular mass of 36.7 kd (137).

PROSTACYCLIN SYNTHASE

The generation of PGI_2 can be stimulated by thrombin, by the mechanical stretching of arterial walls, or by the 5-LO–generated eicosanoid, LTC_4 (133). PGI_2 is an unstable molecule, and its hydrolysis in plasma to the inactive 6-keto-PGF_{1a} occurs within minutes. This characteristic, coupled with its low entry rate (0.1 ng/kg/min) into the circulatory system, makes PGI_2 unlikely to act as a circulating antiplatelet agent (124,138). PGI_2 is formed by PGI_2 synthase, a 56-kd member of the P-450 superfamily of enzymes found predominantly in endothelial cells and vascular smooth muscle cells (139) (Fig. 27.1, Table 27.2). PGI_2 synthase cDNA has been cloned from human and bovine libraries, revealing a 1,500-bp open-reading frame encoding for a 500-amino acid polypeptide of approximately 50 kd (140). The PGI_2 gene is on chromosome 20q13.11-q13.13, is approximately 60 kb in length, contains ten exons, and has a promoter region with consensus sequences for specificity protein 1 (Sp1), activator protein-2 (AP-2), proteins that recognize the consensus target sequence (T/A)GATA(A/G)(GATA), nuclear factor-kB (NF-KB), a CACCC box, and a glucocorticoid response element (GRE) (141) (Table 27.3).

THROMBOXANE SYNTHASE

Thromboxane synthase, which provides a six-membered ring product, catalyzes the formation of this intermediate by rearrangement of the endoperoxide structure of PGG_2 followed by incorporation of a single molecule of water (142). TXA_2 synthase is a microsomal cytochrome P-450 (CYP450) family member with 534 amino acids and a molecular mass of 60.6 kd (Fig. 27.1, Table 27.2). From nucleic acid sequences, five potential sites for *N*-glycosylation and a hydrophobic region may serve to anchor it in the endoplasmic reticulum membrane (143). The enzyme has similar K_m values (approximately 12 µM) for PGG_2 and PGH_2, with a pH optimum of 7.5. The human TXA_2 synthase gene consists of 13 exons and 12 introns and is 193 kb in length, the largest P-450 gene isolated (144). A 1.2-kb segment of the 5'-flanking region contains potential binding sites for several transcription factors, AP-1, AP-2, GATA-1, and a CCAAT box, as well as multiple transcription start sites, but neither a typical TATA box nor a typical CAAT box. The human gene for thromboxane synthase is localized to band q33-q34 of chromosome 7 (145,146) (Table 27.3). The thromboxane synthase transcript is widely expressed in human tissues and is particularly abundant in peripheral blood leukocytes, spleen, lung, and liver (145,146).

The effects of TXA_2 are transient, because it is chemically unstable; its approximately 30-second half-life in the plasma makes it a locally acting mediator. Its hydrolytic metabolism to biologically inactive TXB_2, measured in the blood or urine, can be used as an indicator of TXA_2 synthesis (142).

PHENOTYPIC EFFECTS OF GENE MANIPULATION OF PROSTANOID BIOSYNTHETIC ENZYMES

In mice with either gene disruption or overexpression of eicosanoid biosynthetic enzymes (or receptors), the effects on innate and adaptive immunity have not been fully elucidated, and in many instances, only an unexpected or dominant phenotype has been reported. In addition to the possible aberrant tissue expression of products of gene manipulation, the phenotypes expressed from altering the upstream enzymes must be interpreted with caution. In the case of PLA_2 or PGHS, multiple downstream mediators, often with counterregulatory effects, are affected. Disruption of a receptor may create a different phenotype from that produced by interruption of a biosynthetic pathway with multiple products.

Because neither monocyte/macrophages nor bone marrow–derived mast cells from mice with gene disruption for $cPLA_2$ produce prostanoids or leukotrienes, the role of group IV PLA_2 in eicosanoid generation is secure (147,148). The fertility and parturition defects of these animals (as in PGHS gene-disrupted mice) reflect the lack of $cPLA_2$-provided substrate for the critical downstream prostanoid functions. Similarly, the attenuated decrement in pulmonary function and hyperresponsiveness in sensitized and aerosol antigen-challenged $cPLA_2$ gene-disrupted mice (as in 5-LO or FLAP gene-disrupted mice or PGD_2 receptor gene-disrupted mice) implicates pathobiologic eicosanoids dependent on $cPLA_2$ for provision of arachidonic acid for their synthesis (147,148). These mice are also protected from neurologic damage after transient ischemia (148).

In PGHS-1 knock-out mice, one sees the anticipated effects of a reduction in platelet aggregation and inflammation from exogenous arachidonic acid, and failure of parturition (149), almost certainly from depletion of downstream prostanoids. However, these mice are also protected against gastric lesions caused by nonsteroidal antiinflammatory drugs, an observation that seems to contradict the involvement of the prostanoids formed by PGHS-1 in cytoprotection of the gastric mucosa. One explanation for this observation is the compensatory increase in PGHS-2 that occurs in *ex vivo* stimulated cells from these mice (150). PGHS-2 knock-out mice exhibited a decreased response to pyrogens and exogenous arachidonic acid (151,152 and 153), as well as an increased susceptibility to the effects of experimentally induced bacterial peritonitis. These mice also had multiple female reproductive failures that include ovulation, fertilization, implantation, and decidualization (151,152). PGHS-2 gene-disrupted mice also displayed unexpected developmental renal disease and myocardial fibrosis, with decreased overall survival. In studies of the role of PGHS-2 in tumorigenesis, there did appear to be reduced numbers of polyps in mice cross-bred with a mouse strain with hereditary adenomatous polyposis (151,152 and 153). A mouse model of overexpression of PGHS-2 in the central nervous system potentiates the intensity and lethality of excitotoxicity (154).

Only limited data for the terminal enzymes are available, but much information exists about receptor knock-outs, as discussed later. Mice with brain PGD_2 synthase gene disruption have diminished responses to tactile pain as the prominent phenotypic feature (155). PGI_2 synthase overexpression in a transgenic mouse model provided protection from hypoxic pulmonary hypertension and its consequent arteriolar hypertrophy (156).

Leukotriene and Lipoxin Generation

The name *leukotriene* is derived from leukocytes, the cells from which they were originally discovered, and their structural feature of a conjugated triple bond (-triene). The leukotrienes are composed of two major families, the dihydroxy leukotrienes and the cysteinyl leukotrienes. The dihydroxy leukotrienes consist of LTB_4 and its metabolites, and the cysteinyl leukotrienes include LTC_4 and its metabolites, LTD_4 and LTE_4. Just as in the case of the prostanoids, the initial step in the generation of leukotrienes and lipoxins is the enzymatic release of arachidonic acid from cell membrane phospholipids by group IV PLA_2. The first committed step specific to the biosynthesis of leukotriene biosynthesis is through 5-LO, with subsequent conversion to either the dihydroxy or the cysteinyl leukotrienes.

5-LIPOXYGENASE

Arachidonic acid is converted sequentially by 5-LO to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and by enzymatic dehydration to LTA_4 (157,158 and 159) (Fig. 27.2, Table 27.4). Subcellular assays demonstrated augmented enzyme activity with added phosphatidylcholine, adenosine triphosphate, Ca^{++}, and iron. Although the amino acid sequence motifs within 5-LO reveal no binding domains for ATP or Ca^{++}, site-directed mutagenesis has revealed histidine residues at 368 and 373 that appear integral in the coordination of a catalytically essential iron atom (160,161,162 and 163). Because maximal 5-LO activity is accentuated by hydroperoxides, the enzyme exhibits a biphasic concentration dependence for arachidonic acid, exhibits lag phases, and has rapid turnover inactivation that makes steady-state kinetic values for 5-LO difficult to assess. 5-LO of human leukocytes appears to have a K_m of 10 to 20 µM for arachidonic acid and a V_{max} value of approximately 24 µmol/mg per 5 minutes for HPETE (159) (Table 27.4). In addition to its catalytic function, 5-LO exerts effects in cell remodeling, apparently through interactions of an *src* homology (SH3) domain in the carboxy-terminal of 5-LO with cytoskeletal proteins such as growth factor receptor bound protein-2, actin, and a-actinin (164).

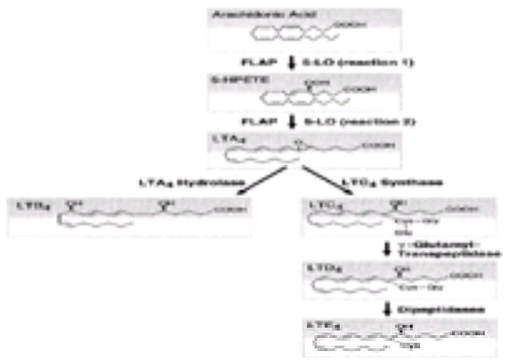

Figure 27.2. Chemical structures and biosynthetic pathways of leukotrienes.

Enzyme/Protein	Size	K_m	Activating/ Cofactor	Subcellular Localization	References
5-lipoxygenase	78-kd, 673 amino acids	10–30 μM for arachidonic acid	Ca^{++}, ATP	Cytosol or nucleoplasm, translocates to perinuclear membranes	157–159, 160–177
5-lipoxygenase-activating protein	18-kd, 161 amino acids	–	–	Perinuclear integral membrane protein	172, 178
LTA_4 hydrolase	70-kd, 610 amino acids	3.5 μM for LTA_4	–	Cytosol	183–185
LTC_4 synthase	18-kd, 150 amino acids	3.5 μM for LTA_4, 1.4 mM for GSH	Mg^{++}	Perinuclear integral membrane protein	183–185

– = not available; ATP = adenosine triphosphate; LTA4 = leukotriene A4; LTC4 = leukotriene C4.

TABLE 27.4. Molecular and Biochemical Characteristics of Leukotriene Biosynthetic Enzymes

In resting cells, 5-LO is localized mainly to the cytosol or nucleoplasm. This localization is cell dependent; in mast cells and basophils, the calcium-dependent movement occurs from the nucleoplasm to the nuclear membrane (165,166 and 167), but in neutrophils, translocation of 5-LO occurs from the cytosol to the nuclear membrane. In neutrophils, this translocation from the cytosolic compartment to the nuclear compartment is associated with increased LTB_4 synthesis, whereas in eosinophils, the similar translocation results in diminished LTC_4 synthesis (167,168). On translocation, 5-LO, in association with a required cofactor, the integral perinuclear protein, FLAP, processes the released arachidonic acid to 5-HPETE and LTA_4 (169,170).

The human 5-LO cDNA encodes a 78-kd protein of 673 amino acids, and the greater than 82-kb, composed of 14 exons and 13 introns, is localized on chromosome 10 (171,172 and 173) (Table 27.4 and Table 27.5). The 5' untranslated region of the 5-LO gene contains a guanosine and cytidine–rich promoter region and lacks TATA and CCAAT motifs. The proximal non–cell-specific promoter element of the 5-LO gene contains a family of alleles in the core promoter characterized by the deletion or addition of consensus Sp1 and early growth response-1 (Egr-1)–binding motifs. Each of the variant alleles binds Sp1 and early growth response-1 (Egr-1) protein, as indicated by electrophoretic mobility shift assays and supershift analyses, and data from reporter assays indicate that these alleles are less effective than the wild-type allele in initiating 5-LO gene transcription (174) (Table 27.5).

Enzyme/Protein	Gene Characteristics	Gene Disruption Mouse Model	References
5-lipoxygenase	>82-kb gene with 14 exons and 13 introns on chromosome 10; 3 tandem Sp1- and Egr-1 sites	Impaired arachidonic acid–induced ear edema, reduced inflammatory cell migration in glycogen and zymosan-induced peritonitis, resistant to platelet-activating factor–induced hypotension, impaired killing of macrophages, and increased lethality in lupus (nzb/w only)	179–184, 214–218
5-lipoxygenase-activating protein	>37-kb gene with 5 exons and 4 introns on chromosome 13q12; modified TMPA box	Blunted inflammatory response to topical arachidonic acid, reduced collagen-induced arthritis, increased resistance to platelet-activating factor–induced shock, and reduced edema in a zymosan-induced model of peritonitis	180, 219, 220
LTA_4 hydrolase	>30-kb gene with 19 exons and 18 introns on chromosome 12q22; TMPA, CAAT-less	Reduced arachidonic acid–induced neutrophil influx and edema, zymosan-induced peritonitis has reduced cellular influx but an intact edematous response	181, 186, 221
LTC_4 synthase	2.5-kb gene with 5 exons and 4 introns on chromosome 5q35; Sp1, cell-specific Hoppel-like factor sites	–	222, 223, 225

– = not available; Egr-1 = early growth response-1; LTA_4 = leukotriene A_4; LTC_4 = leukotriene C_4; Sp1 = specificity protein-1.

TABLE 27.5. Characteristics of CDNAs/Genes of the Leukotriene Biosynthetic Enzymes

Other mammalian lipoxygenases exist in addition to 5-LO. When the products of these lipoxygenases interact, a new family of lipid mediators, the lipoxins, is formed.

12-, 15-, AND 5-LIPOXYGENASE INTERACTION PRODUCTS

The first way in which lipoxins are generated is the neutrophil formation and export of LTA_4, which is then converted by 12-LO in platelets to yield 5S,6R,15S-trihydroxy-7,9,11,13 (E,E,Z,E)-eicosatetraenoic acid (LXA_4) and 5S,14R,15S-trihydroxy-6,8,10,12 (E,Z,E,E)-eicosatetraenoic acid (LXB_4) (175,176) (Fig. 27.3). Alternatively, epithelial cells, monocytes, or eosinophils generate 15S-hydroxyeicosatetraenoic acid (HETE), which is subsequently taken up by neutrophils and is converted to lipoxins by 5-LO. 15R-HETE, which is produced by PGHS-2 after acetylation by aspirin, provides 15-epi LXA_4 (176). Although the lipoxin pathway of human cells has diverged evolutionarily into a two-cell system, lower species such as rainbow trout possess the necessary enzymes to generate both leukotrienes and lipoxins in the same cell in parallel (177).

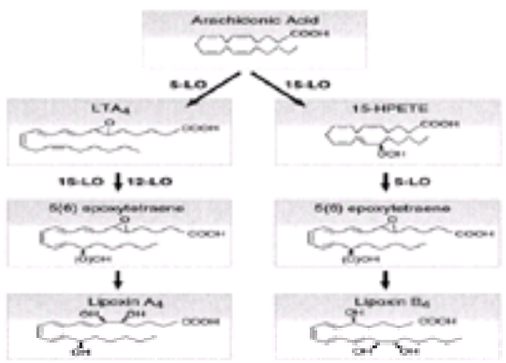

Figure 27.3. Chemical structures and biosynthetic pathways for lipoxins.

5-LIPOXYGENASE–ACTIVATING PROTEIN

FLAP, an 18-kd integral membrane protein of perinuclear membranes, acts in concert with translocated 5-LO to convert released arachidonic acid into 5-HPETE and LTA_4 (178,179) (Fig. 27.2, Table 27.4). FLAP is a 161-amino acid protein with a secondary structure prediction of 3 hydrophobic domains with 2 interspersed hydrophilic loops (179). The carboxy-terminal end of the first loop and the amino-terminal end of the second loop contain amino acid residues responsible for the binding of FLAP-inhibiting drugs. These drugs attenuate cellular leukotriene biosynthesis but not cell-free 5-LO function, which does not require FLAP, a finding implying an effect on intracellular arachidonic acid presentation (180). FLAP has been shown by amino acid sequence and genomic organization to be a member of the MAPEG family of proteins (181). The human FLAP gene is larger than 31 kb, has 5 exons, and is located on chromosome 13q12 (182) (Table 27.5).

LEUKOTRIENE A_4 HYDROLASE

LTA_4 hydrolase, a 70-kd cytosolic metalloenzyme, converts LTA_4 to 5S,12R-(dihydroxy)-6,8,10,14 (Z,E,E,Z)-eicosatetraenoic acid (LTB_4) (Fig. 27.2, Table 27.4). This enzyme requires zinc and contains two overlapping active sites of epoxide hydrolase activity and an aminopeptidase activity; a natural substrate for the latter has not been defined (183,184 and 185). The epoxide hydrolase activity is specific for LTA_4 and, furthermore, is stereospecific for the structure of a 5,6-epoxide with 7,9,11,14 (E,E,Z,Z) double bonds (186). The peptidase activity is activated by chloride ions and, as in the case of 5-LO, is suicide inactivated by the covalent binding of the enzyme to the substrate, LTA_4 (187,188). LTA_4 hydrolase has a K_m value for LTA_4 of 5.8 µM (Table 27.4) and a V_{max} value of 692 nmol/mg per minute for epoxide hydrolase activity (186,187 and 188).

In neutrophils, monocytes and macrophages, and mast cells, 5-LO provides LTA_4 to LTA_4 hydrolase. However, cells such as keratinocytes and type II pneumocytes receive LTA_4 from hematopoietic cells such as polymorphonuclear leukocytes, thus allowing formation of LTB_4 (189,190).

The cDNA of LTA_4 hydrolase encodes a polypeptide of 610 amino acids, and the deduced amino acid sequence shares no significant homology with the nonspecific liver epoxide hydrolase (191). Site-directed mutagenesis studies have revealed that zinc binding depends on histidine 295, histidine 299, and glutamic acid 318; glutamic acid 296 and tyrosine 383 are catalytic residues for the peptidase function; and suicide inactivation by the substrate LTA_4 depends on a tyrosine residue in position 378 (192,193,194 and 195). The human gene for LTA_4 hydrolase is larger than 35 kb, has 19 exons ranging in size from only 24 to 312 nucleotides, and is localized on chromosome 12q22 (196). No definitive consensus sequences for a TATA box are located in the known 5' flanking region, although AP-2 and xenobiotic response elements are present (Table 27.5).

LEUKOTRIENE C_4 SYNTHASE

LTC_4 synthase is an 18-kd integral membrane protein that conjugates GSH to LTA_4 to form the parent cysteinyl leukotriene, 5S-hydroxy-6R-S-glutathionyl-7,9,11,14 (E,E,Z,Z)-eicosatetraenoic acid (LTC_4) (197,198) (Fig. 27.2, Table 27.3). LTC_4 synthase is specific for this enzymatic activity and has no ability to conjugate xenobiotics to GSH. Purified recombinant LTC_4 synthase exhibits K_m values for LTA_4 and GSH of 3.6 µM and 1.6 mM, respectively (Table 27.4), with V_{max} values of 1.3 and 2.7 µmol/mg per minute, respectively (199).

The cDNA for human LTC_4 synthase encodes for a 150-amino acid protein with a secondary structure of 3 hydrophobic domains interspersed by 2 hydrophilic loops (200). Site-directed mutagenesis has suggested that arginine 51 in the first hydrophilic loop is required to open the epoxide by acid catalysis of LTA_4, whereas tyrosine 93 in the second loop provides the thiolate ion by base catalysis of GSH and results in conjugation to form LTC_4 (201). These studies identified additional residues in each loop as being involved in substrate presentation because their mutation increased the K_m without impaired catalytic function (201). The gene for LTC_4 synthase is 2.5 kb and has an intron/exon alignment identical to that of FLAP; however, its chromosomal location is 5q35. This location is distal to the gene cluster for Th2 cytokines (IL-4, IL-5, IL-3, IL-9, and granulocyte-macrophage colony-stimulating factor, which are implicated in allergic and asthmatic inflammation (202). Whereas an SP-1 site and an initiator sequence regulate non–cell-specific function in reporter constructs, upstream motifs for SP-1/-3 and for a Kruppel-like factor govern cell-specific function (203) (Table 27.5).

LTC_4 synthase is also a member of the MAPEG gene family but is distinct in its monospecific function for cysteinyl leukotriene generation. In contrast, FLAP has no known enzymatic function, and the microsomal GSTs possess wider substrate specificities (204,205). Additionally, whereas LTC_4 synthase is expressed in a restricted population of hematopoietic cells including eosinophils, basophils, mast cells, and monocytes and macrophages (206,207,208 and 209), the microsomal GST members are widely distributed, including in cells lacking 5-LO expression, such as those in the liver. This finding implies that the primary function of microsomal GSTs is detoxification, rather than serving as a central source of proinflammatory cysteinyl leukotrienes. Transcellular generation of cysteinyl leukotrienes occurs in platelets and nonhematopoietic cells such as endothelial cells (210), vascular smooth muscle cells (211), synoviocytes (212), and chondrocytes (212,213). Whereas the transcellular conversion of LTA_4 to LTC_4 by platelets occurs by the action of LTC_4 synthase, in the nonhematopoietic cells biosynthesis of LTC_4 appears to occur by microsomal GSTs possessing bifunctional activity (204,205).

PHENOTYPIC EFFECTS OF GENE MANIPULATION OF LEUKOTRIENE BIOSYNTHETIC ENZYMES

Targeted disruption of the 5-LO gene in mice leads to impairment of arachidonic acid–induced ear edema and reduced inflammatory cell migration in glycogen- and zymosan-induced peritonitis (214,215). The shock response to endotoxin remains intact, but these animals are resistant to PAF-induced hypotension, a finding implying a sequential relationship of leukotrienes and PAF (214,215). Although there is no defect in neutrophil recruitment, impaired cellular killing by macrophages appears to be responsible for increased mortality in these mice from *Klebsiella*-induced pneumonia. This particular defect can be restored with the administration of LTB_4 (216).

The role of 5-LO products in the development of the airway reactivity that follows antigen exposure was examined by sensitizing mice lacking 5-LO by intraperitoneal injection of ovalbumin followed by serial exposure to aerosols of ovalbumin. Lung resistance in these challenged mice exhibited an attenuation in methacholine responsiveness and airway eosinophilia (217). A study of 5-LO products in proinflammatory states was undertaken by crossing MRL-lpr/lpr mice, which spontaneously develop autoimmune disease resembling human systemic lupus erythematosus, with 5-LO–deficient mice. The MRL-lpr/lpr mice have sex-related survival differences; female MRL-lpr/lpr mice have significantly higher early mortality rates than males. The mortality rate was higher in male 5-LO–deficient MRL-lpr/lpr mice compared with controls, but not compared with females (218). In male 5-LO–deficient MRL-lpr/lpr mice, renal plasma flow was significantly reduced, although no differences were noted in the severity of renal histopathology, lymphoid hyperplasia, or arthritis. These findings suggest that the presence of 5-LO products confers a survival advantage on male MRL-lpr/lpr mice and that, when 5-LO function is inhibited genetically (or pharmacologically), the gender advantage disappears (218).

Mice with FLAP gene disruption exhibit attenuated responses to inflammatory stimuli similar to those in the 5-LO gene–disrupted mice, including a blunted inflammatory response to topical arachidonic acid, increased resistance to PAF-induced shock, and reduced edema in a zymosan-induced model of peritonitis (219). Additionally, collagen-induced arthritis is significantly reduced in FLAP gene-disrupted mice (220).

Targeted gene disruption of LTA_4 hydrolase in mice ablates only LTB_4 and preserves the cysteinyl leukotrienes (221). Arachidonic acid–induced neutrophil influx and subsequent edema are impaired in these mice, with the vascular edematous response more attenuated than the effects on cellular recruitment. In contrast, with zymosan-induced peritonitis, the reduced cellular influx was not accompanied by any impairment of the edematous response, a finding suggesting that this latter component is an effect solely of the cysteinyl leukotrienes. Furthermore, as in the case of 5-LO and FLAP gene disruption, the mice are resistant to PAF-induced hypotension, a finding suggesting that LTB_4 is the major leukotriene contributing to the hypotension induced by PAF (221).

EICOSANOID RECEPTORS, METABOLISM, AND TRANSPORT

Prostanoid and Platelet-Activating Factor Receptors

Newly generated prostanoids are released by carrier-assisted diffusion to act locally on the cell of origin or on neighboring cells. Two classes of receptors exist to transduce signals for the eicosanoids: the well-characterized G protein–coupled receptor class and the nuclear peroxisome proliferator activator receptors (PPAR) class, which are orphan nuclear receptors acting directly as transcription factors after binding to the appropriate eicosanoid. Thus, in addition to their extracellular functions (Table 27.1), eicosanoids act as intracellular ligands that bind to PPAR-a and PPAR-g to regulate lipid and glucose metabolism, adipocyte differentiation, and inflammatory responses (222,223).

The receptors for prostaglandins are termed P receptors based on the prostanoid to which they bind to transduce a signal; thus, DP, EP, FP, IP, and TP receptors have been classified, including at least four subtypes of EP receptors. These receptors are rhodopsin-type coupling through their respective G proteins to effectors such as adenylate cyclase and phospholipase C (224). Within each of the prostanoid receptors there exists conservation of certain regions, such as in the third and seventh

transmembrane domains and the second extracellular loop. Despite the structural similarities, homology among the prostanoid receptors ranges only from 20% to 30%. The prostanoid receptors have been characterized into three clusters: the relaxant cluster, containing DP, EP2, EP4, and IP; the contractile receptors EP1, FP, and TP; and the inhibitory cluster, containing only EP3 (224) (Table 27.6).

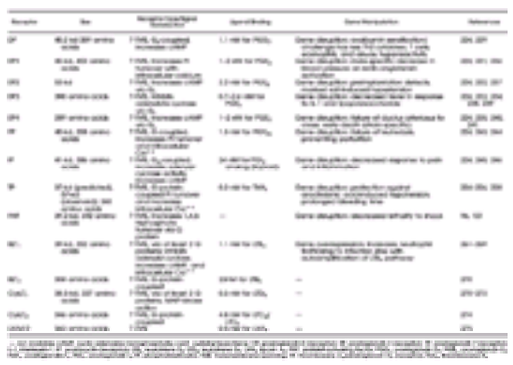

TABLE 27.6. Molecular and Biochemical Characteristics of Eicosanoid Receptors

The TXA_2/PGH_2 (TP) receptor has been isolated from human platelets, and its cDNA and genomic structure have been characterized (225,226) (Table 27.6). This receptor is a 343-amino acid residue, 7-transmembrane domain protein linked to several species of G proteins, and it specifically binds the selective TXA_2 ligand [^{3}H]S-145 with a dissociation constant (K_d) of 0.2 nM. It is encoded by a 3-exon, 15-kb gene on chromosome 19p13.3, containing four potential Sp1 binding promoter sites (226,227). Gene disruption of the TP receptor protects against lethality from arachidonic acid–induced hypotension but it does prolong the bleeding time (228).

The DP receptor is 359 amino acid residues with a predicted molecular mass of 40.2 kd, and it transduces increases in cyclic adenosine monophosphate (cAMP) through G_s protein coupling (Table 27.6). The receptor binds PGD_2 with a K_d of 1.1 nM and shows the highest degree of identity with the IP and EP2 receptors (224,229). Stimulation of the signal transduction pathway of the cloned DP receptor with PGD_2 resulted in elevation of intracellular cAMP and in mobilization of Ca^{++}, but not in generation of inositol 1,4,5-trisphosphate. The DP receptor has limited tissue distribution in humans and is detectable only in retina, brain, small intestine, and lung (224,229). When mice deficient in the DP receptor are sensitized with ovalbumin, the serum concentration of IgE is similar to those in wild-type mice subjected to this model. Aerosol challenge with ovalbumin reveals an attenuation of the markers of a full response, namely, Th2 cytokine concentration in the bronchial fluids, infiltration with T cells and eosinophils into the submucosa of the lung, and appearance of airway hyperresponsiveness to acetylcholine (230).

The human EP receptor subtypes have been cloned (Table 27.6). The EP1 receptor is a 42-kd, 7-transmembrane spanning protein, with a K_d of 1 to 2 nM for PGE_2. Activation of this receptor causes an increase in free intracellular calcium (231). The EP2 receptor is a 53-kd protein with a K_d for PGE_2 of 2.2 nM (Table 27.6); PGE_2 binding is competitively inhibited by misoprostol but not by sulprostone, consistent with the data from mouse EP2. EP2 receptor stimulation increases intracellular cAMP through G_s but fails to transduce a rise in intracellular calcium. As determined by RNA blot analysis, the EP2 receptor is present in lungs, kidneys, pancreas, leukocytes, and immune tissues (232). The EP3 receptor has a k_d of 0.7 to 2.6 nM for PGE_2, but it also binds PGF_{2a}, has a high affinity for sulprostone, and has only an intermediate affinity for misoprostol (233,234). Inhibition of adenylate cyclase occurs through G_s with binding of this receptor, although splice variants transduce differently. The tissue distribution of the EP3 receptor is restricted to the central nervous system, kidney, and pancreas. At least 5 cDNA clones from the human have been detected for the EP3 receptor, and they appear to result from alternate splicing (234). The 5' flanking region of the EP3 receptor possesses a consensus sequence for a classic TATA box. There are no qualitative data for ligand binding for the 289-amino acid EP4 receptor. However, this receptor does transduce increases in cAMP through G_s when bound by PGE_2. It is widely distributed in tissues and is encoded by a 3-exon gene spanning approximately 22 kb, analogous to those of the thromboxane, PGI_2, and PGD_2 receptors (235). The initiation site does not contain a conventional TATA box but contains two CCAAT boxes, Sp1 and AP2 motifs, and motifs consistent with activation by proinflammatory cytokines (235). The EP1, EP3, and EP4 receptors are found on chromosomes 19p13.1, 1p31.2, and 5p13.1, respectively (226).

EP1 receptor-deficient mice have sex-specific changes in blood pressure in that only male mice demonstrate reduced systolic blood pressure with compensatory increases in heart rate and renin-angiotensin activation (236). EP2 receptor-deficient mice are born in small litters because of a preimplantation defect, but their most dramatic phenotype is marked hypertension in response to salt (237). The ablation of the EP3 receptor blunts the fever response to IL-1 and lipopolysaccharide (238,239). Early neonatal death occurs in EP4 receptor gene-disrupted mice because of failure of the ductus arteriosus to close, although this appears in part to be strain specific (240,241). Bone resorption in these mice is also impaired (242).

The FP receptor is a 40-kd 7-transmembrane domain receptor that raises intracellular calcium levels consistent with G protein–coupled transduction (Table 27.6). The K_d for PGF_{2a} is 1.3 nM in the expressed receptor, although mouse studies suggest that this receptor also binds PGD_{2a} (243). The 3-exon gene is located on chromosome 1p31.1 in close proximity to the EP3 gene (224), a finding suggesting evolution by gene duplication. In FP receptor gene-disrupted mice, the inability to transduce the effects of PGF_{2a} prohibited luteolysis, with the consequence that progesterone levels remained elevated, preventing parturition (244).

The PGI_2 (IP) receptor is a 386-amino acid protein with 7-transmembrane domains, a feature that increases free calcium and stimulates adenylate cyclase to raise cAMP; however, it also appears to induce inositol 1,2,5-triphosphate responses, putatively through G_q (Table 27.6). The K_d for PGI_2 is 24 nM in the expressed receptor, and its RNA is abundantly expressed in the luteal cells, aorta, lung, atrium, ventricle, and kidney (245). The 3-exon gene is localized on chromosome 19q13.3 and contains AP-2 sites in its 5' flanking region (224). A gene disruption of the IP receptor revealed the unanticipated effects of PGI_2 as a mediator of pain and inflammation (246).

The PAF receptor is a 342-amino acid polypeptide with a calculated molecular mass of 39.2 kd that possesses 7 putative transmembrane-spanning domains (Table 27.6) (247). Activation of the PAF receptor yields inositol 1,4,5-trisphosphate production through G proteins. PAF receptor mRNA is abundant in leukocytes and heart (247,248), and its intronless gene is localized on chromosome 1 (249). Mice with gene manipulation of the PAF receptor are discussed earlier.

Leukotriene Metabolism

After export from the producing cells, LTB_4 may be metabolized systemically or in the locale of generation. LTB_4 biologic activity is attenuated in neutrophils by serial oxidations of the terminal (C-20) carbon. LTB_4 20-hydroxylase, a microsomal P-450 enzyme, sequentially catalyzes the formation of partially active but unstable intermediates, 20-hydroxy LTB_4 and 20-aldehyde LTB_4 (250,251). The LTB_4 20-hydroxylase cDNA encodes a 60-kd deduced protein of 520 amino acids (252). A 20-aldehyde dehydrogenase converts 20-aldehyde LTB_4 to 20-carboxyl LTB_4 (253), which is functionally inactive. LTB_4 metabolism in keratinocytes occurs by conjugation of LTB_4 to GSH (254). Systemic metabolism of LTB_4 demonstrates b-oxidation (255).

The parent compound of the cysteinyl leukotrienes, LTC_4, either is metabolized to receptor-active products or is inactivated extracellularly. Bioactive derivatives are formed by the sequential removal of glutamic acid by g-glutamyl transpeptidase to form LTD_4, and the cleavage of glycine by dipeptidase to form LTE_4, which is the most stable (256,257). LTE_4 is excreted in the urine directly or after *N*-acetylation. The cysteinyl leukotrienes are locally inactivated in the microenvironment of an inflammatory reaction by hypochlorous acid formed from chloride ions and myeloperoxidase-dependent generation of hydrogen peroxidase. Hypochlorous acid acts at the sulfur bridge to form inactive sulfoxides and then to break the bridge, leaving 6-*trans* diastereoisomers of LTB_4 (258).

Gene disruption of g-glutamyl transpeptidase failed to demonstrate altered leukotriene metabolism; however, the mice displayed glutathionemia and had premature death (259). Membrane-bound dipeptidase gene-disrupted mice retain their ability to form LTE_4, but the dipeptidase cleavage event is not highly selective (260).

Leukotriene and Lipoxin Transport

Specific transporters export LTB_4 and LTC_4 from human granulocytic cells in a temperature-dependent and substrate-specific carrier-mediated process (261,262). The

export of LTB_4 from neutrophils has a Q_{10} value of 3.0 and an energy of activation value of 19.9 kcal/mol; the export of LTC_4 from eosinophils has a Q_{10} value of 3.7 and an energy of activation value of 28 kcal/mol. The transport of LTB_4 exhibits a calculated K_m of 798 pmol/10^7 cells and a V_{max} of 383 pmol/10^7 cells per 20 seconds, whereas K_m and V_{max} values for LTC_4 export are 80 and 38.5 pmol/10^6 cells per minute, respectively (261,262). The LTB_4 carrier export system is distinct from the LTC_4 carrier export system in that no competition for export of LTB_4 occurs when the anionic organic acid transport blocker, probenecid, is present (263). Furthermore, the observation that the export of LTC_4 competes with other GSH conjugates is compatible with a possible role of the adenosine triphosphate–dependent, multidrug resistance–associated protein (264).

LXA_4 is transported into neutrophils by a novel carrier-mediated process that is inducible in human neutrophils and is not present in other hematopoietic cells. At a concentration of 5 nM, uptake of [^{3}H]LXA_4, above that caused by specific binding to receptors, amounted to approximately 0.6 fmol/10^6 cells per minute. This process was specific for LXA_4, was sodium and membrane voltage independent, and exhibited a striking dependence on pH. The LXA_4 carrier did not appear to interact with arachidonic acid, PGE_2, 15(S)-HETE, LTB_4, LTC_4, or LTD_4; it was sensitive to anionic inhibitors, including 3,5-diiodosalicylic acid, pentachlorophenol, and the organomercurial agents mersalyl and *p*-hydroxy-mercuribenzoate (265).

Leukotriene and Lipoxin Receptors

Neutrophils have two different LTB_4 receptors. The high-affinity receptor state, with a K_d of 0.3 nM, transduces the functions of chemotaxis and adhesion; and the low-affinity receptor state, with a K_d of 200 nM, mediates the secretion of granule contents and superoxide generation when it is activated by LTB_4 (266). A cell-surface G protein–coupled receptor for LTB_4 (BLT1) has been cloned and predicts a seven-transmembrane spanning protein of 352 amino acids (267). This receptor is highly expressed in leukocytes and transduces their chemotaxis and chemokinesis. The K_d of this receptor for LTB_4 is 1.1 nM (Table 27.6), and it corresponds to the high-affinity receptor that was biochemically characterized. This receptor increases intracellular calcium and inositol 1,4,5-trisphosphate accumulation with adenylyl cyclase inhibition through both pertussis-sensitive and pertussis-insensitive G proteins (267,268). A transgenic mouse with overexpression of BLT1 has a dramatic increase in neutrophil trafficking to skin microabscesses and lungs after ischemia and reperfusion. BLT2 has 45% amino acid identity to BLT1, has a 23 mN K_d for LTB_4, and is ubiquitously expressed in tissues, whereas BLT1 is leukocyte selective (270).

The cysteinyl leukotrienes mediate their effects through what appear to be at least two distinct receptors. Specific inhibitors of the membrane action of the cysteinyl leukotrienes revealed a receptor for LTD_4/LTE_4 in human lung parenchyma that was blocked, whereas these antagonists were not active against LTC_4 under conditions in which its metabolism to LTD_4/LTE_4 was prevented (271). The former receptor was designated $cysLT_1$, and the latter was $cysLT_2$. A cDNA for the $cysLT_1$ receptor encodes a polypeptide of 337 amino acids with a calculated molecular mass of 38.5 kd (272) (Table 27.6). The transcript for this receptor is widespread, including intestine, heart, spleen, lung, and leukocytes; and within lung, *in situ* hybridization revealed expression in smooth muscle cells and macrophages (272). Pertussis toxin experiments revealed that a $G_{1a/0}$ is the subunit responsible for transduction in the oocyte (272). Previous experiments with the THP-1 monocytic cell line indicated that LTD_4 activates signals through two pathways, with mitogen-activated protein kinase activation using the pertussis toxin–insensitive protein kinase C_a Raf-1 pathway and directed migration transducing through pertussis toxin–sensitive mechanisms (273). $CysLT_2$ has a 38% amino acid identity to $cysLT_1$, has a K_d of 4.8 mN for either LTC_4 or LTD_4, is distributed to lung macrophages and airway smooth muscle as well as to cardiac Purkinje and adrenal medulla cells, and is pharmacologically distinct from $cysLT_1$ in being resistant to the selective $cysLT_1$ antagonists (274).

LXA_4 triggers selective responses with human neutrophils through an inducible, pertussis toxin–sensitive receptor (LXA_4R) with a high-affinity K_d of 0.5 nM (274) (Table 27.6). This receptor in neutrophil membranes is specific, with only LTD_4 competing with LXA_4, with a K_i of 80 nM. In transfection experiments, LXA_4 also stimulated guanosine triphosphatase activity and provoked the pertussis toxin–sensitive release of esterified arachidonic acid, an effect that was also sensitive to pertussis toxin (275).

Chapter References

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28 PRIMARY IMMUNODEFICIENCIES OF LYMPHOCYTES

Rebecca H. Buckley, M.D.

Since defects in human host defense were first discovered five decades ago (1,2), more than 95 different primary immunodeficiency disorders have been recognized (3,4). Naturally occurring immune defects also have been described in animals. The study of these human and murine “experiments of nature” and of mice made immunodeficient by inserting mutant genes for selected immune components has provided unique insights into the intricate workings of the immune system (5). Primary immunodeficiency may affect one or more components, including T, B, and natural killer (NK) lymphocytes; phagocytic cells; and complement proteins. This chapter focuses on some but not all of the genetically determined immunodeficiency diseases that affect lymphocytes.

CLINICAL PRESENTATION OF PATIENTS WITH DEFECTS IN LYMPHOCYTE FUNCTION

Primary immunodeficiency diseases are characterized by undue susceptibility to infection, autoimmunity, and an increased risk of malignancy. Patients who have mutations impairing B- or T-lymphocyte function have three major clinical phenotypes: a deficiency of antibody production, a deficiency of cellular immunity, or deficiencies of both. It is important to point out that different molecular defects can manifest with the same clinical phenotype. Conversely, the same molecular defect can sometimes have different clinical presentations. Although the true incidences of these conditions are unknown, it has been estimated that they occur in one of every 10,000 live births (4). Knowledge of the particular infectious agents involved and the anatomic sites most often affected in a given patient can provide clues as to the most likely type of defect. Patients with B-cell defects have recurrent infections with encapsulated bacterial pathogens. In contrast, patients with T-cell defects have problems with opportunistic infections with viral and fungal agents in addition. The latter patients in general begin to fail to thrive shortly after these problems develop. Excessive routine use of antibiotics by primary care physicians has altered the textbook presentation of many of these conditions so that their detection requires not only a high index of suspicion but also extensive knowledge on the part of the physician about their fundamental causes and the various ways they can manifest.

GENETICS OF PRIMARY IMMUNODEFICIENCY

Until the last decade, little insight had been gained into the fundamental problems underlying these conditions. As a result of remarkable advances in human molecular genetics over the past 8 years, however, the abnormal genes have been identified in a rapidly growing number of defects. The genes essential for immune function are distributed throughout the entire genome; however, there is a dominance of X-linked immunodeficiency resulting from hemizygosity in male subjects for the substantial number of X-encoded immune system genes. There is also a high incidence of new mutations in the latter genes. Carriers of X-linked immunodeficiency have skewed X-inactivation in the cell lineage affected, and this can be used diagnostically to assess whether a female relative is a carrier. Within the past 8 years, the molecular bases of five X-linked immunodeficiency disorders have been discovered: X-linked agammaglobulinemia, X-linked immunodeficiency with hyperimmunoglobulin M (hyper-IgM), the Wiskott–Aldrich syndrome, X-linked severe combined immunodeficiency disease (SCID), and X-linked lymphoproliferative disease (Table 28.1) (6).

Chromosomal Location	Disease
Xp11.22	WAS due to WAS protein deficiency[a] (301000)[b]
Xq13.1	X-linked SCID due to common cytokine receptor gamma chain (γ_c) deficiency[a] (308380)[b]
Xq21.3	X-linked agammaglobulinemia due to Btk deficiency[a] (300300)
Xq25	X-linked lymphoproliferative syndrome due to mutation in SLAM-associated protein SH2D1A[a] (308240)
Xq26.3–q27.1	Immunodeficiency with hyper IgM due to CD154 (CD40 ligand) deficiency[a] (308230)[b]

Btk, Bruton tyrosine kinase; IgM, immunoglobulin M; SCID, severe combined immunodeficiency; SLAM, signaling lymphocyte activation molecule; WAS, Wiskott-Aldrich syndrome.
[a] Gene cloned and sequenced, gene product known.
[b] Numbers represent Online Mendelian Inheritance in Man (OMIM) reference numbers, National Library of Medicine.

TABLE 28.1. Abnormal Genes Known To Cause X-Linked Immunodeficiency Involving Lymphocytes

Other chromosomal regions that carry an increased number of immune function genes include 6p, where histocompatibility genes reside, and 5q, where there are many cytokine genes. In the evaluation of patients with suspected immunodeficiency, inquiry into consanguinity is important because those born to parents from genetically restricted populations are at risk for homozygosity for autosomal recessive immunodeficiency disorders. Some autosomal recessive defects for which the molecular bases have been discovered include leukocyte adhesion deficiency type 1 (LAD-1), adenosine deaminase (ADA) deficiency, purine nucleoside phosphorylase (PNP) deficiency, ataxia–telangiectasia, DiGeorge syndrome, major histocompatability complex (MHC) antigen deficiency, z-associated protein-70 (ZAP-70) deficiency, Janus kinase 3 (Jak3) deficiency, and interferon g receptor deficiencies, among others (Table 28.2). Gene-based understanding now allows them to be viewed according to the types of genetically altered molecules involved. It is important for physicians to determine the molecular causes of their patients' defects for purposes of providing genetic counseling, prenatal diagnosis, and possible future correction by gene therapy.

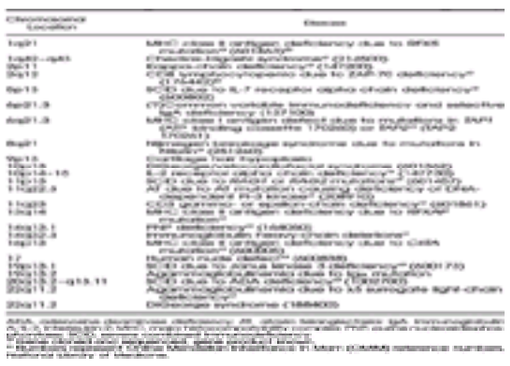

TABLE 28.2. Abnormal Genes Known To Cause Autosomal Immunodeficiency Disorders Involving Lymphocytes

ANTIBODY DEFICIENCY DISORDERS

X-linked Agammaglobulinemia

The clinical problems affecting patients with X-linked agammaglobulinemia (XLA) exemplify the classic phenotype of antibody deficiency. Boys who have deficient immunoglobulin (Ig) and antibody production are protected during the first months of life by maternally transmitted IgG antibodies. Thereafter, they acquire infections with encapsulated organisms, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus*, and with gram-negative organisms such as *Pseudomonas* species. Chronic fungal infections are not usually present, and *Pneumocystis carinii* pneumonia rarely occurs. Usually, viral infections are handled normally, except those with the hepatitis viruses or with enteroviruses, which can cause persistent meningoencephalitis (with or without a dermatomyositislike picture) (7). Paralysis can result from chronic infection with live attenuated vaccine polioviruses. Infections with echoviruses, coxsackieviruses, adenoviruses (8), and *Ureaplasma urealyticum* (9) have been identified in joint fluids of patients even on immunoglobulin replacement therapy. Concentrations of immunoglobulins of all isotypes are quite low. Circulating B cells are extremely few or absent, tonsils are small, and lymph nodes are rarely palpable, which should facilitate early recognition of this disorder. Thymus architecture is normal, as are the thymus-dependent areas of spleen and lymph nodes. Monthly infusions of intravenous immunoglobulin are life saving.

In 1993, two groups of scientists discovered the mutated gene in XLA, now called the *Bruton tyrosine kinase* (*BTK*) gene (Table 28.1, Fig. 28.1) (10,11). Btk is a member of the Tec family of cytoplasmic protein tyrosine kinases. This kinase appears to be necessary for pre-B-cell expansion and maturation into surface immunoglobulin-expressing B cells, accounting for the lack of circulating B cells (12). It has not been detected in T cells but has been found in myeloid cells (11). The latter could be relevant to the intermittent neutropenia seen in boys with XLA (13,14). To date, more than 300 unique mutations in the human *BTK* gene have been recognized, and there has not been any clear correlation between the location of the mutation and the clinical phenotype (15).

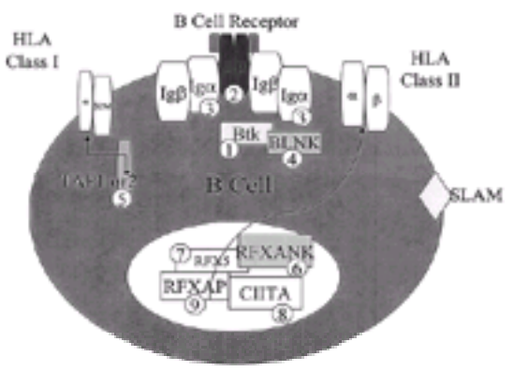

Figure 28.1. Cartoon showing the locations of mutations occurring in B cells in various immunodeficiencies: (1) Bruton tyrosine kinase in X-linked agammaglobulinemia; (2) µ heavy chain; (3) immunoglobulin a; or (4) B-cell linker protein (BLNK) in B-cell-negative, autosomal recessive agammaglobulinemia; (5) transporters of antigenic peptides (TAP-1 or TAP-2) in human leukocyte antigen (HLA) class I antigen deficiency, and HLA class II antigen gene transcription factors: (6) RFX associated protein (RFANK); (7) RFX5; (8) class II transactivator (CIITA); or (9) RFXAP in HLA class II antigen deficiency.

Carriers of XLA can be identified by the finding of nonrandom X chromosome inactivation in their B cells. Prenatal diagnosis of affected or nonaffected male fetuses has also been accomplished by detection of the mutated gene in chorionic villous or amniocentesis samples.

B-Cell Antigen Receptor Gene Deficiencies

These autosomal recessive conditions characterized by a- or hypogammaglobulinemia are caused by mutations in the genes that encode immunoglobulin heavy or light chains or their associated signaling molecules. In µ chain, l5/14.1 (surrogate light chain), Iga (B-cell antigen receptor signaling molecule), and B-cell linker (BLNK, an adapter protein important in B-cell antigen receptor signaling) gene mutations, circulating B cells are also absent (Table 28.1, Fig. 28.1) (16,17,18,19 and 20). Thus, these defects, in which Btk is normal, mimic Bruton agammaglobulinemia clinically and in their absence of B cells. In the case of other heavy-chain gene mutations, deficiencies of individual immunoglobulin classes or subclasses are seen, and circulating B cells are present (21). Mutations in the k-chain gene result in molecules with only l light chains.

Common Variable Immunodeficiency

Common variable immunodeficiency (CVID), also known as *acquired hypogammaglobulinemia*, may appear similar clinically in many respects to XLA (3). The kinds of infections experienced and bacterial etiologic agents involved are generally the same for the two defects. In comparing the two defects further, it is noted that in CVID there is an almost equal sex distribution, generally a later age of onset of infections (which are somewhat less severe), a tendency to autoantibody formation, normal-sized or enlarged tonsils and lymph nodes, and splenomegaly in approximately 25% of affected persons. Lymphoid interstitial pneumonia, pseudolymphoma, amyloidosis, and noncaseating granulomata of the lungs, spleen, skin, and liver also have been seen. There is a 438-fold increase in lymphomas in affected women in the 5th and 6th decades of life (22).

The serum immunoglobulin and antibody deficiencies in CVID may be as profound as in XLA. Despite normal numbers of circulating immunoglobulin-bearing B-lymphocytes and the presence of lymphoid cortical follicles, blood B-lymphocytes from CVID patients do not differentiate into immunoglobulin-producing cells (23,24). From these observations, the defect(s) in this syndrome were postulated to be intrinsic to the B cell (23). In keeping with this are studies showing a lack of protein kinase C activation and translocation to the plasma membrane when CVID B cells are stimulated with phorbol ester or anti-µ (25). However, CVID B cells can be stimulated to both isotype-switch and to synthesize and secrete immunoglobulin when stimulated with anti-CD40 plus interleukin-4 (IL-4) or IL-10 (26,27). T cells and T cell subsets are usually present in normal percentages, but a dominance of gd T cells has been observed in some patients (28) and depressed T-cell function has been reported in others (29,30). In addition, decreased number and function of antigen-specific T cells were noted in CVID patients immunized with keyhole limpet hemocyanin (KLH) (31). One group of CVID patients was reported to have significantly depressed (but not absent) expression of CD40 ligand (CD154) mRNA and surface protein in their activated T-lymphocytes, suggesting that inefficient signaling by poorly expressed CD154 on their T cells could account for failure of their B cells to differentiate (32). Tonsils and lymph nodes are either normal sized or enlarged, and splenomegaly occurs in about 25% of patients with CVID. In addition, there is a tendency to autoantibody formation, and there are now several cases of lupus erythematosus converting to CVID (33). Rarely, CVID has been reported to resolve transiently or permanently when some such patients acquired human immunodeficiency virus (HIV) infection (34).

Because this disorder occurs in first-degree relatives of patients with selective IgA deficiency and some patients with IgA deficiency later became panhypogammaglobulinemic (35), it has long been suspected that these diseases have a common genetic basis (36,37). The high incidences of abnormal immunoglobulin concentrations, autoantibodies, autoimmune disease, and malignancy in families of both types of patients also suggested a shared hereditary influence. This concept is supported by the finding of a high incidence of C4-A gene deletions and C2 rare gene alleles in the class III MHC region in individuals with

either IgA deficiency (38,39) or CVID (40,41), suggesting that there is a susceptibility gene in this region on chromosome 6 (Table 28.2). The abnormal gene has not been identified, however. These studies also have shown that a small number of human leukocyte antigen (HLA) haplotypes are shared by persons affected with CVID and IgA deficiency, with at least one of two particular haplotypes being present in 77% of those affected (41). In one large family with 13 members, two had IgA deficiency and three had CVID (36). All the immunodeficient patients in the family had at least one copy of an MHC haplotype shown to be abnormally frequent in IgA deficiency and CVID: HLA-DQB1 *0201, HLA-DR3, C4B-Sf, C4A-deleted, G11-15, Bf-0.4, C2a, HSP70-7.5, tumor necrosis factor a-5 (TNFa-5), HLA-B8, and HLA-A1 (42). Environmental factors, particularly drugs such as phenytoin, have been suspected as providing the triggers for disease expression in individuals with the permissive genetic background. The prognosis for patients with CVID is reasonably good unless severe autoimmune disease or malignancy develops (22). However, this is a very heterogeneous syndrome, and likely includes some known defects.

Selective IgA Deficiency

An isolated deficiency of serum IgA (i.e., <10 mg/dL) is the most common recognized primary immunodeficiency, with a frequency of 1:333 reported among some blood donors (43,39). This condition has been observed in apparently healthy persons (43), but it also is associated with poor health. As would be expected, infections occur predominantly in the respiratory, gastrointestinal, and urogenital tracts (44). The responsible bacterial agents are essentially the same as in other types of antibody deficiency syndromes. As in CVID, there are frequent occurrences of autoimmune diseases, and there is an increased incidence of malignancy in IgA deficiency (38).

Serum concentrations of other immunoglobulins are usually normal in patients with IgA deficiency, although IgG subclass deficiency has been reported (45) and IgM (usually elevated) may be monomeric. Children with IgA deficiency vaccinated with killed poliovirus intranasally produced local IgM and IgG antibodies. Of the possible causative and important clinical consequences is the presence of antibodies to IgA in the sera of up to 44% of patients with IgA deficiency (43). Some IgA-deficient patients have had severe or fatal anaphylactic reactions after intravenous administration of blood products containing IgA, and anti-IgA antibodies (particularly IgE anti-IgA antibodies) have been implicated (46). For this reason, only normal donor erythrocytes washed five times (in 200-mL volumes) or blood products from other IgA-absent persons should be administered to these patients. Patients with IgA deficiency also frequently have IgG antibodies against cow milk and ruminant serum proteins (47). These antiruminant antibodies often falsely detect “IgA” in immunoassays that use goat (but not rabbit) antisera (44). A high incidence of autoantibodies also has been noted (48).

The basic defect leading to IgA deficiency is unknown. *In vitro* cultures of B cells from some IgA-deficient patients could be stimulated to produce IgA by the combination of anti-CD40 and IL-10; those whose B cells did not produce IgA with these treatments appeared to be more susceptible to infection (49,50). Treatment with dilantin, sulfasalazine (51), d-penicillamine, or gold has been associated with the development of IgA deficiency; the condition also has been known to remit either after discontinuation of dilantin therapy or spontaneously (52,53). IgA deficiency occurs in both male and female patients; in families in whom more than one member is affected, the pattern is that of autosomal dominant inheritance with variable expressivity (36). As already noted, this defect occurs in pedigrees with CVID patients, and some patients with IgA deficiency have gone on to develop CVID (35). Studies suggest that a susceptibility gene for these two defects may reside in the MHC class III region on chromosome 6 as an allelic condition (Table 28.2) (36,37,41,42).

Hyperimmunoglobulin M

The hyper-IgM immunodeficiency syndrome is characterized by extremely low serum IgG, IgA, and IgE concentrations and either a normal or a markedly elevated concentration of polyclonal IgM. Patients with hyper-IgM develop both recurrent pyogenic infections and *P. carinii* pneumonia. There is also an increased frequency of autoimmune disorders and malignancy (3,54,55). Paradoxically, the X-linked form of this condition is not a B cell defect but a T cell defect. Until the T-cell location of the defect was discovered, coexistent neutropenia had been considered a possible explanation for the susceptibility to *P. carinii* pneumonia (3,54).

The abnormal gene in X-linked hyper-IgM was localized to Xq26 (56) and identified by six groups in 1993 (Table 28.1, Fig. 28.2) (57,58,59 and 60). The gene product is a T-cell surface molecule now known as *CD154* (or *CD40 ligand*); it is present primarily on activated $CD4^+$ T cells, and it interacts with CD40 on B cells (61). CD154 is a type II integral membrane glycoprotein with significant sequence homology to TNF (61). Cross linking of CD40 on either normal or X-linked hyper-IgM B cells with a monoclonal antibody to CD40 or with soluble CD154 in the presence of cytokines (IL-2, IL-4, or IL-10) causes the B cells to proliferate and secrete immunoglobulins of various isotypes. Mutations in the gene encoding CD154 result in a lack of B cell signaling by T cells; as a result, the B cells fail to undergo isotype switching and produce only IgM.

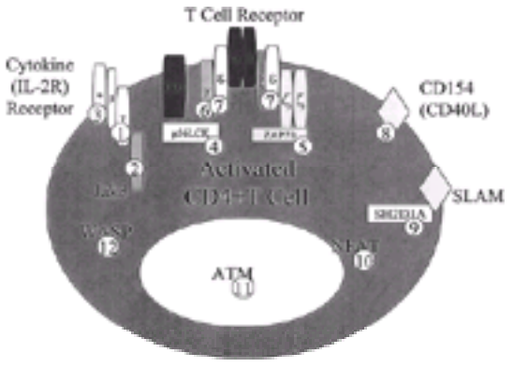

Figure 28.2. Locations of mutations occurring in T cells in various immunodeficiencies: (1) common g chain (gc) shared by multiple cytokine receptors in T^-B^+ natural killer $(NK)^-$ X-linked severe combined immunodeficiency disease X-1 (SCIDX-1); (2) Janus kinase 3 (Jak3) in autosomal recessive $T^-B^+NK^-$ SCID; (3) the chain of the interleukin-2 (IL-2) receptor (CD25) in a lymphoproliferative immunodeficiency syndrome; (4) the tyrosine kinase p56LCK in a SCID-like defect; (5) z-associated protein 70 (ZAP70) in CD8 lymphocytopenia; (6) the g; or (7) e chains of the T-cell receptor complex in CD3 antigen deficiency; (8) the CD40 ligand (CD154) in X-linked hyperimmunoglobulin M (IgM); (9) the adaptor protein SH2DIA in the X-linked lymphoproliferative syndrome; (10) nuclear factor of activated T cells in some T-cell signaling defects; (11) the ataxia telangiectasia mutation; and (12) the Wiskott-Aldrich syndrome protein (WASP).

The lack of cross linking of CD40 also results in failure of the B cells to upregulate CD80 and CD86, important costimulatory molecules that interact with CD28/CTLA4 on T cells (62). The failure of interaction of the molecules of those pathways in the thymus in X-linked hyper-IgM results in defective negative selection of autoreactive thymocytes. Similarly, their lack of interaction extrathymically is postulated to cause defective recognition of tumor cells because of tolerogenic signaling of peripheral T cells. Lymph nodes show only abortive germinal center formation (63).

Many distinct point mutations or deletions in the gene encoding CD154 have been identified (64,65). A highly polymorphic microsatellite dinucleotide (CA) repeat region in the 3' untranslated end of the CD154 gene is useful for detecting carriers and for making a prenatal diagnosis (66).

Numerous examples of autosomal recessively inherited hyper-IgM syndrome (67) indicate that this condition has more than one genetic cause. In such patients, there is an intrinsic B-cell defect; that is, the B cells fail to switch from IgM-secreting to IgG, IgA, or IgE-secreting cells, even when cocultured with monoclonal antibodies to CD40 and cytokines (68). Recently, one defect accounting for non-X-linked hyper-IgM was discovered: mutations in the gene at 12p13 encoding an activation-induced cytidenic deaminase, a messenger RNA editing enzyme (68a).

CELLULAR OR COMBINED IMMUNODEFICIENCIES

DiGeorge Syndrome and the Human Nude Defect

Thymic hypoplasia in the DiGeorge syndrome results from dysmorphogenesis of the third and fourth pharyngeal pouches during early embryogenesis, also leading to hypoplasia or aplasia of the parathyroid glands (3,69). Other structures that form at the same age are also frequently affected, resulting in anomalies of the great vessels (right-sided aortic arch), esophageal atresia, bifid uvula, upper limb malformations (70), congenital heart disease (atrial and ventricular septal defects), a short philtrum of the upper lip, hypertelorism, an antimongoloid slant to the eyes, mandibular hypoplasia, and low-set, often notched ears. There are clinical similarities between the DiGeorge syndrome and the fetal alcohol syndrome (69). Usually, the diagnosis of DiGeorge syndrome is first suggested by the presence of hypocalcemic seizures during the neonatal period. Since the original description of the syndrome, it has become apparent that a variable degree of hypoplasia is more frequent than total aplasia of the thymus and parathyroid glands. Some children have little trouble with life-threatening infections and grow normally; such patients are often referred to as having partial DiGeorge syndrome (71). Those with complete DiGeorge syndrome may resemble patients with SCID in their extreme susceptibility to infections

with opportunistic pathogens (i.e., fungi, viruses and *P. carinii*) and to graft-versus-host disease (GVHD) from nonirradiated blood transfusions.

DiGeorge patients are usually only mildly lymphopenic (71,72); however, the percentage of T cells is variably decreased; as a result, there is a relative increase in the percentage of B cells. B-cell function is impaired only to the extent of needing “helper” T cells. Immunoglobulin concentrations are usually normal, although sometimes IgE is elevated and IgA may be low (71,72). Responses of blood lymphocytes following mitogen stimulation have been absent, reduced, or normal, depending on the degree of thymic deficiency, suggesting that the T-lymphocytes that are present are intrinsically normal (71,72). Thymic tissue, when found, does contain Hassall corpuscles and a normal density of thymocytes; corticomedullary distinction is present. Lymphoid follicles are usually present, but lymph node paracortical areas and thymus-dependent regions of the spleen show variable degrees of depletion.

The DiGeorge syndrome has occurred in both male and female subjects. It is rarely familial, but cases of apparent autosomal dominant inheritance have been reported. Microdeletions of specific DNA sequences from chromosome 22q11.2 (the DiGeorge chromosomal region, or DGCR) have been shown in a majority of patients (73,74 and 75), and polymerase chain reaction (PCR)–based genotyping using microsatellite DNA markers located within the commonly deleted region permits rapid detection of such microdeletions (Table 28.2) (76). Several candidate genes have been identified in this region (74,77,78 and 79). There appears to be an excess of 22q11.2 deletions of maternal origin (80). There are similarities between the DiGeorge syndrome; the velocardiofacial syndrome (VCFS), a condition characterized by craniofacial abnormalities, heart defects, and learning disability) (81); and the conotruncal anomaly face syndrome (CTAFS), which is characterized by congenital heart disease, dysmorphic facies, and cleft palate or hypernasality (82). All three have conotruncal heart defects and 22q deletions. The so-called CATCH 22 syndrome (*c*ardiac, *a*bnormal facies, *t*hymic hypoplasia, *c*left palate, *h*ypocalcemia) includes the broad clinical spectrum of conditions with 22q11.2 deletions. Another deletion associated with DiGeorge and velocardiofacial syndromes has been identified on chromosome 10p13 (Table 28.2) (83,84 and 85).

No immunologic treatment is needed for the partial form. If the patient does not have a severe cardiac lesion, he or she will have few clinical problems, except that some experience seizures and developmental delay. Those with complete DiGeorge syndrome die in infancy unless immune reconstitution can be accomplished.

A clinically similar defect with an entirely different fundamental cause is the human equivalent of the nude mouse (86). In contrast to the DiGeorge anomaly, the defect in nude mice and humans is in their thymic and cutaneous epithelial cells, resulting in hairlessness and a lack of thymic development. The nude phenotype is caused by mutations in a gene on murine chromosome 11 and on human chromosome 17 that encodes a novel winged helix (whn) or fork head domain transcription factor (Table 28.2) (87).

Severe Combined Immunodeficiency

Severe combined immunodeficiency disease is a syndrome of diverse genetic origin characterized by profound deficiencies of T- and B-cell function. In some molecular types, there is also a deficiency of NK cells. In the first few months of life, affected infants have diarrhea and show failure to thrive. Persistent infections with opportunistic organisms, such as *Candida albicans, P. carinii*, varicella, adenovirus, respiratory syncytial virus, parainfluenza 3, cytomegalovirus, Epstein-Barr virus (EBV), and bacille Calmette-Guérin (BCG), lead to death. These infants also lack the ability to reject allografts, leaving them at risk for fatal GVHD (88). This condition is uniformly fatal in the first 2 years of life unless immune reconstitution can be accomplished.

Infants with SCID are lymphopenic; recognition of this characteristic alone can result in early diagnosis, even at birth (89). Their lymphocytes fail to proliferate *in vitro* in response to mitogens, antigens, or allogeneic cells. Serum immunoglobulins and antibodies are diminished to absent. The thymuses are quite small (usually <1 g) and lack thymocytes, corticomedullary distinction, and Hassall corpuscles. Studies have shown that these thymuses are capable of supporting T-cell development when normal stem cells are provided (90). Thymus-dependent areas of the spleen are devoid of lymphocytes, and lymph nodes and tonsils are absent. Flow cytometric studies have shown that there are unique lymphocyte phenotypes for the various genetic forms of SCID. Some have B cells and no NK cells (so-called $T^-B^+NK^-$ SCID), others have no B cells but many NK cells ($T^-B^-NK^+$ SCID), and others have extremely low numbers of all types of lymphocytes ($T^-B^-NK^-$ SCID) (Fig. 28.3) (89,91).

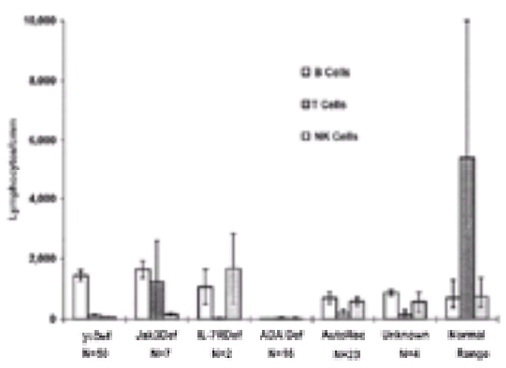

Figure 28.3. Means ± standard error of the mean (SEM) of $CD20^+$ B cells, $CD3^+$ T cells, and $CD16^+$ natural killer (NK) cells at initial presentation of 102 patients with severe combined immunodeficiency disease (SCID), showing the lymphopenia characteristic of all forms of SCID and the different phenotypic characteristics of patients with the various genetic forms of the syndrome compared with mean ± SEM for normal controls.

Enormous strides have been made in identifying the molecular causes of SCID in recent years (Fig. 28.3 and Fig. 28.4) (92,93,94,95,96,97 and 98). An absence of the enzyme ADA was identified in the early 1970s as a cause of SCID, but this defect accounts for only about 15% of patients with SCID (92,89) The gene encoding ADA was mapped to chromosome 20q13.2-q13.11, cloned, and sequenced (Table 28.2). The ADA deficiency caused by mutations in this gene results in marked accumulations of adenosine, 2'-deoxyadenosine and$^{2'}$-O-methyladenosine. The latter directly or indirectly leads to lymphocyte apoptosis. There are certain distinguishing features of ADA deficiency, including multiple skeletal abnormalities of chondroosseous dysplasia on radiographic examination. The latter include flaring of the costochondral junctions and a “bone-in-bone” anomaly in the vertebral bodies. ADA-deficient patients have a more profound lymphopenia than do infants with other types of SCID, with mean absolute lymphocyte counts of less than 500 per mm^3 ($T^-B^-NK^-$ SCID) (89). Milder forms of this condition have been reported, leading to a delayed diagnosis of immunodeficiency even to adulthood (99). The diagnosis should be suspected in any patient with recurrent infections who has severe lymphopenia.

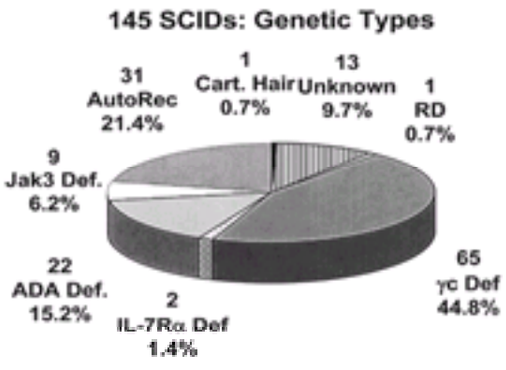

Figure 28.4. Relative frequencies of the different genetic types of severe combined immunodeficiency disease (SCID) among 145 SCID patients seen consecutively by the author over the past three decades. CHH, cartilage hair hypoplasia, a form of dwarfism associated with combined immunodeficiency of varying severity; gene mapped to 9p13 but not cloned as of this writing.

X-linked recessive SCID (SCID-X1) is the most common form of SCID, accounting for about 46% of U.S.cases (Fig. 28.4) (89). The abnormal gene in SCID-X1 was mapped to the Xq13 region and later identified as the gene encoding the common gamma chain (g_c) shared by cell surface receptors for various interleukin molecules (IL-2, IL-4, IL-7, IL-9, and IL-15) (Table 28.1, Fig. 28.2 and Fig. 28.5) (94,100,101). Among the first 136 SCID-X1 patients studied, 95 distinct mutations were identified, resulting in abnormal g_c chains in two thirds of the cases and absent g_c protein in the remainder (102). The finding that the mutated gene results in faulty signaling

through several cytokine receptors explains how T, B, and NK cell types can be affected by a single mutation (Fig. 28.5) (103,93). This was the initial example of $T^-B^+NK^-$ SCID; even though B cells are the dominant lymphocyte type present in the circulation (Fig. 28.3), the B cells do not isotype-switch normally. The single exception to SCID being invariably fatal without marrow transplantation or gene therapy was a patient with SCID-X1 who underwent spontaneous clinical improvement and who was found to have reversion of a documented mutation in the gene encoding g_c, presumably in a T-cell precursor (104).

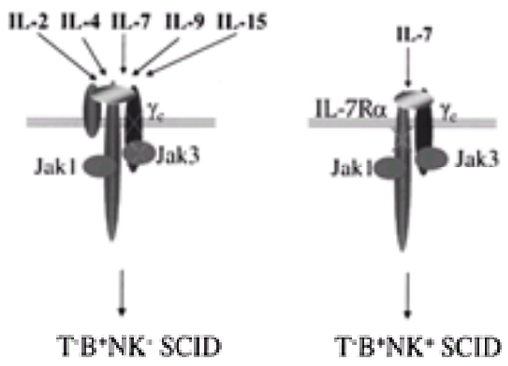

Figure 28.5. Cartoon showing on the left side that Janus kinase 3 (Jak3) is the major signal transducer for the common gamma chain (g_c) shared by multiple cytokine receptors. Mutations in the gene encoding g_c cause SCID-X1 and in the gene encoding Jak3 cause a form of autosomal recessive SCID that mimics SCID-X1 phenotypically. On the right side, it is seen that mutations in the gene encoding the IL-7 receptor a chain also result in another form of autosomal recessive SCID. The latter suggests that the reason for the severe T-cell defect in SCID-X1, and Jak3-deficient SCID is failure to signal through the IL-7 receptor. (Courtesy of Dr. Warren Leonard.) (See Color Figure 28.5.)

Because Jak3 is the only signaling molecule known to be associated with g_c, it was a candidate gene for mutations leading to autosomal recessive $T^-B^+NK^-$ SCID (Fig. 28.5), the identical lymphocyte phenotype seen in SCID-X1 (Fig. 28.3). This proved to be the case, and thus far at least 18 patients who lack Jak3 have been identified (Table 28.2; Fig. 28.4) (89,95,105). Like SCID-X1 patients, they have extremely low NK activity, even after successful marrow transplantation (88,106).

Some of my patients who had previously been shown not to have either g_c or Jak3 deficiency had $T^-B^+NK^+$ SCID (Fig. 28.3). Because mice whose genes for either the a chain of the IL-7 receptor or of IL-7 itself have been mutated are profoundly deficient in T- and B-cell function but have NK function (5), naturally occurring mutations in these genes were sought in human SCID. Mutations in the gene for IL-7Ra on chromosome 5p13 were found in three of my patients (Table 28.2; Fig. 28.4) (97) These findings imply that the T-cell, but not the NK-cell, defect in SCID-X1 and Jak3-deficient SCID results from an inability to signal through the IL-7 receptor (Fig. 28.5).

Infants with SCID due to mutations in recombinase activating genes 1 or 2 (RAG1 or RAG2) fail to rearrange either their T-cell or B-cell antigen receptors and thus have a distinctive lymphocyte phenotype in that they lack both B- and T-lymphocytes and have primarily NK cells in their circulation ($T^-B^-NK^+$ SCID) (Table 28.2) (96). In addition, patients with Omenn's syndrome also have mutations in *RAG1*or *RAG2* genes, resulting in partial and impaired V(D)J recombinational activity (107). Omenn's syndrome is characterized by the development soon after birth of a generalized erythroderma and desquamation, diarrhea, hepatosplenomegaly, hypereosinophilia, and markedly elevated serum IgE levels. The latter are caused by circulating activated, oligoclonal T-lymphocytes that do not respond normally to mitogens or antigens *in vitro* (108,109). Circulating B cells are not found, and lymph node architecture is abnormal due to a lack of germinal centers (110). The condition is fatal unless corrected by bone marrow transplantation.

The most recently discovered molecular defect causing SCID is a mutation in the gene encoding the common leukocyte surface protein CD45 (98). This hematopoietic-cell-specific, transmembrane protein tyrosine phosphatase functions to regulate Src kinases required for T- and B-cell antigen receptor signal transduction. A 2-month-old male infant presented with a clinical picture of SCID and was found to have an extremely low number of T cells but a normal number of B cells. The T cells failed to respond to mitogens, and serum immunoglobulins diminished with time. He was found to have a large deletion at one CD45 allele and a point mutation causing an alteration of the intervening sequence 13-donor splice site at the other (98).

Severe combined immunodeficiency disease is a pediatric emergency (89,88). Nearly all cases could be diagnosed at birth if routine blood counts and manual differentials were done and flow cytometry and T-cell functional studies were performed when lymphocyte counts are below the newborn normal range (i.e., 2,000–11,000/mm^3) (111,89). Marrow transplantation in the neonatal period is highly successful.

Purine Nucleoside Phosphorylase Deficiency

More than 40 patients with combined cellular and humoral deficiencies have been found to have PNP deficiency (112). Although ADA and PNP are both purine salvage pathway enzymes, PNP deficiency does not lead to a combined immunodeficiency that is severe as seen in ADA deficiency. Deaths have occurred from generalized vaccinia, varicella, lymphosarcoma, and GVHD mediated by T cells from nonirradiated allogeneic blood or bone marrow. Two thirds of patients have had neurologic abnormalities ranging from spasticity to mental retardation. One third have developed autoimmune diseases, the most common of which was autoimmune hemolytic anemia. Most patients have normal or elevated concentrations of all serum immunoglobulins. PNP-deficient patients usually have absolute lymphocyte counts less than 500 per mm^3. Analyses of lymphocyte subpopulations with monoclonal antibodies have demonstrated a marked deficiency of T cells but increased numbers of NK cells. T-cell function is low but not absent and is variable with time.

The gene encoding PNP is on chromosome 14q13.1, and it has been cloned and sequenced (Table 28.2). A variety of mutations have been found in the PNP gene in patients with PNP deficiency. Unlike ADA deficiency, serum and urinary uric acid are markedly deficient because PNP is needed to form the urate precursors, hypoxanthine and xanthine. Prenatal diagnosis is possible. This condition is invariably fatal unless immunologic reconstitution can be achieved. Bone marrow transplantation is the treatment of choice but has thus far been successful in only three patients (113,114).

T-Cell Antigen Receptor Gene Deficiencies

These conditions include (a) mutations in the CD3 (cluster of differentiation nomenclature system) g (115) or e (116) chain genes, leading to impaired expression of the T-cell receptor (Table 28.2, Fig. 28.2). Patients have variable susceptibility to infection and autoimmunity. There are few or no circulating CD3+ T cells, poor responses to T-cell mitogens, and variable immunoglobulin and antibody deficiencies.

Wiskott–Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) is an X-linked syndrome characterized by eczema, thrombocytopenic purpura with small defective platelets, and undue susceptibility to infection (3). During infancy, patients usually have bloody diarrhea or excessive bruising. Atopic dermatitis and recurrent infections with pneumococci and other encapsulated bacteria usually also develop during the first year of life. Later, infections with opportunistic agents, such as *P. carinii* and the herpesviruses, become more problematic. Autoimmune cytopenias and vasculitis are common in those who live beyond infancy. Infections and bleeding are major causes of death, but the most common cause of death is EBV-induced lymphoreticular malignancy.

Immunoglobulin concentrations are variable but near normal (117). Despite normal levels of IgG2, patients with WAS have an impaired humoral immune response to polysaccharide antigens, also evidenced by absence of isohemagglutinins (3). In addition, antibody titers to protein antigens fall with time. There are moderately reduced percentages of CD3-, CD4-, and CD8-bearing T cells, and lymphocyte responses to mitogens are depressed.

The mutated gene responsible for this defect was mapped to Xp11.22–11.23 and isolated in 1994 (Table 28.1) (6). It is highly expressed in lymphocytic and megakaryocyte lineages (6). The gene product, a 501 amino acid proline-rich protein that lacks a hydrophobic transmembrane domain, was designated the Wiskott–Aldrich syndrome protein (WASP) (Fig. 28.2). It binds CDC42H2 and rac, members of the Rho family of GTPases (118). WASP appears to control the assembly of actin filaments required for microvesicle formation downstream of protein kinase C and tyrosine kinase signaling. A large and varied number of mutations in the *WASP* gene have been identified in WAS patients (119). Isolated X-linked thrombocytopenia also is caused by mutations in the *WASP* gene (120). Carriers can be detected by the finding of nonrandom X chromosome inactivation in several hematopoietic cell lineages or by detection of the mutated gene (121). Prenatal diagnosis of WAS also can be made by chorionic villous sampling or amniocentesis. Two families with apparent autosomal inheritance of a clinical phenotype similar to

WAS have been reported (122,123). In another report of a girl with WAS, however, extremely skewed X chromosome inactivation combined with a mutation at the X-linked WASP locus on the active X chromosome to produce typical clinical WAS (124).

Some patients with WAS have had complete corrections of both the platelet and the immunologic abnormalities by HLA-identical sibling or matched unrelated donor bone marrow or cord blood transplants after being conditioned with irradiation or busulfan and cyclophosphamide (114,125,126). Several patients who required splenectomy for uncontrollable bleeding had impressive rises in their platelet counts and have done well clinically while on prophylactic antibiotics and intravenous immunoglobulin (IVIG) infusion (127).

Ataxia Telangiectasia

Ataxia telangiectasia is a complex combined immunodeficiency syndrome with associated neurologic, endocrinologic, hepatic, and cutaneous abnormalities (3,128). The most prominent features are progressive cerebellar ataxia, oculocutaneous telangiectasias, recurrent bacterial sinopulmonary disease, a high incidence of malignancy (129), and variable humoral and cellular immunodeficiency. Fatal varicella occurred in one of my patients, and transfusion-associated GVHD has also been reported (130).

Selective IgA deficiency is found in from 50% to 80% of affected individuals. IgG2 or total IgG may be decreased. *In vitro* tests of lymphocyte function have generally shown moderately depressed proliferative responses to mitogens. The thymus is hypoplastic, exhibits poor organization, and is lacking in Hassall corpuscles.

Cells from patients as well as those of carriers have sensitivity to ionizing radiation, defective DNA repair, and frequent chromosomal abnormalities (128,131). The most common causes of death are lymphoreticular malignancy and progressive neurologic disease (128,129). Adenocarcinoma and other forms of malignancy also have been seen.

The gene that is defective in AT, *ATM,* resides on chromosome 11q22.3 (Table 28.2, Fig. 28.2) (128,132). *ATM* encodes a phosphatidylinositol-3-kinase involved in mitogenic signal transduction, meiotic recombination, and cell cycle control.(133,134)

T-CELL ACTIVATION DEFECTS

T-cell activation defects are those in which there are phenotypically mature T cells present, but the cells fail to proliferate in response to mitogens or antigens.

CD8 Lymphocytopenia or Zeta-associated Protein-70 Deficiency

This condition is due to mutations in a gene on chromosome 2 at position q12 encoding ZAP-70, a non-src family protein tyrosine kinase important in T cell signaling (Table 28.1; Fig. 28.2, and Fig. 28.6) (135,136). ZAP-70 has an essential role in both positive and negative selection in the thymus (137). Patients with this condition may have moderate infections or infections as severe as those in SCID. Eight cases have been reported, and most were in Mennonites (135,136). Affected persons have normal or elevated numbers of circulating $CD4^+$ T-lymphocytes but essentially no $CD8^+$ T cells because of lower availability of compensatory signaling pathways for $CD8^+$ cell development within the thymus. The thymus of one patient exhibited normal architecture; there were normal numbers of double-positive ($CD4^+$ $CD8^+$) thymocytes but an absence of CD8 single positive thymocytes. Circulating $CD4^+$ T cells fail to respond to mitogens or to allogeneic cells *in vitro* or to become cytotoxic cells. By contrast, NK activity, B-cell numbers, and serum immunoglobulin concentrations are normal.

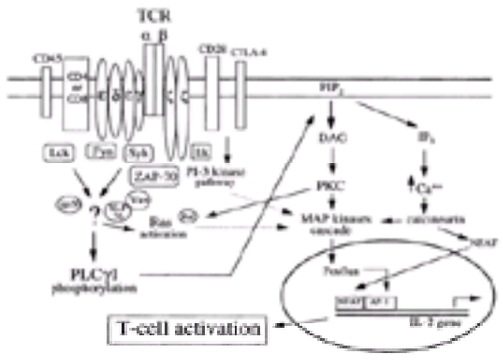

Figure 28.6. T-cell signal transduction pathway. The T-cell receptor (TCR) spans the plasma membrane in association with CD3 and z, CD4 or CD8, CD28 and CD45. Cytoplasmic protein tyrosine kinases (PTK) associated with the TCR are activated on antigen binding to the TCR. These PTK include Lck, Fyn, ZAP-70, and Syk. PTK activation results in the phosphorylation of phospholipase Cg1 and the activation of other signaling molecules. Distal signaling events, including PKC activation and Ca^{2+} mobilization, result in the transcription of genes encoding interleukin-2 (IL-2) and other proteins, culminating in T-cell activation, differentiation, and proliferation. Ionomycin and phorbol myristate acetate (PMA) can be used to mimic distal signaling events. Mutations in the gene encoding z-activating protein-70 (ZAP-70) result in markedly impaired T-cell activation, in addition to abnormal thymic selection resulting in CD8 deficiency. (Modified from Elder ME. Severe combined immunodeficiency due to a defect in the tyrosine kinase ZAP-70. *Pediatr Res* 1996;39:743-748. *Pediatr Res* 31996;39:744, courtesy of Dr. Melissa Elder.)

p56 Lck Deficiency

A 2-month-old male infant who presented with bacterial, viral, and fungal infections was found to be lymphopenic and hypogammaglobulinemic. B and NK cells were present, but there was a low number of $CD4^+$ T cells (138). Mitogen responses were variable. The T cells failed to express the activation marker CD69 when stimulated through the T-cell receptor but did when stimulated with phorbol myristate acetate and a calcium ionophore, suggesting a proximal signaling defect. Molecular studies revealed an alternatively spliced transcript for p56 Lck that lacked the kinase domain (Fig. 28.2 and Fig. 28.6) (138).

GENETIC DEFICIENCIES OF HLA MOLECULES

MHC Class I Deficiency

An isolated deficiency of MHC class I molecules is rare, and the resulting immunodeficiency is milder than in SCID. Class I MHC molecules, normally found on all cells in the body, are absent. There is a deficiency of $CD8^+$ but not of $CD4^+$ T cells. Mutations have been found in two genes within the MHC locus on chromosome 6 that encode the peptide transporter proteins, TAP1 and TAP2 (Table 28.2, Fig. 28.1) (139,140,141,142 and 143). TAP proteins function to transport peptide antigens from the cytoplasm across the Golgi apparatus membrane to join the a chain of MHC class 1 molecules and b microglobulin. The complex then can move to the cell surface; if the assembly of the complex cannot be completed because there is no peptide antigen, the MHC class I complex is destroyed in the cytoplasm (144).

MHC Class II Deficiency

Many persons affected with this autosomal recessive syndrome are of North African descent (145). More than 70 patients have been identified. Affected persons in infancy have persistent diarrhea, often associated with cryptosporidiosis, bacterial pneumonia, *Pneumocystis* infection, septicemia, and viral or monilial infections. Nevertheless, their immunodeficiency is not as severe as in SCID, as evidenced by their failure to develop BCG-osis or GVHD from nonirradiated blood transfusions (145).

Patients who are deficient in MHC class II antigens have a low number of $CD4^+$ T cells but normal or elevated numbers of $CD8^+$ T cells. Lymphopenia is only moderate. The MHC class II antigens, HLA-DP, DQ, and DR, are undetectable on blood B cells and monocytes. The patients have impaired antigen-specific responses caused by the absence of these antigen-presenting molecules. In addition, MHC antigen-deficient B cells fail to stimulate allogeneic cells in mixed leukocyte culture. Lymphocytes respond normally to mitogens but not to antigens. The thymus and other lymphoid organs are severely hypoplastic. The lack of class II molecules results in abnormal thymic selection because recognition of HLA molecules by thymocytes is central to both positive and negative selection. The latter results in circulating $CD4^+$ T cells that have altered CDR3 profiles (146). The associated defects of both B- and T-cell immunity and of HLA expression emphasize the important biologic role for HLA

determinants in effective immune cell cooperation.

Four different molecular defects resulting in impaired expression of MHC class II antigens have been identified (Table 28.2, Fig. 28.1) (147). In one defect, there is a mutation in the gene on chromosome 1q that encodes a protein called RFX 5, a subunit of RFX, a multiprotein complex that binds the X box motif of MHC II promoters (148). A second form is caused by mutations in a gene on chromosome 13q that encodes a second 36-kd subunit of the RFX complex, called RFX-associated protein (RFXAP) (149). The most recently discovered and most common cause of MHC class II defects are mutations in RFANK, the gene encoding a third subunit of RFX (147). In a fourth type, there is a mutation in the gene on chromosome 16p13 that encodes a novel MHC class II transactivator (CIITA), a non-DNA-binding coactivator that controls the cell-type specificity and inducibility of MHC-II expression (150). All these defects cause impairment in the coordinate expression of MHC class II molecules on the surface of B cells and macrophages.

IMMUNODEFICIENCY DISEASES WITH UNIQUE PHENOTYPES

X-linked Lymphoproliferative Disease

X-linked lymphoproliferative disease (XLP) is characterized by failure to eliminate reactive cytotoxic T cells after infection with EBV (151,152). Those affected appear healthy until they experience infectious mononucleosis (151). The mean age of presentation is less than 5 years. The most common form of presentation (75%) is severe mononucleosis, of which 80% of cases are fatal, primarily from extensive liver necrosis caused by activated cytotoxic T cells (151). Most patients surviving the primary infection develop global cellular immune defects, lymphomas, aplastic anemia, and hypogammaglobulinemia. The defective gene in XLP was localized to the Xq26–q27 region and identified as the gene that encodes a T and NK cell adapter protein (Table 28.1, Fig. 28.2) that interferes with the binding to downstream signaling molecules by a high-affinity self-ligand that is present on the surfaces of T and B cells, called signaling lymphocyte activation molecule (SLAM) (153,154 and 155). The adapter protein, called variously *SH2D1A* (the official designation), *SAP* (for SLAM-associated adapter protein), or DSHP (for Duncan syndrome human protein), normally serves to inhibit signal transduction by SLAM so that T cell proliferation does not continue unchecked (151). About half of a small number of patients with XLP given HLA-identical bone marrow transplants are currently surviving without sign of the disease.

Interleukin-2 Receptor a-Chain (CD25) Deficiency

Paradoxically, a mutation in the IL-2 receptor a-chain gene in an infant produced too many, rather than too few, T cells, with extensive infiltrates of the lungs, liver, gut, spleen, lymph nodes and bone (Fig. 28.2) (156). Serum IgG and IgM were elevated, but IgA was low. The infant was lymphopenic, and his T cells responded poorly to anti-CD3, phytohemagglutinin (PHA), and IL-2. This defect and that in XLP are among probably many that are characterized clinically by lymphoproliferation and autoimmunity and are caused by an imbalance of positive and negative signals caused by mutations in genes encoding negative regulatory components.

Hyperimmunoglobulin E Syndrome

The hyper-IgE syndrome is a primary immunodeficiency disorder that was first described in 1972; it is characterized by recurrent staphylococcal abscesses and markedly elevated serum IgE concentrations (157). Those affected have life-long histories of severe recurrent staphylococcal abscesses involving the skin, lungs, joints, and other sites. In addition, there is a unique tendency of these patients to form persistent pneumatoceles following staphylococcal pneumonias. Although usually there is a history of a pruritic dermatitis, it is not typical atopic dermatitis, and respiratory allergic symptoms are usually absent. I noted coarse facial features in the first two patients (157). Hyper-IgE syndrome patients look different from their nonaffected family members. Distinctive facial characteristics were pointed out again by Grimbacher and associates (158). Among the findings reported were a prominent forehead, deep-set eyes, a broad nasal bridge, a wide and fleshy nasal tip, mild prognathism, facial asymmetry, and hemihypertrophy. They also found that the mean nasal interalar distance in these patients was above the 98th percentile ($p < 0.001$). These findings were present in all patients in that study by age 16 years (158). A high incidence of scoliosis also was noted. An interesting observation in that group of patients that had not been previously reported was a 72% incidence of failure or delayed shedding of the primary teeth owing to lack of root resorption (158). This syndrome was mapped to chromosome 4; however, neither the fundamental host defect nor a defective gene has been identified in this condition (159).

TREATMENT

The principal modes of therapy for the primary immunodeficiency disorders include protective isolation, use of antibiotics for the eradication or prevention of bacterial and fungal infections, and attempted replacement of missing humoral or cellular immunologic functions. The complexities of both the immunodeficiency diseases and their treatment emphasize the need for all such patients to be evaluated in centers where detailed studies of immune function can be conducted before therapy is selected or begun.

Antibody Replacement

Regular administration of antibodies in the form of human immune serum globulin (see in later chapter) and judicious use of antibiotics are the only treatments that have proved effective for disorders characterized by antibody deficiency. Patients with agammaglobulinemia, X-linked immunodeficiency with hyper-IgM, antibody deficiency with near-normal immunoglobulins, WAS, all forms of SCID, and any other type of defect in which antibody production is abnormal are candidates for immunoglobulin replacement therapy, most often given as IVIG (160). Anaphylactic reactions caused by IgE antibodies (in the patient) to IgA (in the immunoglobulin preparation) can occur in patients with common variable immunodeficiency who have absent serum IgA (46). All newly diagnosed patients with the latter defect should be screened for anti-IgA antibodies before undergoing IVIG therapy. If such antibodies are detected, IVIG therapy still may be possible by using IVIG containing almost no IgA (Gammagard, Baxter-Hyland, or a similar preparation from the American Red Cross) (160).

Immunoglobulin replacement therapy is contraindicated in patients with selective absence of serum and secretory IgA, owing to the high frequency of anti-IgA antibodies (43,46) and because these patients usually have normal quantities of IgG antibodies (160). IVIG should not be given to infants with transient hypogammaglobulinemia of infancy or to patients with steroid-induced low IgG levels because it could suppress their usually normal innate capacity to form antibodies. There is no indication for the use of gamma globulin therapy in patients with IgG subclass deficiencies unless they have been shown to have a broad defect in antibody-forming capacity, and it is futile to give it to patients with low IgG caused by protein-losing states, such as congenital lymphangiectasia or other protein-losing enteropathies (160).

Thymus Transplantation

The only truly effective therapy for patients with severe forms of cellular immunodeficiency is immunologic reconstitution by means of immunocompetent tissue transplantation (114). Because of variability in the severity of the immunodeficiency in the DiGeorge syndrome, it is difficult to evaluate claimed benefits of fetal thymus transplantation (161,162). Unfractionated HLA-identical sibling bone marrow transplantation was effective in treating three infants with complete DiGeorge syndrome by virtue of adoptive transfer of mature T cells from the donor (163). It is unknown, however, how long that reconstitution will last because they do not have thymi to process stem cells into new T cells. For those infants with complete DiGeorge syndrome who have no HLA-identical siblings, transplantation of unrelated HLA-DR-matched cultured mature thymic epithelial explants is an option. Such transplants have successfully reconstituted the immune function of at least three DiGeorge patients (162).

Enzyme Replacement and Bone Marrow Stem Cell Transplantation

Bone marrow cells from a related donor who is MHC compatible or haploidentical with the recipient are currently the treatment of choice for all other patients with severe cellular immunodeficiency. The only other type of therapy with any efficacy for T-cell deficiency is regular injections of polyethylene glycol-treated bovine adenosine deaminase (PEGADA) for infants with ADA-deficient SCID. Even in those infants, however, bone marrow transplantation remains the treatment of choice because it is more effective and long lasting. PEGADA should not be given unless marrow transplantation has already failed because it will empower the infant's T cells to reject the graft. The major risk to the recipient from transplants of bone marrow or cord blood is GVHD. This risk is much greater if the donation is from a matched unrelated (MUD) adult or cord blood source or if pretransplant chemoablation is given to the recipient. It has been repeatedly shown that pretransplant chemoablation is not necessary to achieve engraftment in SCID patients (88); this also allows the omission of T-cell-toxic drugs like cyclosporine, methotrexate, and steroids in the posttransplant period. The use of the latter drugs defeats the purpose of the transplantation, that is, to achieve immunoreconstitution. The development of techniques to deplete all postthymic T cells from donor marrow has permitted the safe and successful use of haploidentical (half-matched) related bone marrow cells for the correction of SCID for the past 19 years (88,114). Although donor and recipient are only half-matched, rigorous depletion of T cells from the donor marrow also allows the omission of GVHD prophylaxis with cyclosporine and methotrexate. Transplantation of SCID infants in the first 3 months of life with either HLA-identical or HLA-haploidentical related bone marrow stem cells has been shown to offer up to a 96% chance of survival (88). Currently, more than 400 SCID patients worldwide are surviving as a result of successful HLA-identical or -haploidentical bone marrow transplantation (114). Patients with less severe forms of cellular immunodeficiency reject such grafts unless they are given chemoablation before transplantation, and even then there has been a high incidence of resistance to engraftment of T cell-depleted, half-matched marrow cells (164). Several patients with WAS syndrome and other forms of partial cellular immunodeficiency have been treated

successfully with unfractionated HLA-identical related or MUD bone marrow transplants after immunosuppression (114).

Gene Therapy

There is now more optimism than ever that primary immunodeficiency diseases for which the molecular defects have been identified will be correctable by gene therapy. A normal gc-cDNA was successfully transduced into autologous marrow cells of infants with SCID-X1 by retroviral gene transfer, with subsequent full correction of their T and NK cell defects (165). This offers hope that gene therapy eventually will be the treatment of choice for all patients with SCID or other genetically determined immunodeficiency diseases for whom the molecular basis is known. There are still many SCIDs (Fig. 28.4) as well as other types of immunodeficiencies for which the molecular defects have yet to be identified; until then, bone marrow transplantation will remain the treatment of choice (89,88).

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29 PHAGOCYTE DISORDERS

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The neutrophil is the first line of phagocytic defense (see Chapter 15). In that exalted position, few defects are tolerated for long. Although multiple mechanisms of bacterial killing in the neutrophil have been well described, relatively few defects are associated with a demonstrated deficiency in any one of the components (1). This may be because fundamental defects of this system are lethal; that there may be a level of redundancy of killing that makes defects inapparent, or some reactions are relatively efficient even in the absence of specific enzymes. An example of the latter is myeloperoxidase (MPO) deficiency. Although many people who are deficient in MPO have been identified, the number who are clinically symptomatic is quite small, probably because generation of superoxide and hydrogen peroxide occurs at a superabundant level, killing organisms through pathways not dependent on halogenation. Therefore, it takes relatively extraordinary conditions, such as diabetes mellitus, to bring out the phenotype of this genetic disorder (2). In contrast, chronic granulomatous disease (CGD) is caused by a mutation in any one of four genes of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, all of which lead to an absence or a severe reduction of hydrogen peroxide generation and a roughly similar clinical condition (3). There have been multiple individual case descriptions of various defects involving neutrophils or their functional systems, but most of these are single case reports and are not well characterized at the pathophysiologic level. We have chosen here to concentrate on the growing handful of relatively well-characterized syndromes. Where they occur in nature, or have been created by humans, we discuss relevant animal models (Table 29.1).

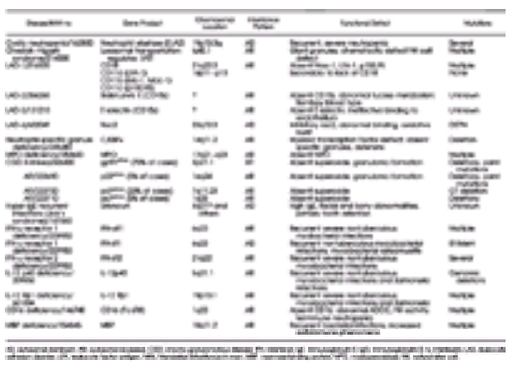

TABLE 29.1. Phagocyte Defects

The incidence of phagocyte defects is formally unknown, but we estimate it to be greater than 1 per 100,000. It is especially important to diagnose phagocyte disorders formally because this leads to specific counseling (e.g., to avoid mulch in CGD), institution of specific therapies [e.g., prophylactic antibiotics and interferon g (IFNg) in CGD], enlistment of other specialists (e.g., dentists for patients with Job syndrome and delayed dental deciduation), and early consideration of bone marrow transplantation [e.g., in leukocyte adhesion disorder (LAD)]. Perhaps more immediately important, the identification of specific phagocyte defects indicates the likely microbiology and therefore leads to appropriate empirical therapy for ongoing infections and will guide the considerations of duration and intensity (Table 29.1).

Symptomatic neutrophil abnormalities fall into several broad categories. They include abnormalities of neutrophil number or production, abnormalities of neutrophil adherence and locomotion, abnormalities of neutrophil granule formation or content, and abnormalities of killing. Several of these syndromes cross the functional categories, share functional defects, and involve abnormalities of nonphagocytic cells as well as phagocytes. A few of these syndromes have been described and characterized at the molecular level. The conditions to be considered in detail are cyclic neutropenia, the Chédiak–Higashi syndrome (CHS), LAD, neutrophil-specific granule deficiency, MPO deficiency, CGD, hyperimmunoglobulin E (HIE) with recurrent infections (HIE or Job syndrome), and defects involving the IFNg and IL-12 receptor pathways (Table 29.2). Although an in-depth discussion of therapeutics is beyond the scope of this chapter, the success of specific agents, notably cytokines, in the treatment of several of these syndromes has shed light on the etiology and pathogenesis of some of these disorders and are discussed herein.

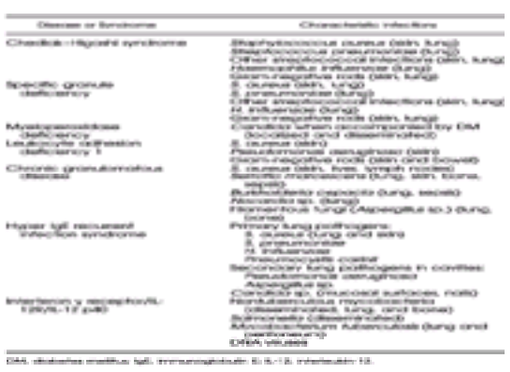

TABLE 29.2. Syndromes and Infections

The clinical presentations of patients with neutrophil disorders share common features. Gingivitis, periodontal disease, and oral ulceration are common in patients with neutrophil dysfunction (4). Cutaneous infections with *Staphylococcus aureus* are recurrent and can be severe. In neutrophil disorders characterized by inadequate inflammation (neutropenia, LAD, CHS, specific granule deficiency), infections can extend locally and subcutaneously with little reaction until marked destruction has taken place. Visceral symptoms, and especially sinopulmonary involvement, are features that help to distinguish neutrophil defects from other syndromes in the differential diagnosis. For instance, patients with HIE experience recurrent pneumonias, whereas patients with atopic dermatitis rarely do. Hepatic abscess is a frequent manifestation of CGD and most often is due to *S. aureus*, an organism rarely encountered at that anatomic site in patients with normal neutrophils. Described abnormalities of neutrophil function are few, and most patients evaluated for the disorders known to date show no defect. Because the management and prognosis of

these diseases vary widely with diagnosis, however, it is critical to search for suspected abnormalities. Updated diagnostic criteria were published (Table 29.2) (5).

As we more fully enter the genomic age of biologic information, we find genes clearly identified as causing disease without understanding their function. The demonstration of a genetic defect is only the first, and in some cases the easiest, step in the march toward understanding diseases, their pathophysiology, and rational therapy.

CYCLIC NEUTROPENIA

Cyclic neutropenia or cyclic hematopoiesis (m/m 162800[1]) is a rare disease occurring in autosomal dominant, spontaneous, and acquired forms. The autosomal dominant form is characterized by regular 21-day oscillations in the levels of blood neutrophils, monocytes, eosinophils, lymphocytes, platelets, and reticulocytes (6). The defect has been identified as being due to point mutations around the active site in ELA2, the gene for neutrophil elastase, one of the components of the neutrophil secondary granules (7). Neutrophil elastase is involved in tissue destruction, presumably to allow neutrophil entry into areas of infection and to allow for neutrophil transit across tissue planes. It is inactivated by a_1-antitrypsin, one of the serpin (serine protease inhibitor) family of proteins. How the loss of normal neutrophil elastase inactivation leads to the phenotype of cyclic neutropenia is still unclear, but presumably the altered neutrophil elastase disrupts the balance of activities in the bone marrow that allow for consistent and constant hematopoiesis. Along these lines, neutrophil elastase has been shown to be a competitive ligand for the binding of CD18/CD11b to intercellular adhesion molecule (ICAM), suggesting that neutrophil elastase may have a crucial role in getting neutrophils off of endothelium once they have adhered (8). The precise role of neutrophil elastase in hematopoiesis is yet to be characterized, however.

Patients usually present in childhood and have recurrent episodes of fever, malaise, mucosal ulcers, and occasionally life-threatening infections associated with periods of neutropenia (<200/µL) (6,9,10). Patients with adult-onset disease have been described with an associated clonal proliferation of large granular lymphocytes (LGL) as well (11,12). Neutrophil number is transiently impaired, but function is normal. Chronic neutropenia as seen in adults has been associated with abnormalities of Fas ligand expression and metabolism (13).

Wright et al. (14) performed bone marrow culture on patient-derived cells in the presence and absence of recombinant human granulocyte-microphage colony-stimulating factor (rhGM-CSF). Numbers of myeloid progenitors (granulocyte-macrophage colony-forming unit [GM-CFU]) were normal to increased and did not vary with cycle time but were markedly hyporesponsive to rhGM-CSF. This relative refractoriness was seen most dramatically in the neutrophil-committed colonies. Mononuclear cells derived from the marrow of patients with cyclic neutropenia and CD34+ progenitor cells also showed abnormally low responses to granulocyte colony-stimulating factor (G-CSF) but not to interleukin (IL)-3 (15).

The gray collie dog has an autosomal recessive condition similar to cyclic neutropenia except that cycling of cell counts occurs every 12 days instead of every 21 days. Bone marrow transplantation can be used to transfer the disease or to cure it in these dogs, confirming the stem cell as the critical component. Treatment of affected gray collies with recombinant human G-CSF (rhG-CSF) increases both peak and nadir neutrophil counts and shortens the frequency of cycling (16). Administration of rhG-CSF to patients has been successful in dramatically decreasing the number of days of neutropenia per month, increasing the peak and nadir polymorphonuclear (PMN) cell counts, shortening the periodicity of the cycle to 14 days from 21 days, and decreasing the symptoms of patients (17,18).

The diagnosis is suspected in children with recurrent stomatitis, gingivitis, cutaneous infections, lymphadenopathy, and fever. The clinical diagnosis can be established only after blood cell counts with differentials are repeated at least three times per week for at least 6 weeks. With the recognition of mutations in neutrophil elastase as the underlying cause of cyclic neutropenia, specific diagnosis requires demonstration of elastase gene mutations. Because this is an autosomal dominant mutation, patients are heterozygous, and mutation detection must be done at the molecular level. In congenital agranulocytosis (the Kostmann syndrome) (19), neutrophil counts are consistently low from birth and show no periodicity. Dale et al. (20) found that not only do patients with cyclic neutropenia have these mutations in neutrophil elastase, but so do most patients with congenital neutropenia; however, the mutations cluster in different areas of the molecule.

Management of the infections in cyclic neutropenia is simplified by the returning neutrophils and need not be as prolonged as in patients whose neutrophils are abundant but dysfunctional. Therapy for cyclic neutropenia is rhG-CSF administered subcutaneously (17). Although rhG-CSF does not eliminate the cycling of cell counts, it does lift the nadirs out of the neutropenic range and thereby prevents infections. Interestingly, rhG-CSF is also the treatment for congenital agranulocytosis (21). Treatment of large granular lymphocyte-associated neutropenia depends on immunosuppression, such as with methotrexate, steroids, or cyclosporine (22).

AUTOIMMUNE NEUTROPENIA

Autoimmune neutropenia is caused by peripheral destruction of neutrophils as a result of granulocyte-specific autoantibodies. Primary autoimmune neutropenia is the most common form of neutropenia seen in infants and young children and is not associated with other systemic immune disorders such as systemic lupus erythematosus (SLE) (23,24). Most patients have mild skin and upper respiratory tract infections, but a small minority may have severe infections such as pneumonia, meningitis, or sepsis. The discovery of primary autoimmune neutropenia may be coincidental because some patients remain asymptomatic despite low neutrophil counts. At diagnosis, most patients have a neutrophil count greater than 500 per microliter. The neutrophil count may transiently increase during a severe infection and return to neutropenic levels following resolution (24,25). Bone marrow examination is normal or shows increased cellularity; maturation arrest may occur, but myeloid precursors reach at least the myelocyte/metamyelocyte stage. Phagocytosed granulocytes may be seen in the bone marrow, indicating that the removal of sensitized granulocytes begins there.

Granulocyte-specific antibodies are found in primary autoimmune neutropenia, but detection may require repeated testing. Granulocyte immunofluorescence testing (GIFT) is one of the most sensitive methods available for the detection of antigranulocyte antibodies (25). The vast majority of these antibodies are immunoglobulin G (IgG), and are most commonly directed at the neutrophil antigen (NA) determinants in FcgRIIIb (CD16), the neutrophil and natural killer (NK) cell surface receptors for immunoglobulin. The NA antigens are recognized in more than 70% of cases of primary autoimmune neutropenia, but in 21% to 28% of cases, the autoantibodies are directed against the leukocyte integrin CD18/CD11b (26). The disease is typically self-limited, with neutropenia disappearing within 1or 2 years. Disappearance of the autoantibodies precedes normalization of neutrophil counts. Symptomatic treatment with antibiotics for infections is usually sufficient. Prophylactic antibiotic treatment should be reserved for those with recurrent infections: Cotrimoxazole, ampicillin, or first-generation oral cephalosporins are the most commonly used. For severe infections and in the setting of emergency surgical interventions, high-dose intravenous immunoglobulin (IVIG), corticosteroids, and rG-CSF are considered; G-CSF is the most effective at increasing the absolute neutrophil count (ANC).

Secondary autoimmune neutropenia can be seen at any age and has a more variable clinical course. It is typically associated with other systemic or autoimmune diseases such as hepatitis, SLE, or Hodgkin disease. Patients are at risk for developing other autoimmune problems, even if these are not evident at diagnosis. The antineutrophil antibodies have pan-FcgRIII specificity and are not specific for the NA. CD18/CD11b antibodies have been detected in a subset of patients with secondary autoimmune neutropenia (24). This neutropenia responds poorly to most therapies (27).

Alloimmune neonatal neutropenia was first described by Lalezari and Bernard (28) and is caused by the transplacental transfer of maternal antibodies against the fetal neutrophil antigens NA1, NA2, and NB1, leading to immune destruction of neonatal neutrophils, as reviewed by Dale (29). These complement activating antineutrophil IgG antibodies can be detected in 1:500 live births. Maternal absence of expression of FcgRIII (CD16) has been reported to be associated with this form of neutropenia in the neonate (30). Antibody-coated neutrophils are removed from the circulation, leaving the neonate neutropenic and at risk for infections. Omphalitis, cellulitis, and pneumonia typically occur within the first 2 weeks of life in the setting of neutropenia. The diagnosis can be made by detection of CD16-directed neutrophil-specific antibodies in maternal serum. Alloimmune neonatal neutropenia responds to G-CSF or high-dose IVIG, but most patients improve without specific treatment in a few weeks to 6 months (31).

CHÉDIAK–HIGASHI SYNDROME

This rare autosomal recessive disorder is characterized by recurrent bacterial infections, partial oculocutaneous albinism, photophobia, nystagmus, and peripheral neuropathy (MIM 214500). Many patients die in childhood from infection. About half of those who survive into adolescence develop an aggressive “lymphoproliferative” phase with diffuse organ infiltration and death. Several patients have lived into adulthood, at which time an aggressive, severe, debilitating peripheral neuropathy is a common feature (32). Pathologically, giant abnormal granules are found in PMN cells, melanocytes, hair, Schwann cells, the central nervous system (CNS), peripheral ganglia, capillary epithelium, renal tubular epithelium, erythroid precursors, fibroblasts, and other granule-containing cells (33) (Fig. 29.1). In neutrophils, the granules are formed mainly by fusion of azurophilic or primary granules to each other and, to a lesser extent, to specific or secondary granules (34). Whereas some features of the CHS cellular phenotype are reversible in cell culture in association with normal cells, granule fusion in CHS cells is not preventable (35). Multiple examples of the CHS phenotype exist in lower animals, making this disease amenable to *in vivo* experimentation.

Figure 29.1. Chédiak–Higashi syndrome. **A:** Polymorphonuclear leukocyte from a patient with Chédiak–Higashi syndrome. The grant granules are easily appreciated. Unfused granules are visible in the cytoplasm as well. **B:** A light microscope view of hair from a patient with Chédiak–Higashi syndrome is shown on the left. Clumps of pigment are seen irregularly distributed in the hair shaft as a result of giant melanosomes. **Right panel:** A normal hair with evenly distributed pigment for comparison.

The syndrome was described in 1943 by Beguez Cesar (36), in 1948 by Steinbrink (37), in 1952 by Chédiak (38), and in 1954 by Higashi (39). It was Sato (40) who identified the cases of Chédiak and Higashi as being one and the same entity and created the eponym Chédiak–Higashi syndrome. Using two-color immunofluorescence in peripheral blood and bone marrow, Rausch et al. (34) showed that in CHS, primary granules are normal in appearance after genesis during the promyelocyte stage, but they are abnormal in cellular distribution. With progressive maturation, primary granules condense into giant abnormal bodies with a few unfused normal primary granules nearby. At the myelocyte stage, genesis of normal-appearing secondary granules occurs. A few secondary granules fuse with the giant lysosomes, giving rise to double staining of the giant lysosomes. By the time of neutrophil maturation, essentially no unfused primary granules remain, and almost all remaining granules are secondary granules. These findings reflect a generalized abnormality of neutrophil granules affecting the earlier arising primary granules more severely and the later arising secondary granules less. The relative deficiency of primary granules and their associated functions may account in part for the abnormal bactericidal activity in CHS cells and the propensity for infection in patients with CHS.

Bone marrow aspirates and biopsy specimens show normal cellularity or hypercellularity despite peripheral neutropenia (32). Normal circulating granulocyte half-life, poor responses to stimuli of bone marrow release, and elevated serum lysozyme levels all indicate that the neutropenia associated with CHS is due to increased intramedullary destruction of PMN cells (41). Chemotaxis to a variety of stimuli and accumulation onto Rebuck skin windows are delayed and diminished in CHS (42). The *in vitro* chemotactic defect may be in part an artifact of interference by the giant lysosome with the ability of the PMN cell to traverse the filter that is frequently used (43). Rates of phagocytosis by CHS cells are increased over normal, but levels of some primary granule enzymes are below normal (44), leading to a decrease in bactericidal activity (45). Resting glucose oxidation and stimulated oxygen consumption are supranormal (45). The discrepancy in killing between CHS and normal phagocytes is most apparent in the first 20 minutes after ingestion and is more marked for *S. aureus* than for streptococci or gram-negative rods; candida-killing activity is normal (45).

Natural killer cell activity has been shown to be deficient in CHS patients. Antitumor NK activity is absent in patients with CHS, but binding of NK cells to tumor targets is normal (46,47). Despite extremely low lymphocytic antibody-mediated cellular cytotoxicity (ADCC), neutrophil and monocyte ADCCs are intact (48). CHS cells stain normally for the NK antigen HNK-1, which is present in higher density than normal (49). In keeping with the kinetic abnormalities observed in the bactericidal activity of CHS neutrophils, Targan and Oseas (50) coined the term *lazy NK cell of Chédiak–Higashi syndrome* to describe the restoration of NK function to CHS cells after prolonged exposures or incubations. Exposure of CHS NK cells to targets for four times longer than normal resulted in 10- to 100-fold increases in the rates of CHS NK cell lysis. Incubation with IFN-a at a high concentration resulted in an increase in lytic activity after 24 hours. CHS NK cells also were able to release normal amounts of soluble cytotoxic factors after 24 hours of target contact. Holcombe et al. (51) demonstrated elevated levels of g-d cells in CHS patients and were able to clone one such line and show it to be fully capable of tumor lysis, thus confirming the presence of a functional lytic mechanism in these cells. CHS cells have a quantitative defect in their killing of both intracellular and extracellular targets, which is most apparent when examined at early time points.

Features of CHS include CNS and peripheral nervous system (PNS) involvement with peripheral neuropathy, myopathy, autonomic dysfunction, and leptomeningeal involvement (33). Lymphohistiocytic infiltration of axons and myelin sheaths occurs in patients with peripheral neuropathy and in ganglia in patients with autonomic neuropathy. Melanin granules seen in the neurons of the substantia nigra are large, irregular, and clumped. These aggregates of melanosomes seem to increase in size and number with age. Low intelligence was noted in some series (32).

The accelerated phase of CHS is a diffuse lymphohistiocytic infiltrative process associated with adenopathy, organomegaly, and pancytopenia in about 85% of patients (32,52). Most of these abnormalities respond to therapy with vincristine and prednisone. Ascorbate is not protective against the accelerated phase (53). Similarities of the accelerated phase to hemophagocytic syndromes associated with defects of the granule protein perforin suggest that the underlying cause of this lymphoproliferative process may be due to the abnormal granule maturation and mobilization in CHS. In addition, abnormal presentation of the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4; CD152) may be important in the evolution of the lymphoproliferative disease (54). Bejaoui et al. (55) reported 18 episodes of accelerated phase in 11 patients. Treatment with etoposide (VP-16), steroids, and intrathecal methotrexate induced remission in seven of seven patients. Compatible bone marrow transplantation induced apparent cure in all three patients so treated, whereas all other patients eventually relapsed and died.

The protein defect in CHS has been identified as *lysosomal trafficking regulator* (*LYST*), encoded by the gene CHS1 located at 1q42 in humans (56,57 and 58). This gene was positionally cloned in the mouse, and mutations in it have been found to cause CHS-like syndromes in rodents, cattle, and humans. The gene is clearly involved in endosomal trafficking and various aspects of surface molecule display, but the mechanisms by which *LYST* works, and therefore the mechanisms of disease, remain elusive (59).

Many mammalian mutants have the pigmentary dilution characteristic of CHS. These include mink (60), cattle (61), mice (62), killer whales (63), cats (64), blue foxes (65), silver foxes (66), and rats (67). The *beige* mouse has been used extensively as a model for the neutrophil dysfunction of CHS (68). NK cell defects in the *beige* mouse closely resemble those in the human (69,70).

Diagnosis of CHS is made easily by inspecting the peripheral smear for giant lysosomes or microscopic examination of hair for characteristic melanin clumps. Management of CHS is challenging. The small numbers of patients followed at any one center have made design and execution of therapeutic trials difficult. Aggressive diagnosis and treatment of infections are of paramount importance. As in other diseases of defective neutrophil function, the extent of infection is often underestimated by clinical history and examination. This is the result of defective chemotaxis and bacterial killing. Prophylaxis with cloxacillin did not seem to have any significant role in reducing infections in four patients studied at the National Institutes of Health (NIH) (71).

The role of ascorbate has been difficult to clarify. Boxer et al. (72) showed improvement in *in vitro* parameters after *in vivo* administration of ascorbate to an 11-month-old girl with CHS. They found improvements in bactericidal activity, granule release, chemotaxis, and cyclic nucleotide levels. Weening et al. (73) also found improvements in bactericidal activity, chemotaxis, degranulation, and clinical course in a patient treated with ascorbate. Gallin et al. (74) found a slight improvement in the survival of lethally challenged *beige* mice with ascorbate therapy, but could demonstrate no benefit in two patients. The benefit of ascorbate is unreliable, but it is virtually without toxicity.

Bone marrow transplantation has been successful in several patients with CHS and should be pursued when appropriate donors are available (55,75). Although this cures the immunodeficiency and the accelerated phase, it does not alter the progressive neuropathy that is seen in many long-term survivors.

LEUKOCYTE ADHESION DEFICIENCY

Leukocyte adhesion to endothelium, other leukocytes, and bacteria is a critical step in the ability of leukocytes to communicate, travel, inflame, and fight infection. These processes are mediated by several sets of molecules on the leukocytes and the endothelium, critical among which are the integrins and selectins (see Chapter 15). Defects in either of these two intercellular adhesion pathways can lead to somewhat overlapping clinical phenotypes (76). In addition, transduction of the signal from the receptor on the leukocyte surface to structural proteins in the leukocyte permitting movement is dependent on the intracellular molecule *rac2* (77).

Type 1

Type 1 LAD (LAD 1; MIM 116920) is an autosomal recessive disorder that involves the leukocyte integrins, the molecules required for leukocyte adherence to endothelium, other leukocytes, and bacteria (78,79,80,81 and 82). Each of the leukocyte integrins is a heterodimer with a common b chain, CD18 (see Chapter 15). The a–b heterodimers of the integrin family include CD11a/CD18 [lymphocyte function antigen (LFA-1)], CD11b/CD18 (Mac-1 or CR-3), and CD11c/CD18 (p150,95, or

CR-4) (83). CD18 is a 95-kd protein expressed on phagocytes, monocytes, macrophages, and lymphocytes and is obligatory for expression of the respective a chains (84). The CD18 gene is located at chromosome 21q22.3, whereas the a chain genes, CD11a–c, are all located at chromosome 16p11–p13.1, presumably the result of a distant gene duplication event (85). The dysfunctional and nonfunctional mutations of CD18 are clearly multiple and fall into at least five categories (86). To date, no autosomal dominant mutations have been described.

Deficiency of CD18 leads to a corresponding deficiency of the complexes LFA-1, Mac-1, and p150,95 and resulting abnormalities of cellular adhesion (87). These abnormalities are predictable from the basic defect. Patients with LAD-1 have chronic leukocytosis, in part due to the inability of neutrophils to adhere to endothelium through the integrin pathways. Other marginated pools, such as those mediated by selectins and in the lung, mediated in part by the narrow capillaries there, are still likely to be intact. The poor inflammatory response leads to recurrent infections. Since 1974, patients who have recurrent life-threatening bacterial infections, chronic leukocytosis, and delayed and impaired wound healing in the setting of neutrophils defective in chemotaxis and phagocytosis have been described (88,89). Crowley et al. (90) proposed that this abnormality was due to a defect in cellular adhesion. By the mid-1980s, it was appreciated that these patients were all defective in the high-molecular-weight proteins LFA-1, Mac-1, and p150,95 (87,91). Kishimoto et al. (86) subsequently showed that all three molecules exist as a–b heterodimers in association with a common b chain, CD18, which is the defective moiety in LAD.

Type 1 LAD falls into two broad categories, severe and moderate, depending on the degree of CD18 deficiency (87,91). Patients with severe deficiency (<0.5%) of normal protein expression have characteristic delayed umbilical stump separation, umbilical stump infection, persistent leukocytosis in the absence of active infection (>15,000/µL), and severe, destructive periodontitis with associated loss of dentition and alveolar bone. Recurrent infections of the skin, upper and lower airways, bowel, perirectal area, and septicemia are common and usually due to *S. aureus* or gram-negative rods, most notably *Pseudomonas* species. Infections tend to be necrotizing and may progress to ulceration but demonstrate an almost complete absence of neutrophil invasion on histopathology. Aggressive medical management with antibiotics and prompt surgery when indicated are requisite. In the absence of therapy directed at correction of the severe defect, such as bone marrow transplantation or perhaps gene therapy in the future, death before age 2 is common (87,91).

Patients with the moderate form of disease (3%–10% of normal expression) tend to have normal umbilical stump separation, to be diagnosed later in life, and less commonly have life-threatening infections. Leukocytosis is still the rule, as is delayed wound healing and periodontal disease. These patients are less ill and tend to live beyond childhood, but several deaths of young adults from infection have been reported (87,91). Hogg et al. (92) identified a patient with relatively high-level expression of CD18 and its associated complexes but a lack of function resulting from a mutation in the activation domain. Therefore, expression of CD18 alone is not adequate to exclude LAD-1.

Laboratory findings in general reflect the clinical differences between severe and moderate phenotypes of the disease, with the severe form showing more profound deficiencies. Abnormalities include grossly defective granulocyte and mononuclear cell mobilization into Rebuck skin windows *in vivo* and diminished neutrophil migration in response to the bacterial chemoattractant formyl-methionine-leucine-phenylalanine (fMLF) *in vitro*, despite normal numbers of fMLP receptors (87,91). These laboratory abnormalities are demonstrated *in vivo* in histologic sections of infected tissues, showing the presence of some mononuclear cells but extremely low numbers of invading neutrophils. Adherence of granulocytes from patients to glass, plastic, nylon wool, and to other LAD granulocytes is greatly reduced and is not stimulated by exposure to fMLF or phorbol myristate acetate (PMA) (87,91) (Fig. 29.2). The absence of CD18 leads to the absence of Mac-1 (also known as the iC3b receptor, CR3). It is therefore expected and observed that complement-mediated phagocytosis is severely impaired, whereas IgG-mediated phagocytosis is normal. Although viral infections are not usually special problems in LAD, antibody-dependent cell-mediated cytotoxicity (ADCC) by cells from patients is also diminished. Oxidative metabolism in response to PMA or calcium ionophore is normal in granulocytes from patients as measured by nitroblue tetrazolium (NBT) reduction or chemiluminescence. Primary and secondary granule release in response to PMA or chemoattractants is normal in LAD cells, whereas the response after zymosan particle ingestion is depressed (87,91).

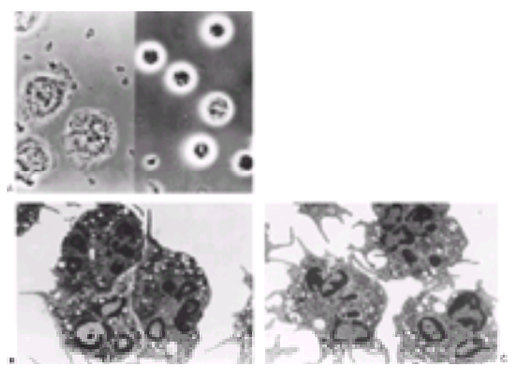

Figure 29.2. Leukocyte adhesion deficiency (LAD) type 1. **A:** Normal neutrophil adhesion to plastic is seen in the **left panel;** note the flat, spread-out cells with cytoplasmic projections. **Right panel** shows LAD-1 cells; note the rounded, refractile, unattached cells, which are characteristic of the adhesion-defective cells of LAD. **B:** Normal neutrophils change shape and clump when stimulated with formyl peptide. **C:** LAD-1 cells change shape but fail to clump in response to the same stimuli.

Animal models of LAD in the dog (93) and mouse (94,95) are currently described. Since 1975, the canine form of this disease has been described at least twice in Irish setters. They have the characteristics of human LAD with neutrophil dysfunction, leukocytosis, and frequent infections. Bovine LAD (BLAD) (96) is a more recently recognized homolog of LAD. The artificial insemination of cows with sperm from a particularly prolific bull, *Osborndale Ivanhoe*, and his progeny led to the current rate of about 14% of American bulls and 6% of American cows being heterozygous for the BLAD gene defect. The BLAD mutation is at nucleotide 383 and results in the change of amino acid 128 from aspartic acid to glycine (D128G). The affected calves have gingivitis, periodontitis, tooth loss, mild diarrhea, and poor growth. Most affected calves die within the first year of life. CD18 expression on neutrophils was less than 2%, and the neutrophil count was more than 47,000 per microliter (normal cow, about 4000/µl) (96).

The diagnosis of LAD1 is established through a thorough history, with special attention to depressed inflammation in the neonatal period, delayed umbilical stump separation, and recurrent infections. A dental history is helpful because most of these patients have severe problems with gingivitis, periodontal disease with tooth loss, and alveolar bone erosion. Wounds often heal abnormally, leaving atrophic, paper-thin scars. The diagnosis is confirmed by fluorescent-activated cell sorting (FACS) which shows reduction or absence of CD18, CD11a, CD11b, and CD11c. Therapy to date has consisted of bone marrow transplantation, when available, for severely affected patients. Engraftment has occurred in most of the few patients reported (91,97). Moderate disease is usually managed by scrupulous attention to infections with surgical débridement and drainage when necessary. Prophylactic antibiotics and cytokine administration, although they may have theoretic merit, have not been studied in a prospective manner. Wilson et al. (98) reported correction of this defect in immortalized patient B cells with a retroviral vector carrying the CD18 gene. Gene therapy may offer the long-term benefits for this disease (99). Animal-model systems should facilitate the next series of gene correction experiments as well as make exploration of other therapeutic approaches possible.

Because neutrophil adhesion is clearly important in the mediation of certain aspects of inflammation, especially in the ischemia–reperfusion period, study of this receptor system is important for understanding the role of neutrophils in inflammation and provides an important site for potential modulation (100).

Type 2

In LAD type 2 (LAD 2; MIM 266265), neutrophil adherence to endothelial cells is defective, on the basis of the absence of the sialyl-Lewis X antigen (CD15s) on the neutrophil surface, which is the binding site for E- and P-selectins (101) (see Chapter 15). The reported patients were products of consanguineous marriages and had neutrophilia; recurrent pulmonary, periodontal, and cutaneous infections; abnormal chemotaxis; mental retardation; short stature; distinctive facies; and the Bombay (hh) blood phenotype (101,102). The underlying defect is autosomal recessive and appears to be in fucose metabolism. This defect in fucosylation has much broader implications than neutrophil function alone (102,103). Despite this apparently severe defect, the infection susceptibility phenotype appears to moderate over time and to present very little problem in adolescence (104).

Type 3

DeLisser et al. (105) recently reported a child with a history of *Pseudomonas* omphalitis, recurrent otitis, and urinary and severe soft tissue infections who had impairment of pus formation and neutropenia. They found normal CD18 and CD15s expression but no E-selectin on endothelium; however, the sequence of the E-selectin cDNA was normal and E-selectin levels in the circulation were twice normal, suggesting that there may be a problem in E-selectin tethering or secretion. The neutropenia is somewhat difficult to explain, but it did respond to G-CSF therapy. This child had lost a full sibling to *in utero* infection but has two normal half-siblings,

leaving the pattern of inheritance unclear.

Type 4

Ambruso et al. (106) described a child with failure of umbilical cord separation at 5 weeks and a perirectal abscess who had neutrophilia but normal CD18 and CD15s levels. They showed that this child had an autosomal dominant mutation in *Rac2*, the predominant *rac* molecule in the neutrophil. This defect led to failure of coupling of the CD18 ligation signal to cellular actin, thereby disrupting the ability of the cell to respond to a variety of stimuli. In addition, *Rac* is needed for superoxide production, degranulation, and chemotaxis, all of which were severely defective in this child. A mouse model of *Rac2* deficiency predicted a similar phenotype (77). This mouse model shows abnormalities in T-helper cell-1 (Th1) activation as well, indicating a critical role for *Rac2* in activation of interferon gamma secretion and Th1 differentiation (107).

NEUTROPHIL-SPECIFIC GRANULE DEFICIENCY

Neutrophil-specific (secondary) granule deficiency (MIM 245480) is a rare, heterogeneous, autosomal recessive disease characterized by the profound reduction or absence of neutrophil-specific granules and their contents (108,109). Associated abnormalities in the few patients reported include bilobed or trilobed notched neutrophil nuclei, mononuclear eosinophils, and absence of some primary granule proteins.

In 1974, Strauss et al. reported a boy with recurrent bacterial infections without sepsis whose neutrophils had nuclear structural abnormalities and appeared to be without specific granules. Nuclei were predominantly bilobed with rare trilobar forms, and eosinophils were mononuclear. Platelet function and results of chromosomal analysis were normal. Specific granules appeared late in neutrophil ontogeny and were few in number and abnormal in shape. Rebuck skin window studies showed no accumulation of PMN or mononuclear cells over 24 hours, and neutrophil migration *in vitro* was markedly diminished. Staphylococcicidal activity was reduced as a result of inadequate phagocytosis, but candidacidal activity was normal. NBT reduction and superoxide production were normal. This was the first report of a selective defect in secondary granules (109). Four subsequent patients have been described, and all share the cardinal features of this boy's syndrome: paucity or absence of neutrophil-specific granules, predominantly bilobed neutrophil nuclei (pseudo-Pelger–Huët anomaly), and increased susceptibility to infections (108,110,111 and 112). Where measured, the cell surface–volume ratio was elevated in specific granule deficiency cells, consistent with either augmentation of plasma membrane surface by premature discharge of specific granules or immaturity (108,113).

The specific granule protein lactoferrin is diminished or absent in these patients' neutrophils (109,113,114). In one patient, despite the low total amount of neutrophil lactoferrin, the percent released in response to stimulation was normal (113). The defective production of lactoferrin is relatively tissue specific. Studies of bone marrow show no lactoferrin protein and extremely low levels of messenger RNA (mRNA) (114). In contrast, lactoferrin protein was more abundant than normal in nasal secretions. The existence of only one lactoferrin gene and the presence of grossly normal copies of it in the affected patients confirmed that the expression of lactoferrin was tissue specific and raised the possibility of a transcriptional factor defect underlying the host of defects seen in specific granule deficiency (114).

Interesting differences between some of these patients suggest that it is not a single entity but perhaps a variety of defects along a pathway that have a final common expression. The patient described by Gallin et al. (108) had no eosinophils detected on visual inspection of peripheral blood or bone marrow smear and delayed disaggregation following stimulation. Subsequently, however, it was shown that cells staining positive for eosinophil peroxidase were present in normal numbers despite the absence of classic eosinophils on smear, and they increased markedly with GM-CSF therapy (115). The patient described by Strauss et al. (109) and Boxer et al. (113) had normal numbers of eosinophils and increased disaggregation. The patient first reported by Gallin et al. (108) developed joint hyperextensibility as a child and diffuse osteopenia and severe aortic regurgitation as an adult.

Other granule contents also are affected in specific granule deficiency. The primary (azurophil) granule products, defensins, are low or absent (116), as is the tertiary granule product, gelatinase (117). Although normal platelet function was noted in the patient reported by Strauss et al. (109), abnormalities of hemostasis were associated with reduced levels of platelet-associated, high-molecular-weight von Willebrand factor and reduced levels of platelet fibrinogen and fibronectin in the patient reported by Parker et al. (118). These anomalies indicate a defect in platelet a granules as well as in the primary, secondary, and tertiary granules. Eosinophil granules also are affected in specific granule deficiency (115). Sibling deaths at early ages, consanguineous marriages in parents of patients, and occurrence of the syndrome in females have led to the assumption of an autosomal recessive pattern of inheritance for specific granule deficiency.

The recognition that the mouse deficient in the CCAAT/enhancer binding protein e (C/EBPe) had a clinical phenotype similar to that of specific granule deficiency prompted our investigation of the human C/EBPe locus in the case reported by Gallin et al. (108). A homozygous, autosomal recessive, disabling mutation was found in C/EBPe, located at 14q11.2 (119). Therefore, at least one case of specific granule deficiency is due to C/EBPe deficiency, confirming the prediction that a transcription factor defect underlies specific granule deficiency, thereby uniting the defects in primary, secondary, tertiary, and platelet a granule contents. Two subsequent cases examined do not have mutations in C/EBPe, suggesting that this may be a genetically heterogeneous disease (Lekstrom-Himes, Gallin, and Casanova, unpublished observations).

The diagnosis of specific granule deficiency (SGD) rests on clinical suspicion bolstered by careful inspection of the peripheral smear (Fig. 29.3). Nuclear morphology is abnormal in both PMN cells and eosinophils with bilobed or trilobed PMN cells and mononuclear eosinophils. In some cases, eosinophils may not be detectable on routine smears. Demonstration of absent or low specific granule contents helps confirm the diagnosis. Electron microscopy shows absent peroxidase-negative granules in some patients and empty peroxidase-negative granules in others. Management is complicated by these patients' impaired inflammatory response and impaired killing of some intracellular organisms. Gram-positive cocci are the most common offenders but not exclusively so. As in other patients with defective inflammatory responses, clinical examination often understates the extent of infection. Aggressive diagnosis of infection, prolonged and intensive therapy, and early use of surgical excision and débridement are necessary. Although the C/EBPe gene is responsible for at least one case of SGD, other cases of this immunodeficiency may reflect defects at other sites along this pathway of early myeloid maturation (120).

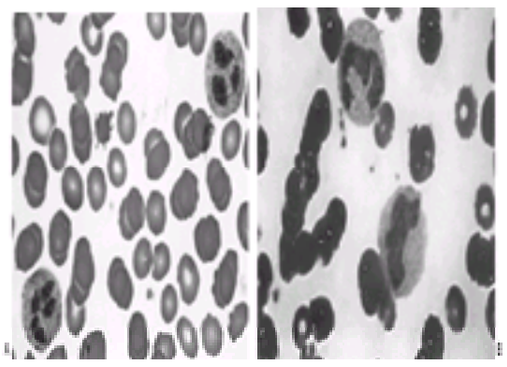

Figure 29.3. Neutrophil-specific granule deficiency. **A:** Wright's stain of normal neutrophils shows granularity of the cytoplasm and well-defined lobulations of the nucleus. **B:** Cells from a patient with neutrophil-specific granule deficiency show a pale cytoplasm with poorly defined nuclear lobulations. Note that the patient's cells are larger than the normal neutrophils. This is easily appreciated if red blood cells are used in comparison.

MYELOPEROXIDASE DEFICIENCY

Also called *verdoperoxidase*, MPO is the heme binding protein that makes pus green. It catalyzes the marriage of hydrogen peroxidase and halide ion into hypohalous acid. In neutrophils, this results in hypochlorous acid or bleach; in eosinophils this results in hypobromous acid. MPO is synthesized and packaged into azurophil granules only during the promyelocyte phase of neutrophil development. From the mature neutrophil, it is released either into the phagosome or into the extracellular space (2). The complementary DNA (cDNA) for MPO has been cloned and the gene localized to 17q21.3–q23 (121,122). The presence or absence of MPO is affected in certain hematologic malignancies and may serve as a tumor marker (123).

Myeloperoxidase is synthesized as an 89-kd precursor molecule that is cleaved to 59-kd and 13-kd moieties in the prelysosomal compartment. The mature enzyme complex, which consists of two heavy and two light chains of MPO, migrates at about 150 kd under nonreducing conditions. Individuals homozygous for myeloperoxidase deficiency are completely deficient in the mature MPO protein product, whereas heterozygotes have half the normal amount of normal MPO in their neutrophils (124) (MIM 254600). Some deficient patients have detectable precursor without mature protein (124), and others have detectable mRNA for MPO but no detectable protein (125). Nauseef (126) demonstrated at least two separate defects in five unrelated patients with MPO deficiency, by protein, mRNA, and genomic

analysis. The most common defect affects the arginine at 569, usually converted to tryptophan (127).

With the advent of automated cytochemical systems for determining differential counts of peripheral blood cells in the late 1970s, MPO deficiency was transformed from the status of a rare syndrome (15 reported cases in the literature) to the most common genetic defect of neutrophils, at 1 in 2,000 persons (2,124). Most patients are entirely asymptomatic and are found only incidentally or through family screening (128). Of the MPO-deficient patients who had difficulty, most were infected with *Candida* organisms (2). Most of these patients also had diabetes mellitus, suggesting that further compromise of host defense exacerbates the phenotype of MPO deficiency.

Neutrophil function is affected by MPO deficiency in a variety of ways. The respiratory burst in MPO-deficient PMNs is prolonged, resulting in exaggerated amounts of superoxide (129). Phagocytosis is normal to increased in MPO-deficient PMNs, whereas bactericidal activity is somewhat slower than normal. Killing of *Aspergillus* conidia by MPO-deficient PMNs is retarded, whereas the combination of MPO-deficient PMNs with CGD PMNs (see later), which are unable to generate hydrogen peroxide but do produce MPO, results in normal killing of *A.* conidia (130). Candidacidal activity is markedly defective against the more pathogenic *Candida* species such as *C. albicans, C. krusei*, and *C. tropicalis*. In contrast, candidacidal activity is preserved against the less pathogenic *C. parapsilosis* (2). This is quite remarkable in view of the rare occurrence of *Candida* infections in MPO-deficient persons.

CHRONIC GRANULOMATOUS DISEASE

This genetically heterogeneous disease is characterized by recurrent life-threatening infections with bacteria and fungi and dysregulated granuloma formation (MIM 306400, 233690, 233700, 233710). CGD is caused by a defect in the NADPH oxidase, which is responsible for the respiratory burst and the generation of superoxide (see Chapter 15). The functional NADPH oxidase is a six-protein complex. In the basal state, it exists as two components: a membrane-bound complex embedded in the walls of secondary granules, and distinct cytosolic components (3). The granule membrane contains the heme and flavin-binding cytochrome b_{558}, which is composed of a 91-kd glycosylated a chain (gp91^{phox}) and a 22-kd b chain (p22^{phox}). The cytosolic components are p47^{phox}, p67^{phox}, p40^{phox} and *rac*. All these components are necessary for the generation of superoxide except p40^{phox}, which is thought to have a regulatory role. On cell activation, the cytosolic components translocate to the cytochrome in the membrane, resulting in an active NADPH oxidase. Pathologic mutations causing CGD in all the required components have been documented (131,132 and 133). The inability to generate superoxide leads to a failure to make the reactive oxygen species hydrogen peroxide and hydroxyl radical. This defect in turn manifests as defective microbial killing and recurrent infections with catalase-positive bacteria and fungi. No spontaneous animal models exist, but homologous recombinants to inactivate gp91^{phox} and p47^{phox} have been created (134,135). *In vitro* techniques are advanced in terms of both cell-free systems and functional cell lines.

The disease was first described by Janeway et al. (136) and by Berendes et al. (137) and Landing and Shirkey (138), but it was not well characterized until 1959 (139). Several cases and cohorts in male subjects were described over the ensuing years, suggesting an X-linked pattern of inheritance (140). The demonstration of cases in three sisters in 1968 led to the recognition of autosomal recessive forms of CGD as well (141). Subsequently, each of the genes required for mounting the respiratory burst has been cloned, sequenced, and localized.

In a classic display of positional cloning or reverse genetics, Royer-Pokora et al. (142) cloned the gene encoding gp91^{phox} by a subtraction hybridization strategy from a boy with an interstitial deletion of Xp21. This led to the description of several mutations in this gene, most of which result in a failure to produce protein (143). The gp91^{phox} promoter element is quite unusual and shows unique phagocyte-specific regulation (144). Failure to produce either member of the cytochrome heterodimer prevents expression of the other (145,146). There are cytochrome-positive cases described in which a defective gp91^{phox} is expressed (143,147,148).

Chronic granulomatous disease is characterized by a host of different mutations in the different genes. The most common gene affected, accounting for about 70% of U.S. cases, is the X-linked gp91^{phox} (149). Defects include those that result in reduced or absent specific mRNA, normal mRNA amount without protein, immunoreactive protein without function, and immunoreactive but profoundly hypofunctional protein (3). One corollary of an X-linked gene is that lyonization of the gene occurs in females. Heavy lyonization of the functional gene resulting in female patients with the X-linked form of CGD has been reported (143). Defects in p47^{phox} account for about 25% of cases (147,149). Most p47^{phox}-deficient patients who have been examined molecularly have at least one allele that has a deletion of a GT couplet at the first intron–exon boundary, leading to improper splicing (150). This common mutation is due to the conversion with a highly homologous p47^{phox} pseudogene (151). There was a suggestion of autosomal dominant transmission in one family, but molecular analysis confirmed only the recessive GT deletion. The GT deletion allele is fairly common in the normal population, about 1:2,000 (S.J. Chanock, personal communication). Deficiency of p67^{phox} was documented in relatively few patients and accounts for fewer than 5% of cases (3,149). Mutations of the b chain of the cytochrome, p22^{phox}, account for the remaining fewer than 5% of cases (3,149). Comprehensive reviews of mutations in CGD have been published (131,152).

Homologous genes for the NADPH oxidase system have been described and characterized to different extents in several species. The mouse NADPH oxidase genes are highly conserved compared with those of humans (153,154). The biochemical and functional dissection of the defect in CGD led to an impressive body of information about the role of both oxidative and nonoxidative mechanisms in bacterial and fungal killing. Quie et al. (155) first noted normal phagocytosis associated with a defect in intracellular killing of bacteria in CGD. Baehner and Nathan (156) developed a quantitative assay using NBT to detect patients and carriers with CGD. This assay was modified and simplified by Ochs and Igo (157) to essentially the NBT slide test that is used today for detection of both patients and carriers of CGD. In an elegant and classic paper, Klebanoff and White (158) showed that CGD cells were able to halogenate bacteria that produced hydrogen peroxide but they could kill organisms that produced catalase. This demonstrated that the defect in CGD cells was at the level of production of hydrogen peroxide and that mechanisms distal to that in the bactericidal pathway, most notably MPO, were intact. Recognition of this defect provided the pathophysiologic explanation for the clinical observation that patients with CGD are subject to infections almost exclusively with catalase producing organisms. Organisms that produce and do not degrade hydrogen peroxide supply the substrate for the formation of hypohalous acid. Other functional defects have been observed in CGD cells, including abnormal membrane potentials in response to PMA or fMLF stimulation, abnormal ADCC, and reduced tubulin tyrosinolation (159). Another corollary of an inability to form superoxide is the failure to oxidize NADPH. In this event, there is no mechanism to regenerate the NADP+, which is the terminal electron acceptor for the hexose monophosphate (HMP) shunt. Therefore, HMP activity is also defective in CGD cells.

Clinically, CGD is quite variable, ranging in time of presentation from infancy to late adulthood, with the most patients diagnosed as toddlers and young children; however, a significant number of patients are diagnosed in later childhood or adulthood (149). Children with CGD tend to be short and small for their age, a finding that may cause anxiety to patients, parents, and physicians. This may be in part due to chronic or recurrent infections or to some other aspect of defective oxidase function. Children with CGD tend to achieve the height predicted by their parents' heights. Bone and chronologic ages are most disparate in early childhood but achieve equivalence in late adolescence (160). It is not uncommon for children with CGD to continue growing into their early twenties.

In the absence of the NADPH oxidase, other host defense genes may be left with more discernible roles. Foster et al. (161) examined assorted host defense genes in patients who have CGD and showed a striking influence of polymorphisms in myeloperoxidase and FcgRIII on the occurrence of gastrointestinal complications and an effect of polymorphisms in mannose binding lectin on the development of autoimmune disorders.

The frequent infections are pulmonary, cutaneous, lymphatic, and hepatic. Osteomyelitis, perianal abscess, and gingivitis are also common (3,149). The microbiology of the infections of CGD is remarkable for its relative specificity. The overwhelming majority of infections in CGD are due to only five organisms: *S. aureus, Burkholderia cepacia, Serratia marcescens, Nocardia,* and *Aspergillus* species. Whereas the typical liver abscess in the immunologically normal patient involves enteric organisms and is liquid and easily drainable, the liver abscesses encountered in CGD are dense, caseous, and staphylococcal. This dense, undrainable abscess material is the reason that surgery is needed in almost all cases of CGD liver abscess. In the preprophylaxis era, most lung, skin, and bone infections were staphylococcal. Now, however, in the setting of trimethoprim-sulfamethoxazole (TMP-SMX) prophylaxis, staphylococcal infections are essentially confined to the liver and lymph nodes. Bacterial infections with the gram-negative organisms listed here are most commonly pneumonia and cellulitis. Bacteremia is uncommon, but when it occurs is due to *B. cepacia, S. marcescens*, or one of the gram-negative rods that inhabits warm, brackish water, *Chromobacter violaceum*. Bacterial and *Nocardia* infections in CGD tend to be symptomatic and associated with elevated erythrocyte sedimentation rates and may be associated with elevated leukocyte counts. Normal values and no symptoms offer scant reassurance that a patient is not significantly infected.

Aspergillus species and some of the rarer fungi such as *Exophiala dermatitidis* (162) and *Paecilomyces* species (163) are encountered in CGD. Fungal infections are now the leading cause of mortality in CGD (149). Bony involvement can occur by direct extension in the case of *Aspergillus* and other fungi. *A. nidulans* is an organism virtually exclusive in its occurrence in CGD. It carries a much higher rate of osteomyelitis than other fungi, and a much higher rate of mortality than *A. fumigatus* or other fungi (164,165). Involvement of the vertebral bodies with fungus, typically by direct extension from the adjacent infected lung, is a grave finding. The advent of antibiotic prophylaxis has altered the frequency of infections in CGD and reduced the frequency of staphylococcal infections in particular (149). The rate of fungal infections in CGD is lower than that for bacterial infections and apparently has not changed in the setting of prophylactic antibiotics (166).

The gastrointestinal and genitourinary tracts are frequently involved in CGD, sometimes as the site of the presenting complaint, sometimes asymptomatically. Ament and Ochs (167) described the clinical manifestations and laboratory and pathologic findings of gastrointestinal involvement in CGD. They noted frequent malabsorption, intrinsic factor-unresponsive vitamin B12 deficiency, abundant lipid-pigmented histiocytes in small-bowel biopsy specimens, and pigmented histiocytes and granulomas in rectal biopsy specimens. These findings were present in both autosomal and X-linked patients. Esophageal, jejunal, ileal, cecal, rectal, and perirectal involvement

with granulomas often mimicking Crohn disease have been described (168,169,170,171,172 and 173). Gastric outlet obstruction is an especially common manifestation and may be the initial presentation of CGD (174,175 and 176). In a comprehensive review of the genitourinary manifestations of CGD, Walther et al. (177) found that 38% of patients had some kind of urologic event. These included bladder granulomas, ureteral obstruction, and urinary tract infection. All patients with granulomas of the bladder or stricture of the ureter had defects of the membrane component of the NADPH oxidase; eight had gp91^{phox} defects, and one had a p22^{phox} defect. A previous report showed this same case bias in favor of membrane component defects (178). Steroid therapy in combination with antibiotics is quite effective and surprisingly well tolerated when used for resolution of obstructive lesions (Fig. 29.4). Several reports and many anecdotes confirm the benefit of steroids given at about 1 mg per kilogram for a brief initial period and then tapered to a low dose on alternate days (178,179,180 and 181). Prolonged low-dose maintenance is often necessary.

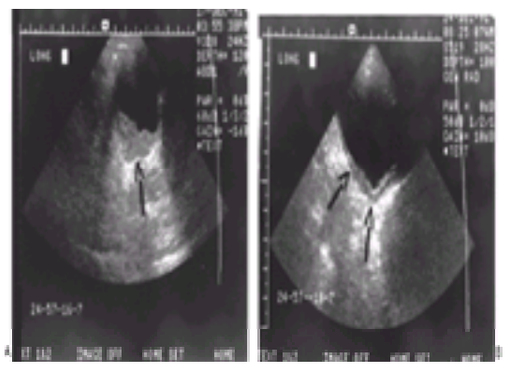

Figure 29.4. Obstructive granulomatous cystitis lesions in chronic granulomatous disease (CGD). **A:** Dysuria and hydronephrosis caused by granulomatous cystitis led to evaluation of this 2½-year-old boy with X-linked CGD. The bladder wall is thickened and irregular. The arrow indicates the bladder wall. Granuloma formation is seen between the bladder wall and the bladder lumen (*black*). **B:** After 1 week of corticosteroid treatment, the boy's symptoms were gone. The bladder wall diminished in thickness.

Cutaneous inflammatory responses are often dysregulated in CGD such that patients with membrane component defects may form exuberant granulomas and granulation tissue (182). This can manifest as wound dehiscence or delayed wound healing. Male CGD patients had an abnormal persistence of PMN cells in Rebuck skin windows compared with female CGD patients and normal subjects. This suggested a defect in “turnoff” of the acute inflammatory response in CGD (182). This abnormal persistence of PMN cells was normalized in one patient who was receiving white blood cell transfusions simultaneously. Blepharokeratoconjunctivitis, pannus formation, and stable chorioretinal scars also have been associated with CGD (183,184). The scars rarely involve the macula, but further study is needed to determine how often these lesions are sight threatening.

The X-linked carrier state for gp91^{phox} is not entirely silent. Lyonization of the X chromosome leads to two populations of phagocytes in carriers. One population has normal oxidase function and is detected as NBT reducing or DHR oxidizing; the other population, which has inactivated the normal X chromosome and has only the defective one functioning, is unable to produce superoxide (Fig. 29.5). Therefore, carriers give a characteristic mosaic pattern on oxidative testing. Discoid lupus erythematosuslike lesions, aphthous ulcers, and photosensitive rashes have been seen in female relatives of gp91^{phox}-deficient patients and screening of patients with discoid lupus erythematosus has detected unsuspected CGD carriers (185,186). The ease of determining which X chromosome is inactivated in a given phagocytic cell has been used to determine the presumed number of stem cells (exceeding 400 cells) necessary to support human hematopoiesis (187). Characteristic infections usually are not seen in female carriers until the normal cells fall below 10% (143,149). Several cases of extreme lyonization of the X chromosome carrying the functional gp91^{phox} have been reported, leading to the rare appearance of X-linked CGD in female patients. This is due to skewed random inactivation in some cases but may be due to selected mutations in the gene controlling X chromosome inactivation, *XIST*, in others. We have had an otherwise healthy highly lyonized X-linked carrier present with *Serratia* lymphadenitis as levels of her normal neutrophils fell below 10% (Malech and Holland, unpublished). Other investigators noted progressive decline in the number of normal neutrophils in some lyonized X-linked carriers over time, leading to the occurrence of CGD-type infections later in life (J. Roesler, personal communication).

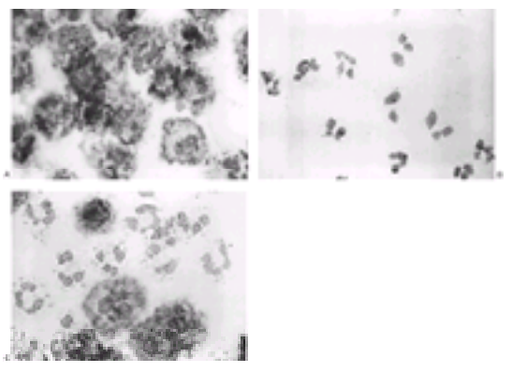

Figure 29.5. Nitroblue tetrazolium (NBT) reduction by purified neutrophils following stimulation with phorbol ester and calcium ionophore.**A:.** NBT is reduced by all stimulated normal neutrophils yielding a blue–black deposition. **B:.** Neutrophils from a patient with chronic granulomatous disease (CGD) fail to reduce NBT and retain a clear cytoplasm. **C:** Neutrophils from an X-linked carrier of CGD show two populations of cells (mosaicism), normal NBT-reducing (blackened), and affected non-NBT-reducing (clear) neutrophils. The presence of an NBT mosaic pattern in the neutrophils of a mother of a CGD patient confirms the X-linked form of CGD.

Advances in recent years drastically altered the morbidity and mortality rates in patients with CGD. Prophylactic TMP-SMX reduces the frequency of major infections from about once every year to once every 3.5 years (159). In addition, prophylactic TMP-SMX changes the type and site of serious infections in patients with CGD to fewer staphylococcal and skin infections (188) but no increase in the frequency of serious fungal infections (166). Ketoconazole is ineffective at preventing *Aspergillus* infection (189), but itraconazole prevents fungal infections in CGD (Gallin, unpublished). In a longitudinal analysis of 47 patients, Muoy et al. (189) found an 8-year survival rate of 70.5% for children born before 1978 and 92.9% percent for those born later. More recently, Winkelstein et al. (149) showed that the mortality rate is about 5% per year for the X-linked form of the disease and 2% per year for the autosomal recessive varieties. The largest causes of mortality are *Aspergillus* pneumonia, followed by *B. cepacia* pneumonia or sepsis.

Treatment of infections in CGD must be aggressive and prolonged for preservation of end-organ integrity and survival. In severe infections, leukocyte transfusions are often used, although the efficacy is anecdotal. The principle is that the deficient product in CGD cells, superoxide, is highly diffusible, such that supplementation of defective cells with a few normal cells leads to complementation of the defective pathway and reconstituted bacterial and fungal killing (190). Therefore, benefits accrue to granulocyte transfusions, even though the overall number of peripheral blood leukocytes is not significantly altered. The number of infused granulocytes averages about 10^9 to 10^{10} per transfusion. Buescher and Gallin (191) showed transfused phagocyte penetration into and persistence in pulmonary secretions in a patient with *Nocardia* pneumonia. Using NBT reduction, they were able to detect transfused neutrophils in peripheral blood for up to 1 hour after transfusion, but they detected normal monocyte derived cells up to 5 days in sputum. At 2 days after transfusion, half of the NBT-positive cells in sputum were monocytes. Emmendorffer et al. (192) conducted a similar study in a boy with *Aspergillus* pneumonia using FACS analysis for p22^{phox} in peripheral blood and pulmonary secretions. Although it is common practice to irradiate white blood cells before transfusion to prevent graft-versus-host disease (GVHD) in the recipient, this reduces the bactericidal activity, growth, and survivability of both neutrophils and monocytes in the transfused product (193,194). CGD patients have no defect in lymphocytic immunity, and there have been no reports of GVHD in recipients of unirradiated leukocytes who have CGD.

In 1983, IFN-g was unequivocally identified as the lymphokine responsible for enhanced HMP activity and hydrogen peroxide generation in macrophages (195). Ezekowitz et al. (196) and Sechler et al. (197) showed that IFN-g could produce some of these same effects *in vitro* in cells from patients with an X-linked variant of CGD. When IFN-g was administered to patients, Sechler et al. (197) and Ezekowitz et al. (198) found similar augmentation of bactericidal activity and superoxide production. In some patients, this was concomitant with an increase in gp91^{phox} levels. The augmentation of superoxide production in neutrophils was demonstrable for several weeks, suggesting that IFN-g was working at the progenitor cell level as well as on more differentiated cells (199). A large multinational, multicenter, placebo-controlled study proved a marked clinical benefit for patients receiving IFN-g. Recipients had 70% fewer and less severe infections than did placebo-treated patients. These benefits held true regardless of inheritance pattern of CGD, sex, or use of prophylactic antibiotics. Interestingly, no significant difference could be

detected in terms of *in vitro* superoxide generation, bactericidal activity, or cytochrome$_b$levels (200) although IFN-g was able to augment neutrophil activity against *Aspergillus* conidia (130).

Bone marrow transplantation leading to stable chimerism was successfully performed in a patient with CGD (201). The patient remained infection free despite having only 15% to 20% of his cells derived from the donor. Ozsahin et al. (202) performed successful bone marrow transplantation on a child with CGD and active incurable *Aspergillus* infection. More recently, Horwitz et al. (203) performed low-intensity ablation (mini–bone marrow transplantation) from HLA-identical siblings into CGD patients. This approach may be promising for patients with HLA-matched relatives. Clinical trials of p47^{phox} gene therapy showed prolonged marking of cells in the periphery for several months, but clinical benefit has not been shown because of the low numbers of corrected cells in the circulation (< 0.01%) (204).

Diagnosis of CGD is made by assays for superoxide production. These include direct measurement of superoxide production, ferricytochrome *c* reduction, chemiluminescence, NBT reduction, or dihydrorhodamine oxidation (DHR). Currently, we prefer the lattermost assay because of its relative ease of use and the ability to distinguish X-linked from autosomal patterns on the flow cytometry assay (205,206). Immunoblot, flow cytometry, or molecular techniques can be used to determine the specific genotype. The history will usually suggest whether a patient has autosomal recessive or X-linked disease, based on sex, age of presentation, and severity. The subtype of CGD is important since AR CGD has a better prognosis than X-linked disease (149). Glucose 6-phosphate dehydrogenase (G6PD) deficiency may mimic certain aspects of the neutrophil dysfunction of CGD, such as the decreased respiratory burst and increased susceptibility to bacterial infections (207). G6PD deficiency is most often associated with some degree of hemolytic anemia, whereas CGD is not.

The index of suspicion should be raised by severe or recurrent infections in children, especially those involving pulmonary or hepatic parenchyma. Infections with unusual organisms such as *B. cepacia, S. marcescens, Chromobacterium violaceum, Nocardia,* or *Aspergillus* species should initiate a search for CGD in patients of any age without other predisposing factors. Granulomatous events such as gastrointestinal or genitourinary obstruction likewise are suggestive of the diagnosis. Management of the infections in CGD must be extremely aggressive. Patients often fail to display symptoms commensurate with the extent of their disease and may present for care late in the course of infection. The erythrocyte sedimentation rate (ESR) is the most sensitive laboratory test to monitor the presence of ongoing infection; a newly elevated ESR should provoke active concern. The importance of a microbiologic diagnosis cannot be overemphasized because the differential diagnosis for infection in these patients includes bacteria, higher bacteria, fungi, and granulomatous processes.

Bronchoscopy, transthoracic needle biopsy, and thoracotomy should be first-line diagnostic procedures, not procedures of last resort. Hepatic abscesses are usually staphylococcal and require open excavation, débridement, and drainage. In occasional cases, percutaneous catheter placement has been successful, but because of the consistency of the lesions, it cannot be routinely recommended. Intralesional instillation of granulocytes has been used and may be helpful in particularly difficult cases (208). Therapy for infections in CGD should be intravenous and prolonged by using more than one agent. The reduction in mortality and morbidity rates seen in recent years is attributable to the recognition and following of these principles. The next generation of advances in molecular and genetic therapeutics should make these therapeutic guidelines obsolete.

HYPERIMMUNOGLOBULIN E AND RECURRENT INFECTION SYNDROME (JOB SYNDROME)

The rare autosomal dominant disorder HIE (MIM 147060) is characterized by recurrent infections, typically of the lower respiratory system and skin, eczema, extremely elevated levels of IgE, eosinophilia, and abnormalities of the connective tissue, skeleton, and dentition. Most patients have facial abnormalities, including ocular hypertelorism; a prominent, protruding, triangular mandible; and a broad, somewhat bulbous nose (209,210,211 and 212). Failure of primary dental deciduation, leading either to failure of secondary dentition eruption or retention of both sets of teeth, is common (212,213). Many patients also have abnormalities of bone formation and metabolism, which may result in fractures, severe scoliosis, kyphosis and short stature, and craniosynostosis (212,214,215,216,217 and 218). HIE occurs spontaneously in all racial and ethnic groups and in many cases is transmitted as an autosomal dominant trait (209,212,219,220 and 221).

This syndrome was first described by Davis et al. (222) in two red-haired, fair-skinned girls who had frequent sinopulmonary infections, severe dermatitis, and recurrent staphylococcal skin infections that were remarkable for their lack of surrounding warmth, erythema, or tenderness. These “cold” abscesses harkened these investigators back to the biblical figure Job, who as part of a celestial wager between God and Satan, was afflicted with boils over his entire body. The syndrome was further defined and clarified by Buckley et al. (223), who noted similar infectious problems in two boys with severe dermatitis, characteristic facies, and elevated IgE levels. Subsequent studies demonstrated abnormalities of the phagocytic, T-cell, and B-cell arms of the immune system (224). Recently, we showed the complex multisystem nature of this disease and documented its effects on skin, bone, teeth, lung, immunity, and infection susceptibility (212). These abnormalities suggested aberrant regulation of some cell or molecule common to all these tissues, such as the monocyte and its derivatives.

Several features of the syndrome have been considered central enough to be of potential etiologic significance. IgE is greatly elevated at most points in the life of patients with HIE (209,210,212). Elevation of IgE was noted in the cord blood of a child born to a mother with HIE, and the child subsequently developed HIE herself (220). Because IgE does not cross the placenta, this must reflect a rate of endogenous synthesis of IgE before exposure of the newborn to the outside world, which suggests that IgE dysregulation is part of the primary problem, not entirely a response to exposure to specific extrauterine environmental stimuli. Although these findings suggest an important role for IgE in the genesis of HIE, it is unclear whether it is a central one. A few patients have been observed to drop their IgE levels below 2,000 IU per milliliter as they get older (212) and yet retain their high susceptibility to infection. Studies by Buckley and Becker (209) showed that unstimulated B cells from HIE patients had maximal *in vitro* rates of IgE synthesis that could not be augmented by exposure to pokeweed mitogen. In contrast, coculture of patient B cells with normal mononuclear cells resulted in significant reductions of IgE synthesis. Geha et al. (225) examined T-cell subsets in children with HIE and found a relative deficiency of CD8+ T cells compared with normals. They also noted a deficit of concanavalin A-inducible suppressor cell activity. *In vitro* IgE synthesis by HIE B cells was greatly reduced by coculture with parental, partially human leukocyte antigen (HLA)-matched lymphocytes. Lymphocyte fractionation identified the IgE-suppressive properties to the CD8+ subset. These data suggest that the abnormality of IgE in HIE lies in the regulation of IgE synthesis, not in the synthesis of IgE *per se*. Dreskin et al. (226) showed that IgE catabolism was also defective in HIE, providing another mechanism for the hyper IgE aspect of the disease.

Interferon-g specifically inhibits the IgE-enhancing activity of a concanavalin A-stimulated T_H cell line supernatant (227) and inhibits the effect of IL-4 on cell expansion and induction of DNA synthesis (228). In HIE patients, there appears to be a reduction in lymphocytes producing either IFN-g or tumor necrosis factor-a (TNF-a) (229). Synthesis of IL-2 and IL-4 appear to be normal, however. A correlation between elevated *in vitro* IgE synthesis and diminished IFN-g production in T cells has been found in HIE patients (229). Vercelli et al. (230) were unable to confirm *in vitro* modulation of IgE synthesis by IFN-g and suggested that T-cell-independent mechanisms of IgE regulation were possible. King et al. (231) examined effects of IFN-g on HIE peripheral blood mononuclear cell IgE production *in vitro* and gave subcutaneous IFN-g to five patients with HIE for 2 to 6 weeks. They found that IFN-g was able to suppress spontaneous IgE production *in vitro* in most patients. This effect was also seen in patients when peripheral blood mononuclear cells were isolated and assayed for spontaneous IgE production before and after IFN-g administration. Serum IgE levels fell in two of five patients treated, one of whom received 0.05 mg/m^2 and the other received 0.1 mg/m^2. Serum IgE levels eventually returned to their previous high levels in both patients. The other three patients experienced no decline in serum IgE. No patient was noted to have any clinical change as a result of the brief course of IFN-g administration.

Grimbacher et al. (212) confirmed autosomal dominant transmission in seven families extending over several generations with variable penetrance. The rate of spontaneous occurrence is unknown, but sporadic cases account for at least half of those recognized so far. The disease in several of the families described by Grimbacher et al. (232) is linked to proximal chromosome 4q. Attention was directed to this region of the genome by a patient with HIE and a ring chromosome involving 4q (232). In several families with autosomal dominant inheritance, the disease is not linked to 4q, suggesting that at least two genetically distinct types of autosomal dominant HIE exist. There may also be autosomal recessive forms of HIE, as suggested by several consanguineous kindreds in which there are multiple affected persons (Grimbacher and Beloradsky, personal communication). Therefore, HIE is likely a complex autosomal disorder with both dominant and recessive forms.

The clinical manifestations of HIE are distinct. Patients usually present within the first days to months of life with severe eczema. Other early infections include mucocutaneous candidiasis and severe diaper rash. Sinus or pulmonary infections, predominantly with *S. aureus* or *Hemophilus influenzae*, are common. Postinflammatory pneumatoceles often are noted early in life. Otitis media and externa are common. Infections occur less frequently in bone and joints and infrequently in the liver, kidneys, and the gastrointestinal tract. Documented sepsis is rare. Deep-tissue infections are frequently extensions of cutaneous or dental infections, such as paronychia or apical abscesses.

Other pathogens that have been recovered include *Aspergillus* species, *Pseudomonas aeruginosa, Streptococcus pneumoniae*, group A streptococci, *Cryptococcus neoformans*, and *C. albicans*. Lung abscesses with empyema require thoracotomy (Fig. 29.6). Although some patients with HIE may have years of relatively low disease activity, the average frequency of infections that required therapy was calculated by Donabedian and Gallin (210) to be 1 every 3.6 months. The incidence of major infections in this same group (requiring intravenous antibiotics or an inpatient surgical procedure) was about 1 per year. The life expectancy of these patients has not been formally determined. Although several patients have survived into adulthood and middle age, there is often severe pulmonary destruction. Cerebrovascular events (strokes, reversible ischemic neurologic deficits, unidentified bright objects/T2 hyperintensities on magnetic resonance imaging (MRI) scanning, carotid artery aneurysms) have been seen in an unusually high number of our patients (unpublished observations). Neoplasms, including Hodgkin disease (233), histiocytic lymphoma of the brain in a 10-year-old girl (234), carcinoma of the tongue, nodular sclerosing Hodgkin disease, and Barrett esophagus (Holland, Puck, and Gallin, unpublished observations) also have occurred in HIE patients.

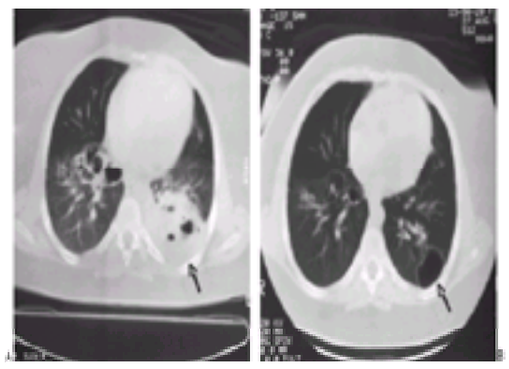

Figure 29.6. Pulmonary disease in hyperimmunoglobulin E (HIE) and recurrent infection syndrome (HIE or Job). **A:** Computed tomography reveals a staphylococcal left lower-lobe lung abscess in a 19-year-old patient (*arrow*). Central bronchiectasis from previous infections is present in the right lung. **B:** Six months after chest tube drainage and antibiotics in the same patient, pneumatoceles persist (left lower-lobe pneumatocele indicated by*arrow*).

Frequent occurrence of bony abnormalities has been noted in HIE. Osteoporosis of the spine and long bones with thin metaphyseal cortices was found in several patients (217). Brestel et al. (214) found osteogenesis imperfecta tarda of the “slender” type in a young girl with HIE. Of the nine patients reported by Geha and Leung (211), five had experienced at least one bone fracture. Of the six patients they tested by single-photon absorptiometry, all showed reduction in bone density. Likewise, the frequency of pathologic fractures in the population reported by Grimbacher et al. (212) was 58%. Although the general mechanism for these abnormalities is unknown, Leung et al. (235) demonstrated increased *in vitro* bone resorption by monocytes from patients with HIE. They also showed that monocytes from patients had substantially elevated rates of spontaneous prostaglandin (PG) E_2 release compared with cells from controls. This increased rate of bone resorption was normalized *in vitro* with indomethacin and *in vivo* with aspirin. Craniosynostosis has been documented in four male patients with HIE (215,218), yielding a rate much greater than what would be expected for the coincidence of one uncommon and one very rare disease.

The characteristic facies that are so often associated with the syndrome may reflect the same underlying abnormality of bony or soft tissue metabolism. The facies of HIE can develop later in life, as evidenced by two patients, described by Donabedian and Gallin (210), who did not have HIE facies at the time of publication but subsequently developed them. We noted the rate of characteristic facies to be 100% for patients over the age of 16 years (213,233). Prominent clubbing is also common. Unique to HIE is the failure of primary teeth to deciduate, leading to retained primary teeth and unerupted secondaries.

The laboratory findings in HIE cover a broad spectrum. The syndrome is defined by marked elevations of IgE (>2,000 IU/mL), with levels of more than 50,000 IU per milliliter reported (209). IgE levels fell below 2,000 IU per milliliter, close or into the normal range in 20% of the patients followed at the NIH. Therefore, although a high IgE level is part of the diagnostic criteria for HIE, authentic cases may lack this particular feature at the later stages of the disease. The catabolism of IgE is abnormally reduced in both HIE and atopic dermatitis, contributing further to the elevated levels of IgE seen in these syndromes (227).

The tropism of the IgE in HIE has been a source of great interest. A high proportion of the IgE in HIE binds to *S. aureus* and *C. albicans* (236). The lack of similar binding activity in other patients infected frequently with *S. aureus* indicates that this abnormality is host, and not organism, driven. Elevated anti-*S. aureus* IgE is thought to be specific for HIE and can help in the discrimination of this syndrome from severe atopic dermatitis with high IgE levels. HIE patients have normal levels of anti-*S. aureus* IgG and modestly elevated levels of anti-*S. aureus* IgM. In contrast, serum anti-*S. aureus* IgA is depressed in patients with HIE compared with normal control subjects and patients with other types of immune defects (237). Total salivary IgA is also reduced. Quinti et al. (238) reported high-molecular-size (>900,000 Da) immune complexes composed of IgG anti-IgE Fc. Buckley and Becker (209) found low antibody titers to diphtheria and tetanus, with only 1 of 11 patients who received booster inoculations responding. *In vitro* responses to the mitogens phytohemagglutinin, concanavalin A, and PWM have been essentially normal. In contrast, responses to *Candida* and tetanus have been low or absent. Some HIE patients also showed a defect in mixed lymphocyte culture proliferation to intrafamilial but not unrelated mononuclear cells. There is no clear HLA association with HIE (209,210).

White blood cell counts are typically in the normal range but have been reported to range from 60,000 to 1,700 per microliter (209,210). Chronic leukopenia with borderline neutropenia has been observed in several patients (210). Mild to moderate eosinophilia is the rule, although occasional patients do not show this. There is no correlation between IgE levels and degree of eosinophilia or clinical disease (212). There is a slight enrichment of eosinophils in drainage from sites of infection, such as abscesses. ESRs tend to be elevated. Complement levels have been normal when studied. Elevated levels of urinary histamine have been reported in some patients and correlate with the extent of the eczematoid dermatitis (226).

Chemotaxis is abnormal in this syndrome at least some of the time in most of the patients. The failure to demonstrate a consistent chemotactic defect even in the same patient over time suggests that this finding is not a central part of the syndrome. Even when present, the defect is not so severe as in well-defined disorders with defects in chemotaxis. A 61-kd protein inhibitor of chemotactic activity has been described as being produced by lymphocytes from HIE patients (210). The role of this protein in the fleeting chemotactic defect of this disorder remains unknown.

Mucocutaneous candidiasis with involvement of mouth, vagina, intertriginous areas, fingernails, and toenails affects about 50% of patients with HIE (210,212,233). Despite this enormous *Candida* burden, lymphocytes have depressed proliferative responses to *Candida in vitro* (209). A remarkable feature of the patient with HIE is the enormous infectious burden with which he or she may present. By the time the patient admits to illness, there is usually nothing subtle about the diagnosis. Indeed, one of the most difficult problems in the long-term management of HIE is the fact that even in the presence of obvious auscultatory and radiographic evidence of pneumonia, patients continue to feel well and may be unwilling to submit to a prolonged course of therapy. As in most of medicine, the firm establishment of a microbiologic diagnosis cannot be overemphasized. Bronchoscopy can be especially helpful in this regard and may help to mobilize secretions. Lung abscess is not uncommon and may require thoracotomy for adequate drainage. High-dose intravenous antibiotics for a prolonged course are required for eradication of infection and to prevent bronchopleural fistula formation and bronchiectasis. Empirical acute coverage should consider *S. aureus, H. influenzae,* and *S. pneumoniae*. The former two organisms account for most acute infections. Colonization of pneumatoceles and bronchiectatic lung with *P. aeruginosa* and *Aspergillus* species can be especially problematic. Cases of esophageal and colonic cryptococcosis have been reported (239,240).

The role of prophylactic antibiotics has not been rigorously investigated in this setting, but there is general consensus for their use. Most investigators direct coverage at *S. aureus* with a synthetic penicillin. The use of oral antifungals, such as ketoconazole or fluconazole, has made an enormous difference in the prevalence and severity of mucocutaneous candidiasis. The role for immune modulators is undefined. Levamisole was tried in a double-blind placebo-controlled trial, which showed an increase in infectious complications in the treatment arm (241). Patients with a severe condition have received plasmapheresis with apparently dramatic, if short-lived, results (242).

The differential diagnosis from atopic dermatitis can be difficult, and indeed the two are often confused when IgE levels are elevated. Points that rule in favor of HIE include an IgE level above 2,000 IU per milliliter and a history of or sequelae of severe deep tissue infections such as pneumatoceles, scoliosis, hyperextensibilty, and failure of primary dental deciduation. Although IgE levels in atopic dermatitis can be extremely high, those patients are usually not affected with deep tissue infections, do not have the associated skeletal and soft tissue anomalies seen in HIE, and do not have the classic facies. A scoring system has been devised to aid in the formal diagnosis of HIE patients (232).

MONOCYTE/MACROPHAGE DEFECTS

Interferong Production and Receptor Deficiencies

The mononuclear phagocyte is critical in defense against virulent and avirulent environmental pathogens alike, among them mycobacteria, as shown by patients with defects affecting this limb of host defense. The most glaring susceptibility recognized in these patients so far is to nontuberculous mycobacteria and the low virulence vaccine, bacille Calmette–Guérin.

Mycobacteria infect macrophages leading to the production of IL-12. This stimulates T cells and NK cells through the IL-12 receptor (IL-12R) to produce IFNg, which then acts through its receptor (IFNGR) on macrophages to upregulate genes and activities. IFNg increases the killing of mycobacteria, the upregulation of IL-12, and the production of TNFa.

Iterferon g binds to the ligand-binding chain, IFNgR1, as a homodimer, leading to receptor dimerization. Following dimerization of IFNgR1, the “signal transducing”

chains, IFNgR2, join the receptor complex. IFNgR1 and IFNgR2 are constitutively associated with their respective Janus kinases, Jak1 and Jak2. Mutual transphosphorylation of the Jaks leads to tyrosine phosphorylation of the intracellular domain of IFNgR1 at tyrosine 457. Y457 is the docking site for latent cytosolic signal transducer and activator of transcription-1 (STAT1). STAT1 phosphorylation leads to homodimerization of STAT1P, which then translocates to the nucleus and upregulates the transcription of IFNa-regulated genes. Patients with defects in the IFNg receptors, the IL-12 receptor b1, and IL-12 p40 were identified through their susceptibility to mycobacteria as well as *Salmonella* and some viruses (243) (Fig 29.7).

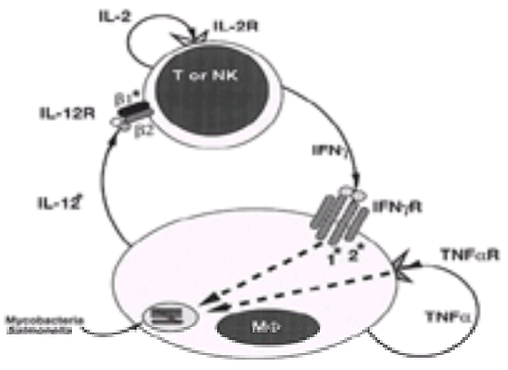

Figure 29.7. Cytokine interactions between mycobacteria-laden macrophages and responding T or natural killer (NK) cells. Macrophage-produced interleukin-12 (IL-12) leads to T-cell production of IL-2, interferon g (IFNg), tumor necrosis factor a (TNF-a), and granulocyte macrophage–colony-stimulating factor (GM-CSF), IFNg and GM-CSF stimulate macrophages to produce TNF-a and kill intracellular mycobacteria through mechanisms not yet characterized. Both IFNg and TNF-a drive the production of IL-12. Asterisks indicate genes in which specific mutations have been identified, as mentioned in the text.

There are several levels of defect in the IFNgR. Mutations in both IFNgR chains have been identified and characterized. Autosomal recessive complete IFNgR1- or IFNgR2-deficient patients tend to develop mycobacterial disease in infancy or early childhood, leading to death or severe disease requiring continuous antibiotics. In countries administering BCG vaccination, these children present with disseminated BCG. These patients typically fail to form well-circumscribed tuberculoid granulomas (244). Although this genetic defect has been associated predominantly with mycobacteria and *Salmonella*, the recognized phenotype has been expanded to include increased susceptibility to a number of viruses such as cytomegalovirus, respiratory syncytial virus, varicella, parainfluenza virus (245), as well as the opportunistic pathogen *Listeria monocytogenes* (246). These patients' cells, both myeloid and somatic, cannot respond to IFNg or signal through the IFNgR. Partial defects in both IFNgR1 and IFNgR2 have been described in which IFNg signal transduction is impaired by 2 to 3 logs, but it is not abolished (247,248). These patients tend to present later in life with more circumscribed disease and to be curable with antibiotics, with or without added IFNg. The autosomal recessive mutations have been in the extracellular domain and lead either to complete loss of protein expression, partial deficiency, or nonbinding but normally expressed receptors due to loss of critical epitopes (249).

Even more common than the autosomal recessive forms of IFNgR defects is the autosomal dominant form of IFNgR1 deficiency (243,250). In this defect, the mutation is in the intracellular domain just beyond the transmembrane segment. This common 4-base-pair deletion leads to a frame shift and premature truncation of the IFNgR1 before the Jak1 and STAT1 binding sites. In addition, the receptor recycling domain is removed, severely impairing the normal removal and recycling of the receptor from the cell surface. Therefore, displayed on the cell's surface is an overabundance of the extracellular domain of IFNgR1. This truncated receptor's IFNg binding is largely preserved, leading to sequestration of IFNg onto impotent receptors as well as the engagement of IFNgR2 molecules. This autosomal dominant mutation occurs relatively frequently as a result of mispairing of DNA at a mutational hotspot around base 818 of the IFNgR1 (250). These patients tend to present later in childhood with circumscribed disease that is antibiotic responsive. In all North American cases identified so far, the children have gone on to develop mycobacterial osteomyelitis. These cases are IFNg responsive and are well controlled by a combination of antimycobacterials and IFNg. The duration of therapy should be prolonged, but the precise duration is undefined. These patients are susceptible to other infections as well, including histoplasmosis and herpes viruses (243).

Interleukin-12 Receptor b1 Mutations

Patients with disseminated nontuberculous mycobacterial (NTM) and *Salmonella* infections in the setting of IL-12 unresponsiveness have been identified in Europe and Turkey. These cases have been due to mutations in the IL-12 receptor b1 (IL-12Rb1) chain (251,252). The genetic lesions are in the extracellular domains of the IL-12Rb1, leading to premature stop codons. All reported patients with IL-12Rb1 mutation show an autosomal recessive inheritance pattern, and heterozygous carriers are clinically healthy with normal IL-12 signaling and IFN-g production. Patients lack cell surface expression of IL-12Rb1 on T and NK cells as detected by FACS. As a result of defective IL-12R signaling, IFNg is not produced by T cells and NK cells. These patients develop severe, recurrent, disseminated *Salmonella* and NTM infections or progressive BCG infection following vaccination. Granulomata are well contained and well organized.

Interferong therapy may provide increased benefit, especially in patients for whom antimycobacterials alone have been incompletely successful. Recombinant IL-12 is being studied in those for whom IFNg is not effective and whose IL-12R is somewhat responsive, perhaps through novel, parallel pathways.

INTERLEUKIN-12P40 DEFICIENCY

The reported patient with IL-12p40 deficiency had disseminated BCG infection and *S. enteritidis* sepsis (253). The mutation was a homozygous autosomal recessive deletion leading to a loss of two exons of IL-12p40. Neither the IL-12p40 subunit nor the IL-12p70 heterodimer was detectable. The patient's lymphocytes secreted IFNg at a reduced capacity, which could be corrected with recombinant IL-12 *in vitro*.

This is a milder clinical phenotype than complete IFNgR1 or IFNgR2 deficiencies because residual, IL-12-independent IFN-g secretion pathways persist, as reflected in this patient's capacity to form organized granulomas.

DEFECTS IN PATTERN RECOGNITION MOLECULES

All organisms encounter other organisms as a matter of daily life, and as such they need to have ways to reject, kill, or contain them immediately that are not dependent on the generation of delayed, specific responses. Innate or natural immunity is the term applied to the panoply of these mechanisms that recognize certain general patterns in the molecules of invading organisms and respond to them on the basis of general and not specific structures (254). The acute-phase response is composed of the early inflammatory responses initiated by ligation of these pattern recognition molecules. The types of cytokine and cellular responses elicited during the acute-phase response then set the stage for the development of the specific or adaptive response mediated by B cells and T cells (255,256). These molecules include the endotoxin-binding molecules of the *Toll* family, CD14, mannose-binding proteins, C-reactive protein, serum amyloid A, complement, integrins, and many others. In addition, Fc receptors are relatively nonspecific receptors that bind classes of host molecules (antibody) that are bound to invading organisms or antigens. The acute-phase response should be seen as the opening salvo in the concerted host response to potential infection. Failure to mount any of this effort correctly may lead to severe infections or blunted responses. The association of autoimmunity with these defects (e.g., complement deficiencies) (257) suggests that these pathways are important in many ways as yet not well understood.

Endotoxin Signaling Pathway

Recognition and response to bacterial lipopolysaccharides (LPS) or endotoxin and other bacterial antigens are critical components of the host defense armamentarium and occur via *Toll*-like receptors and their downstream signaling pathway (258). Only one patient has been identified with a defect in the phagocyte LPS/IL-1 pathway (259); however, her clinical history underscores the importance of this pathway. During her first 15 years of life, she developed 13 life-threatening infections, including two episodes of *S. pneumoniae* meningitis, endophthalmitis, otitis media, cellulitis, septic shock, and a *Clostridium septicum* infection requiring disarticulation of her leg at the hip. All episodes were characterized by minimal to no febrile response. Following experimental challenge with intravenous LPS, she failed to develop fever or neutrophilia and expressed little to no TNFa, G-CSF, or IL-8, compared with normal volunteers. The precise defect remains elusive because the sequences of the patient's genes that encode many of the known components of the LPS/IL-1 pathway appear normal (J. Blanco, A. MedDev, D. Kuhns, S. Vogel, and J. Gallin, unpublished observations). Analysis of these and other signaling molecules involved in this shared pathway is currently ongoing. This condition is consistent with a defect in the *Toll* pathway, which is involved in mediating the binding and response to endotoxin and similar bacterial products (260,261).

Mannose-binding Molecules

Mannose-binding protein is synthesized as a monomer in the liver, but it forms a trimer, which in turn forms multimers of trimers, up to hexamers of trimers. This molecule binds a wide spectrum of gram-positive and -negative bacteria, mycobacteria, fungi, viruses, parasites, and protozoa. Once the mannose-binding protein complex has bound its target, it serves to activate complement by substituting for C1q and to enhance phagocytosis through the C1q receptor, as reviewed by Fraser et al. (262). Polymorphisms and mutations have been identified in the mannose-binding protein (MIM 154545). There are significant population differences in specific alleles, but their functional effect is uncertain (263). Case reports of increased susceptibility to *Neisseria meningitidis* infection (264), as well as more generalized susceptibility to infections (265), suggest that under certain circumstances, mannose-binding protein defects may be important. Garred et al. (266) examined the rates of different mannose-binding protein alleles in patients with cystic fibrosis and correlated that with infection history and survival. They showed a striking association between variant mannose-binding protein alleles and reduced survival, *P. aeruginosa* infection, and *B. cepacia* infection.

The macrophage has many surface binding molecules or lectins that mediate interaction with a variety of pathogens as well as host molecules in various states (267). The best characterized of these is the mannose receptor. The mannose receptor molecule is expressed on macrophages and some lymphatic endothelia. It binds to glycoproteins, especially those with high mannose contents. These molecules may be pathogen associated, such as those on the surface of yeasts and fungi, or they may be host associated, such as cathepsin D. The receptor can circulate in a cleaved form as well (268). Ligation of the mannose receptor initiates several activities of the macrophage, including phagocytosis, inflammatory cytokine production, and generation of reactive oxygen species, as reviewed in Linehan et al. (267). There are discrepancies between mouse and human macrophage mannose receptor-induced activities that may reflect true species differences or differences in activation and developmental states of the cells used. Koziel et al. (269) showed downregulation of the mannose receptor on the surface of alveolar macrophages in patients with *Pneumocystis carinii* and human immunodeficiency virus infection with low $CD4^+$ T lymphocytes.

FcgRIIIb (CD16) Deficiencies

The molecule that makes up two of the major neutrophil antigens, NA1 and NA2, is CD16, the Fcg receptor III (FcgRIII) (270). It binds IgG and, through mechanisms still not entirely clear, induces signaling including calcium flux, granule fusion, and respiratory burst activity. Mutations in CD16 (MIM 146740) leading to CD16 null granulocytes (NA null granulocytes) were first recognized as the basis of isoimmune neonatal neutropenia. Mothers who are deficient in CD16 develop antibody against fetally expressed CD16, leading to a transient neutropenia in the newborn (30,271). Subsequently, it was appreciated that there is a somewhat higher than expected incidence of recurrent bacterial infections in individuals with CD16 deficiency (270). Biron et al. (272) reported a patient with CD16 and NK cell deficiency and severe herpes virus infections. de Vries et al. (273) identified a child with bacterial and viral infections who had a dysfunctional mutation in CD16. Autoimmune phenomena, including Hashimoto thyroiditis and SLE also have been associated with CD16 deficiency (274).

CONCLUSIONS

Detection of neutrophil disorders is fundamentally dependent on a high index of suspicion based on history, physical findings, or suggestive microbiology. All the syndromes discussed in this chapter are capable of certain and specific diagnoses, which carry great importance as to therapy and prognosis. With the exception of MPO deficiency, which is largely clinically silent, the intrinsic functional neutrophil disorders share several key features. The extent of infection is grossly underestimated by clinical assessment. Computed tomography is valuable in examining lungs and livers damaged by recurrent, destructive infections. Recommended therapy that is adequate for normal hosts is associated with high rates of relapse in patients with impaired host defense. High-dose, prolonged, preferably intravenous therapy is indicated. Patients and their families must be educated as to the importance of early diagnosis and treatment.

Characterization of neutrophil defects of clinical importance has come a long way since the first description of abnormal neutrophils by Beguez Cesar in 1943. The features of neutrophil function that have proved most informative are still quite few, however. Superoxide production, chemotaxis, bacterial killing, degranulation, and granule contents have been the staples. Facility in describing and examining newly recognized features of phagocyte function, such as cytokine production and response and phagocyte-endothelial cell interaction, should greatly augment the detail and complexity of the picture we can paint in the future.

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[1]MIM, Online Mendelian Inheritance in Man. www.ncbi.nlm.nih.gov/MIM/

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30 Complement In Disease: Inherited And Acquired Complement Deficiencies

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As discussed in Chapter 26, complement (C)-mediated host defense processes include two basic mechanisms: first, alteration of microbial membranes by opsonization and lysis; second, promotion of the inflammatory response through cell activation. Complement proteins are being increasingly recognized for their important role in linking innate and adaptive immunity, particularly relative to antigen processing and the development of autoimmunity.

Often, in disease states, the C system functions normally but has been activated by an aberrant immune response. For example, if an autoantibody is formed to a basement membrane protein and activates C (Goodpasture's syndrome), it damages the lung and kidney, which express the targeted antigen. In this setting, C is functioning normally but acts as a misguided bullet. Measurement of the C system's overall activity and its individual proteins can serve as a guide in the diagnosis and, more commonly, to monitor a patient's response to therapy. The inherited deficiencies of C-pathway components, receptors, and inhibitors, although rare, are instructive in teaching about normal C function.

INCIDENCE OF INHERITED COMPLEMENT DEFICIENCY SYNDROMES

Genetic deficiencies in C proteins are rare but instructive anomalies [reviewed in (1)]. In an extensive review of all reported cases up to 1984, only 244 individuals were documented with genetically controlled total deficiency (2). In 1991, 521 cases of inherited component or inhibitor deficiencies, excluding cases of hereditary angioedema, were reported (3). An exception is mannan-binding lectin (MBL) deficiency, wherein some studies homozygous deficiency may be present in as many as 3% of the population (4) and heterozygosity in as many as 30% of the population. This is important because, unlike other complement deficiencies in which heterozygous persons have one third to one half normal level, heterozygous MBL-deficient persons may have 10% of the normal level. Moreover, additional genetically controlled transcription-controlling defects may lead to low MBL levels. The prevalence of inherited C deficiency, excluding MBL deficiency in the general population, is estimated at 0.03%. C2 deficiency is most common in the white population, that is, in 0.009% to 0.010% of normal individuals (4,5). Although total C4 deficiency is rare, the incidence of heterozygosity for one of the four C4 genes is high, approaching 40% (4). C9 deficiency, common among a subset of the Japanese population, is present in ~0.1% of the normal population (4,6,7).

In populations defined by certain disease entities, the incidence of C-component deficiencies is much higher. In rheumatologic disorders, such as systemic lupus erythematosus (SLE), the incidence of C2 deficiency in the white population may approach 1.0% (8). The terminal component C deficiencies in patients with *recurrent*, *disseminated*, neisserial infections, especially meningococcal, have been estimated to be as high as 20%. Although one study demonstrated a 15% incidence of late-component deficiency in patients with even a single meningococcal infection, usual estimates are 5% to 10% (3).

GENETICS OF COMPLEMENT PROTEINS

In Chapter 26, Table 26.2 lists chromosomal location, chain structure, and plasma concentration of each of the C proteins. Many genetic polymorphisms also have been reported. In most cases, these allotypic variants show autosomal codominant inheritance and function normally.

The C proteins C4, C2, and factor B are referred to as *class III gene products* because they are encoded by genes within the major histocompatibility complex (MHC) on chromosome 6 in humans. C4 is encoded at two separate closely linked loci, and its two highly homologous gene products possess subtle differences in their binding to substrates. Because of their proximity to class I and II genes (see Chapter 3, Chapter 26), linkage of C4, C2, and factor B allelic variants with particular histocompatibility phenotypes has been noted. Given the location of these C genes, the association of deficiency in these proteins with autoimmune disorders is notable (9,10 and 11).

COMPLEMENT DEFICIENCIES

As reviewed in Chapter 26, the major pathways of C activation merge at the level of C3 and the later steps of C activation are common to all pathways. The alternative pathway (AP) acts as a first line of defense against invading organisms and functions in the absence of specific antibody. The lectin pathway and the classical pathway (CP) [via natural antibodies (Ab)] allow more specific activation of C in the nonimmune host and provide a measure of recognition to the innate antimicrobial response. In the immune individual, the CP serves as a major effector arm of humoral immunity. Given the presence of an intact AP, the absence of one of the early CP proteins does not usually predispose to overwhelming infection; however, some patients with C1, C4, or C2 deficiency have had serious infectious complications (2,3). Because the early components of the AP and MBL pathways and C3 are critical for control of microorganisms, patients with deficiencies in these components often have infectious disease problems (2,3). This increase in susceptibility to infections is predictable. The surprise in the analysis of inherited C deficiencies is the association of early CP component deficiency with autoimmunity, especially SLE. These results imply an important role for C in immune complex handling and immunoregulation. Finally, a deficiency of a regulatory protein commonly results in excessive activation of the system and a disease process related to a secondary deficiency (C3 in factor H or I deficiency) of a component or liberation of activators to produce a pathologic event [i.e., hereditary angioedema (HAE)].

DEFICIENCIES IN CLASSICAL PATHWAY COMPONENTS

C1 Deficiency

Patients who lack each of the C1 subunits (C1q, C1r, C1s) (Table 30.1) and combined C1r and C1s deficiencies have been identified (2,3). C1q deficiency is the most common of this group. These persons usually produce no identifiable C1q, although the synthesis of a dysfunctional protein has been noted in several families and, when found, is almost always present with SLE (12,13 and 14). SLE in C-deficient persons has some features that are distinct from that of lupus in the general population. For the former group, the age of onset is usually earlier and the male-to-female ratio is more nearly equal. Also, the frequency of renal disease and antinuclear antibodies, especially anti-DNA and ANA, are lower and occasionally absent, although the frequency of the anti-Ro Ab is increased. There is an increased susceptibility to viral and bacterial infections, but it is not nearly as pronounced as in C3 deficiency.

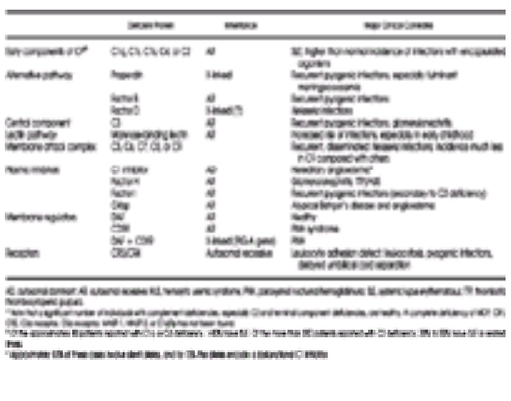

TABLE 30.1. Inherited Complement Protein-Deficiency States[a]

A depressed level of C1q occurs in patients with hypogammaglobulinemia (15). The synthetic rate of C1q is normal; in the presence of reduced immunoglobulin (Ig) concentrations, however, removal of C1q from the intravascular pool is accelerated. Administration of Ig restores the C1q levels to normal.

Antibodies to C1q are found in 15% to 30% of SLE patients and correlate with hypocomplementemia, renal disease, anti-DNA Abs and a poorer prognosis. The Abs are of the IgG class and tend to fluctuate with the course of the disease. Abs to C1q also can be present in patients with the hypocomplementemic urticarial vasculitis syndrome (HUVS MIM C1q deficiency 120790; cis deficiency 216950; cis deficiency 120580).*

C4 Deficiency

There are two separate C4 loci in a 55-kb region of the human MHC that give rise to two protein products: C4A and C4B. Although highly homologous, C4A and C4B differ in molecular weight, electrophoretic mobility, hemolytic efficiency, and types of covalent bonds formed (4). Multiple allelic variants exist for both C4A and C4B. Thus, most persons possess four genes that code for C4, and they may be heterozygous at either or both of the two loci, yielding up to four C4 variants in a given plasma. A deficiency-resulting allele at either C4 locus is termed *Q0* (*quantity zero*) for the gene product in question. The frequency of persons who are deficient in 1, 2, or 3 C4 proteins is about 30%, 15%, and 5%, respectively (16).

Among C-component-deficient patients, those completely lacking C1q and C4 (~40 reported of each) have the highest incidence (80%–90%) of SLE or closely related syndromes (2,3,8,9,12). Infections have been documented in three homozygous C4-deficient patients, one of whom died as a result of disseminated herpes simplex and cytomegalovirus infections (2,3). The absence of either C4A or C4B also is associated with the development of autoimmune disease (3,8,17,18). The relative risk of SLE is increased 16.6-fold for white persons who are homozygous for C4AQ0 and the presence of even a single copy of the C4AQ0 correlates with an increased risk of SLE in both whites and blacks (14,17,18). A portion of this increased risk may be related to the autoimmune diathesis of the human leukocyte antigen (HLA)-B8, -DR3 haplotype with which the C4AQ0 allele is in linkage disequilibrium. The C4AQ0 allele, however, appears to represent an independent risk factor for SLE because, even in the setting of other MHC haplotypes (a situation observed in blacks and in Asians), the incidence of lupus is increased in C4A deficiency (14,16,17 and 18). Other diseases reportedly associated with silent alleles at one or both C4 loci include Graves disease, insulin-dependent diabetes mellitus, Sjögren's syndrome, subacute sclerosing panencephalitis, and chronic active hepatitis (MIM C4 deficiency 120810).

C2 Deficiency

The incidence of C2 deficiency is about 1:10,000 (1% frequency of null allele) in persons of European descent. Many of these persons appear to have a normally functioning immune system. Autoimmune disorders and, less commonly, infections are observed with increased frequency in C2-deficient patients (1,2 and 3,8,9). Infectious complications, including pneumonia, bacteremia, and meningitis, are generally caused by pyogenic bacteria that require opsonization and phagocytosis for eradication. Autoimmune or rheumatic diseases have been reported to occur in 30% to 50% of C2-deficient individuals (1,2 and 3,8). The most common disorder is SLE. C2-deficient individuals tend to be characterized by early age of onset, photosensitivity, modest antinuclear Ab titers but elevated Ab titers to Ro, and relatively mild renal involvement. As in heterozygous C4 deficiency, an increased risk for the development of autoimmune disorders has been reported with heterozygous C2 deficiency (14,17,18). Type 1 deficiency is due to a 28-base-pair gene deletion that leads to aberrant RNA splicing and the deletion of exon 6. In type 2 deficiency, the gene is transcribed, but for unknown reasons, the protein is not secreted (14,19). C2 deficiency sometimes is associated with below-normal factor B levels; this appears to be an acquired defect (2,3,5,19). Two C2-deficient patients with relatively treatment-refractory SLE have had remission of their disease in association with plasma transfusions (MIM C2 deficiency 217000) (18).

IMMUNOREGULATION, ANTIBODY RESPONSES, AND COMPLEMENT DEFICIENCY

Autoimmune disease is the most prominent manifestation of early C-component deficiencies of the CP. The underlying mechanisms are not clear but may include aberrant immune complex handling, increased susceptibility to unusual (especially viral) infections, linkage of C deficiency producing alleles to other disease susceptibility genes inside or outside the MHC, and immunoregulatory defects in the immune response. Numerous spontaneously arising animal models of CP C deficiency, especially C4- and C2-deficient guinea pigs, demonstrate serologic abnormalities (such as rheumatoid factors) suggestive of autoimmunity, but these animals are in good health otherwise (20). In addition, animals deficient in C4, C2, or C3 have repeatedly shown impaired antibody responses to prototypical test antigens and, in particular, failure of isotype switching from IgM to IgG. These abnormalities are correctable by infusion of the missing component (21). Similar abnormalities in Ab responses have been documented in CP-deficient humans (21). C1q, C4, C3, and CR1/CR2 knockout mice have further established a critical role for C in the development of normal immune response, especially to low levels of T-cell-dependent antigens, and have led to a novel hypothesis to explain the association with autoimmunity (14,22,23). The role of complement in the induction of tolerance to self is unclear, but there is a breakdown of tolerance induction in several complement-deficient mouse models, which should allow further dissection of this phenomenon.

DEFICIENCIES IN ALTERNATIVE PATHWAY COMPONENTS

Properdin Deficiency

Properdin deficiency and possibly factor D deficiency are the two C-component deficiency states that demonstrate sex-linked inheritance (2,3,19). The deficiency often comes to medical attention because of fulminant meningococcal infections (2,3). This is in contrast to the late component deficiencies, which tend to have recurrent but less severe neisserial infections. Female carriers also appear to be at greater risk for overwhelming meningococcal infection (2,3). In type 1 properdin deficiency, the most common, the gene defect is such that there is no protein product secreted. In type 2 deficiency, a single gene defect leads to normal plasma levels of a nonfunctioning protein. A type 3 deficiency has been described in which patients have normal levels of a protein that retains some but not all of the functions of properdin (OMIM Properdin deficiency 312060) (24).

Factor D Deficiency

Partial factor D deficiency has been reported in three families. One family had adult twin sisters, both of whom suffered from recurrent upper and lower respiratory tract infections and unexplained infertility. Family studies failed to provide data to suggest a mode of inheritance (3). One person with a complete deficiency suffered from recurrent *Neisseria* infections. X-linked mode of inheritance was suggested, but the location of the factor D gene has not been determined (OMIM factor deficiency

12940).

Factor B Deficiency

There is only one report in abstract form of a 6-year-old white boy with functional factor B deficiency. Meningococcemia was the initial manifestation, and the patient had no alternative pathway hemolytic activity and appeared to be missing epitopes on factor B as identified by specific monoclonal antibodies (MIM factor B deficiency 138470) (25).

C3 Deficiency

Given the central role of C3 in both C activation pathways and the multiple biologic roles that C3 fragments subserve, it is not surprising that C3 deficiency has severe consequences. For example, no homozygous C3-deficient patient thus far reported has been disease free. Also, the molecular basis of human C3 deficiency is specific for each of the six kindreds, indicating that most represent new mutations. This probably reflects the fact that, until the use of antibiotics, patients died before reaching reproductive age. Infectious complications, predominately pyogenic in nature, including meningitis, bacteremia, pneumonia, urinary tract infections, and peritonitis, occur frequently and recurrently (2,3,19). *Streptococcus pneumoniae* and *Neisseria meningitidis* have been the major pathogens reported. In addition, SLE, vasculitic syndromes, and glomerulonephritis have been documented in 15% to 21% of C3-deficient patients (2,3,19). Some patients have detectable, albeit extremely low, amounts of C3 in plasma. Mononuclear phagocytes from such persons synthesize reduced but readily measurable amounts of C3 *in vivo* (19). Other patients have no detectable protein. The genetic defects have included mutations leading to stop codons, major gene deletions, or a failure to secrete an abnormal protein (MIM C3 deficiency 120700) (26).

DEFICIENCIES OF MANNOSE-BINDING LECTIN

Three point mutations of the MBL gene in exon 1 are common, leading to substitutions at codons 54, 57, or 52. MBL contains three identical polypeptide chains, and these substitutions lead to the formation of chains that do not interact normally. Thus, persons with mutations of both MBL alleles have undetectable or extremely low levels of MBL. They constitute about 3% to 5% of the population. Individuals with one normal and one abnormal allele have one sixth to one eighth the normal functional level of MBL and constitute as many as 30% of the population. In addition, mutations in the promotor region of the gene can lead to low levels of protein synthesis. In a number of studies, MBL deficiency has been reported to lead to infection, particularly in neonates and infants (27). Even heterozygous persons appear to be at risk. In a recent study of older children with cystic fibrosis, those who were homozygous deficient for MBL or heterozygous-deficient had a more rapid progression of their lung disease and a shorter life span (28).

The situation with regard to the development of autoimmunity is more complex and less clear. For example, it is reported that patients with rheumatoid arthritis who are either heterozygous or homozygous for MBL deficiency have earlier onset of disease but less rapid progression of their disease (29). Perhaps MBL deficiency predisposes to the development of rheumatoid arthritis. It is known that rheumatoid arthritis is associated with the synthesis of IgG with abnormal glycosylation. It is hypothesized that such IgG can interact with MBL, activate complement, and further promote joint destruction. Presently, there is enormous interest in MBL, and further work will help decide the role of the MBL in acute and chronic infection and in the inflammation that accompanies autoimmune disease (MIM Mannose-Binding Lectin 154545).

DEFICIENCIES OF TERMINAL COMPONENTS

Lysis by terminal C components is important as a host defense mechanism against gram-negative organisms, especially *Neisseria* species. The risk of neisserial infections in a patient with a late component deficiency may be increased as much as 10,000 times above that of the general population (2,3). Patients lacking C5, C6, C7, or C8 present a relatively homogeneous clinical picture with recurrent episodes of meningococcemia, meningococcal meningitis, or disseminated gonococcal infection. More than 50% of such patients reported have had significant neisserial infections (2,3). In addition, other infectious complications, including brucellosis, toxoplasmosis, chronic pyelonephritis, and pneumonia, have been reported. Of the patients with terminal component deficiencies, 11% had autoimmune disorders (often SLE-like syndromes or Raynaud phenomenon) without infectious complications (2,3).

C8 deficiency is particularly interesting. C8 is a three-chain molecule with a, b, and g chains. The genetic locus encoding for the b chain is separate from the locus coding for the a and g chains (19). Thus, two general types of C8 deficiency are recognized: C8b deficiency and C8a-g deficiency. Whereas all reported C8b-deficient patients are white, those with a C8a-g deficiency have been largely black or Hispanic (2,3). A mixture of serum from a-g and b-chain-deficient patients permits the reassembly of functional C8 and restores C activity. A dysfunctional b chain variant has also been described (3,19).

Densen and Ross and colleagues (2,3) pointed out several issues regarding disseminated Neisserial infections in patients with late component deficiency. Perhaps the most striking is that, although the relapse (6.3%) and recurrence rates (45%) of infection in these individuals are considerably higher than those in the normal population (0.6% and 0%, respectively), the mortality rate (2.9%) is lower than that in the normal population (19%). Moreover, fulminant onset of meningococcal disease is not characteristic of patients with late-component deficiency (unlike properdin-deficient patients), and most such individuals have had prodromal symptoms. Two other unusual aspects are an increase in the incidence of infection with serogroup Y organisms and a higher median age at onset of the first infection. It is interesting to speculate about possible reasons for these differences. Although C-mediated bactericidal activity is clearly important in dealing with these organisms, other mechanisms contribute to host defense. Given these figures, it is apparent that patients with terminal-component deficiency acquire systemic Neisserial infections readily, but are capable of at least partial containment of the organisms, presumably via phagocytic cells. In older persons, specific Ab is probably present as a result of previous exposure to these or related organisms, which provides an additional measure of protection. It is striking that the pattern of serum resistance seen in isolates from patients with terminal component deficiency exactly parallels that seen in *Neisseria* isolated from C-sufficient individuals with systemic disease. Thus, terminal component deficiency does not simply confer pathogenicity on a broad spectrum of innocuous *Neisseria* strains that otherwise would have been killed by serum lytic activity (MIM C5 deficiency 120900; deficiency 217050; C7 deficiency 217079; C8 deficiency 120950 X chain 1290 R class; C9 deficiency 120940) (3).

Because killing of some bacteria may occur in the absence of C9, albeit slowly, it has been suggested that C9 deficiency may be of little consequence. Many patients reported to date have not had infectious complications (30). In Japan, screening studies have shown that C9 deficiency is the most common C deficiency, with a gene frequency of approximately 3.5% in some regions, and is present in a relatively large number of individuals (6,7). Initial reports indicated that C9 deficiency occurs with identical frequency in healthy blood donors and in hospitalized populations; however, more carefully controlled studies indicated that there is a higher than expected incidence of meningococcal infection in persons with C9 deficiency (31). There are also reports in the American literature of patients with C9 deficiency who developed meningococcal meningitis (3). Mim C5 deficiency 120900; deficiency 217050; C7 deficiency 217079; C8 deficiency 120950 × chain, 290 R class; C9 deficiency 120940.

DEFICIENCIES IN REGULATORY PROTEINS

Inherited C1 Inhibitor Deficiency: Hereditary Angioedema

Hereditary angioedema is inherited as an autosomal dominant trait (32,33 and 34). The product of one normal C1 inhibitor gene is not sufficient to maintain homeostasis and regulate the various protease functions. Also, there is considerable evidence for a negative feedback by the abnormal gene product on synthesis of the normal gene. For these reasons, plasma levels are below a critical threshold and symptoms result. These patients experienced recurrent attacks of nonpainful, nonpruritic, and nonerythematous swelling of the extremities, face, trunk, lining of abdominal viscera, and, most critically, the upper airway. Bowel wall edema commonly presents as small bowel obstruction syndrome with bouts of colicky abdominal pain, nausea, and vomiting. It should not be confused with an acute abdomen because of the absence of fever, leukocytosis, and clear signs of peritoneal irritation (32,33). Angioedema typically subsides by 48 to 72 hours. The episodes occur spontaneously or, in about half the cases, in response to trauma, such as dental manipulation or pressure. Psychological stress may precipitate attacks in some individuals.

The biochemical mediator(s) of angioedema have not been definitively elucidated. C1-INH is an important regulator not only of C1r and C1s but also of Hageman factor and its enzymatically active fragments as well as clotting factor XIIa, plasma kallikrein, and plasmin (34). Thus, the mediators responsible for angioedema could arise from the C, coagulation, fibrinolysis, kinin-generating system, or a combination of these. There are two principal schools of thought about the mediator of the angioedema. In HAE, unregulated activation of the classical C pathway leads to the excessive formation of C2 fragments (33,35). Some believe that one of these fragments, following interaction with plasmin, is further cleaved into small peptides that induce angioedema. Others proposed that uncontrolled activation of the kinin-generating pathway leads to the formation of bradykinin and that this peptide causes the edema (33). Evidence exists to support both positions, but neither is entirely satisfactory. The quantity of C2 peptide that can be generated seems insufficient for the massive edema sometimes seen. Bradykinin injections cause pain, yet pain is not present (except with intestinal episodes where small bowel obstruction has taken place). Although fluid-phase C consumption is chronic in HAE, acute alterations of both prekallikrein and high-molecular-weight kininogen can be detected in temporal relation to swelling attacks (34). Whereas the activation of C1 is unregulated, the multiplicity of control mechanisms at the levels of C4 and C2 prevents substantial C3 cleavage. Thus, patients with HAE manifest depletion of the early CP components (C4 and C2), but C1, C3, AP, and terminal components are normal (32,33).

Patients with HAE are not at increased risk for infection. Detailed studies, however, suggested that, analogous to the effects of deficiencies in the CP components, patients with HAE have an immunoregulatory abnormality and are probably at increased risk for developing autoimmune disorders such as SLE, Sjören's syndrome, Crohn's disease, and scleroderma (36).

About 85% of HAE patients have one normal and one abnormal C1-INH gene (type 1) that produces no detectable protein (34,37). The remaining 15% have one normal and one gene that codes for a functionless inhibitor (type 2). Thus, in such patients C1-INH antigen levels are in the normal or high range, and functional testing is required if HAE is a strong diagnostic consideration. In these individuals, of course, C4 and C2 levels are low, as in type 1.

The C1-INH gene maps to chromosome 11 in humans and contains eight exons (34,37) as well as a large number of Alu repeat sequences (particularly in the region 3' and 5' to exons 4 and 7, respectively). More than 50 different mutations leading to deficiency or dysfunction have been described. The Alu sequences may be hot spots for unequal crossovers. In analysis of a large number of HAE kindreds with the type 1 abnormality, deletions and, in some cases, duplications of a part of the C1-INH gene, often in the Alu repeat rich regions, have been noted. Kindreds with point mutations are also common. About 20% to 30% do not have a positive family history, and most represent new mutations.

The C1-INH is a *ser*ine *p*rotease *in*hibitor (serpin). It presents a bait sequence to the protease for cleavage. On cleavage of the inhibitor, a reactive site is exposed to the enzyme to be inhibited. The inhibitor then covalently attaches to the active site of the serine protease to cause irreversible inhibition. The usual cleavage point is between Arg 444 and Thr 445. Most type 2 abnormalities represent point mutations that affect the amino acids at or around the active site of the inhibitor, leading to the synthesis of an inhibitor with negligible or absent function.

Angioedema tends to be infrequent and mild before but accelerates in frequency and severity at puberty. Attacks usually are unprovoked but may be precipitated by local trauma, emotional stress, or, in a few patients, menses. The attack frequency varies widely (several times a week to several times a year) from patient to patient, with rare individuals possessing the biochemical defect but having no attacks. This angioedema does not respond to epinephrine, antihistamines, or glucocorticoids, the mainstays of treatment of allergic angioedema (33,38). Laryngeal swelling necessitates careful monitoring in a facility where endotracheal intubation or a tracheostomy can be performed. FFP can be given to persons in nonemergency situations to prevent attacks, for example, and to preoperative patients, particularly those undergoing dental surgery (39). The usual dose of fresh frozen plasma (FFP) is 2 units, an amount that has been used extensively and has proved effective. Infusion of purified C1-INH reliably terminates attacks (34), but this protein is not yet available in the United States. FFP is not usually given in life-threatening situations because it supplies substrate for the various mediator enzymes as well as the C1-INH, and swelling may at times become more severe before it improves.

Long-term prophylactic therapy is used to treat patients with frequent attacks or those with recurrent life-threatening attacks (33,38). Patients respond to impeded, acetylated artificial androgens with increased levels of C1-INH. This is not a direct effect on the promoter of the C1-INH gene, and the mechanism of this response is unknown, although several cytokines also can increase the synthesis of the C1-INH. With such treatment, C1-INH levels partially correct, as do serum levels of C4 and C2. Concomitantly, there is a marked amelioration of symptoms. Adverse effects of long-term androgen therapy include virilization, weight gain, myalgias, headache, microscopic hematuria, abnormal liver-function tests, anxiety, altered libido, and nausea (34). Although the list of side effects is long, they usually do not present major problems. In the rare patient for whom androgens are ineffective or for whom drug toxicity is a problem, the plasmin inhibitor e-aminocaproic acid has been found to be effective (33,38). Its mechanism of action is unknown, and there is no change in the C profile. Antifibrinolytic therapy may potentially be complicated by thrombotic episodes and often is accompanied by myalgias, with or without elevation of muscle enzymes. With each of these agents, there is a high degree of patient-to-patient variation in dosage, and the lowest dose that controls symptoms is chosen. Women are often treated with danazol, an impeded androgen that has few masculinizing side effects, and other attenuated androgens, such as stanozolol are used as well. The usual dosage of danazol is 200 to 600 mg daily or stanozolol 2 to 6 mg daily. Men often are treated with the less expensive but more androgenic agents, such as methyltestosterone at a dose of 10 to 30 mg daily orally (MIM hereditary angioedema 106100, with normal levels of protein 300268).

Acquired C1-inhibitor Deficiency

Low levels of C1q, lack of a family history, later age of onset, and association with other conditions, particularly malignancies, distinguish these patients from those with the hereditary form of C1-INH deficiency (34). Acquired C1-INH deficiency may be associated with lymphoproliferative disorders and in most cases represents development of an autoantibody that binds to and neutralizes the C1-INH (34). Individual patients have been treated successfully with androgens, antifibrinolytic agents, glucocorticoids, and cytotoxic agents, but a uniform, effective approach to therapy is not available.

Factor I Deficiency

Factor I, in the presence of a cofactor protein, cleaves C3b. Cleaved C3b (iC3b) is no longer active in the sense that it cannot react with factor B to form a C3 convertase. Thus, factor I prevents unrestrained activation of the AP by C3b. The absence of factor I will lead to unrestrained consumption of C3 secondary to accelerated spontaneous AP turnover. Patients with factor I deficiency are clinically similar to those with C3 deficiency in that their C3 is low to barely detectable. They suffer from recurrent infections with pyogenic organisms, including meningococcal meningitis (2,3). Some individuals have had glomerulonephritis with lupuslike illness. Infusion of factor I corrects the C3 abnormality and normalizes C levels (19), but its long-term clinical usefulness is not clear (MIM factor I deficiency 217030).

C4b-Binding Protein (C4BP)

There is only one reported case with C4BP, and this patient had a syndrome resembling Behçet's and angioedema (40).

Factor H Deficiency

Like factor I deficiency, these persons cannot regulate C3b and therefore have excessive AP turnover (2,3,19). They usually present with glomerular-based kidney disease. Because of the abnormality in C3 regulation, these patients have low plasma levels of C3 and a high incidence of infection, especially neisserial meningitis. Other patients have more problems with glomerulonephritis. A strain of Yorkshire pigs deficient in factor H develops renal failure a few weeks after birth (41). The kidney lesion is membranous glomerulonephritis, and large C3 fragments are deposited in the glomeruli. Familial deficiency of factor H in humans has been associated with thrombotic thrombocytopenia purpura/hemolytic uremic syndrome (42,43). In one series, the infants at birth manifested severe hemolytic uremic syndrome, and the condition was often rapidly fatal. The association of hemolytic uremic syndrome and factor H abnormalities is being examined in a number of laboratories, and patients with low C3 should have their factor H examined (MIM factor H deficiency 134370).

DEFICIENCIES IN CELL SURFACE COMPLEMENT REGULATORY PROTEINS AND RECEPTORS

CR1 Deficiency

In patients with SLE, as well as in patients with other immunologically mediated diseases featuring immune complexes, such as Sjögren's syndrome, human immunodeficiency virus (HIV), and autoimmune hemolytic anemia, the quantity of CR1 expressed on E is below normal (44). The magnitude of the decrease in CR1 correlates with disease activity and returns to normal as disease activity subsides. As for the role of this partial deficiency in the pathogenesis of SLE, it has been speculated that soluble immune complex handling may be altered when levels of CR1 on the blood cells are low; this can lead to the deposition of immune complexes in organs where their presence is undesirable, such as the kidney (45). No report has been made of a patient with a total deficiency of CR1 or CR2; however, persons with fewer than 50 copies of CR1/E are healthy (46,47). These individuals have normal numbers of CR1 on phagocytes and B-lymphocytes. The copy number of CR1 expressed per E is under genetic control. Despite early reports indicating that persons with a low copy number have a predisposition to development of SLE, such an association has not been confirmed (48).

CR3/CR4 Deficiency

CR3/CR4 are receptors for C3bi and are members of the family of b_2 integrins (4,49). The latter family is composed of four members: CR3, lymphocyte function antigen (LFA)-1, p150,95 or CR4, and a_Db_2, each possessing a common b subunit. Patients with a b_2 integrin deficiency suffer from recurrent severe pyogenic infections and have defects in multiple neutrophil and mononuclear phagocyte microbicidal and adhesive functions. Lymphocyte functions related to adhesion also are impaired, especially cytotoxic activity. The clinical correlates of these *in vivo* findings are delayed separation of the umbilical cord, persistent leukocytosis, recurrent pyogenic infections (particularly of the skin and mucous membranes), and aggressive periodontal disease. Two phenotypic variants have been identified on the basis of the severity of the recurrent infections. These appear to correlate with reduced (in the moderate phenotype) versus absent (in the severe phenotype) CR3 expression (4,49,50). The biochemical basis of this disorder is an inability to synthesize the chromosome 21-encoded b subunit, resulting in failure of these cellular adhesion proteins to insert into cell membranes (see Chapter 15). There is one report of an a-subunit defect in a patient with SLE (MIM leukocyte adhesion defect 116920) (51).

CD59

In the single case of isolated, complete deficiency of this glycolipid attached inhibitor of C8 and C9 insertion into membrane-bound MAC, the initial manifestation was the syndrome of paroxysmal nocturnal hemoglobinuria, including hemolytic and thrombotic complications (see later) (52,53).

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem cell disorder characterized by chronic intravascular hemolysis and propensity to thrombosis [reviewed in (54,55)]. It exemplifies the critical role of membrane regulatory proteins in C homeostasis. The clinical manifestations of PNH are due to the absence of multiple proteins, all of which are attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor. This lipid anchor presumably allows for rapid lateral movement of regulatory proteins in the plane of the membrane. This anchor consists of, in order, phosphatidylinositol, *N*-glucosamine, three mannoses, and an ethanolamine. Posttranslationally, the ethanolamine is linked through an amide bond to the carboxyl amino acid of the protein. The fully formed anchor is attached en bloc to the protein in the endoplasmic reticulum (56). This “pigtail,” or so-called “greasy foot,” is not synthesized because of a somatic stem cell mutation in a gene (Pig A) on the X-chromosome responsible for the initial step in its biosynthesis. As a result, the proteins are translated but cannot be attached to the membrane, and all hematopoietic cells derived from this stem cell possess this defect. Thus, GPI-linked C-inhibitory proteins CD59 and CD55 are absent from cells. CD59 was described already. CD55 inhibits the C3 and C5 convertases.

In the absence of CD55 and CD59, more C complexes than normal are added to the E membrane, resulting in lysis when even small amounts of C are activated. Human E, in contrast to most all other cell types, do not express a third C-membrane regulatory protein, MCP or CD46. Consequently, PNH E is devoid of membrane regulators, and the outcome is chronic hemolysis. On platelets that also lack GPI-linked proteins, the addition of polymeric C9 results in the generation of procoagulant vesicles, which may account for the marked tendency to thrombosis in this disease.

Other immunologically relevant proteins deficient on hematopoietic cells in PNH include the FcgIIIa receptor, CD58 (LFA-3), and CD14 (plasma endotoxin-binding protein) (57). Surprisingly, the lack of these moieties results in minimal or no immune deficiency in PNH, and these patients are not highly susceptible to infections. Current evidence indicates that it is the ability of PNH stem cells to survive in bone marrow injury syndromes compared with normal stem cells that accounts for their preferential appearance in PNH (50%–70% of the time PNH arises in an aplastic marrow) (54). Various other proteins are also missing, including several receptors (urokinase receptor, folate receptor), enzymes (acetylcholinesterase, leukocyte alkaline phosphatase), and several proteins of unknown function. Presumably, the deficiency of one or more of these GPI-linked proteins provides a survival advantage to stem cells in the setting of marrow injury (54).

The diagnosis of PNH is no longer made by demonstration that Es have an unusual susceptibility to C lysis but is established by the demonstration of the absence or reduction by fluorescence-activated cell sorter (FACS) analysis of proteins bearing GPI-anchors on hematopoietic cells. CD59 is often the protein monitored and it tends to be lacking on a greater percentage of leukocytes than E.

MANAGEMENT OF THE INHERITED COMPLEMENT DEFICIENCIES

In most clinical situations, it is the management of the complications (infections and autoimmune syndromes) that dominates the clinical picture. Little attention has been paid to correcting the C defect. Plasma infusion treatment has been attempted in a few patients. In several cases, the transient reversal of clinical or biochemical abnormalities has been of sufficient magnitude and duration to suggest the clinical usefulness of plasma infusion in the setting of acute infectious diseases (1,14,58). Because the *in vivo* half-life of most C proteins is short, however, plasma therapy requires evaluation on a case-by-case basis. For a variety of reasons, including inconvenience, possible sensitization to the replaced protein, and the attendant risks of plasma infusions, this approach usually is not recommended for long-term therapy.

Two patients with C2 deficiency have had successful resolution of a relatively refractory lupus syndrome by long-term infusion of plasma. Vaccination with polyvalent meningococcal and pneumococcal vaccines has been recommended (3) for many C-deficient patients, especially those with properdin and C5-8 deficiency. Chronic administration of prophylactic antibiotics also has been suggested and has, in some patients, reduced the incidence of infections (1,3). Given the past failure of penicillin in the prophylaxis of meningococcal disease, this approach may have only limited usefulness in persons who are deficient in terminal components (3). Although a considerable number of C-deficient individuals may be clinically well or have primarily autoimmune syndromes, infection, usually bacterial, accounts for substantial morbidity and mortality rates. A persistently high index of suspicion for infection is thus appropriate in the long-term management of all patients deficient in C components. Therapy of rheumatologic or other autoimmune conditions in C-deficient persons has not been studied extensively but does not appear to differ from optimal treatment of the same syndromes in C-sufficient patients. The influence of replacement therapy (for example, aggressive androgen therapy to achieve normalization of C4 and C2 levels in HAE) on autoimmune disorders in this population has not been assessed in a controlled manner.

EVALUATION OF HYPOCOMPLEMENTEMIA

General Principles

To develop a differential diagnosis of hypocomplementemia in the clinical setting (Table 30.2), the pathophysiology of C activation and the factors that influence the level of the circulating proteins must be considered. The proteins that constitute components of the C cascade generally circulate in a functionally inactive form. Activation of the C sequence leads to utilization of components. The resulting level is the sum of the rates of synthesis, activation, and degradation. Consequently, blood levels may be normal in situations in which C is activated, such as in most infectious diseases and many autoimmune diseases. In pneumococcal pneumonia, C activation occurs at the site of infection, but the magnitude of this localized process is more than balanced by the acute-phase response-mediated increase in C3 synthesis.

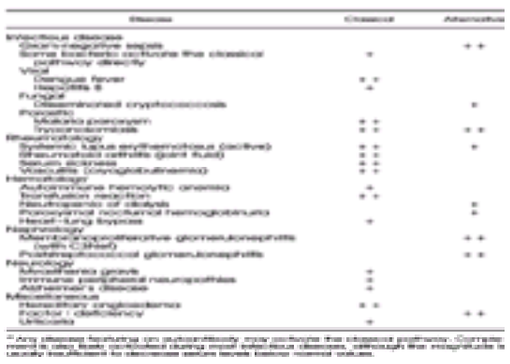

TABLE 30.2. Pathway Primarily Activated By Disease States[a]

Certain cytotoxic, opsonic, and ischemic-reperfusion injury syndromes are associated with minimal lowering of the C titer, even though C is involved in mediating the damage. Thus, in hemolytic anemias, transfusion reactions and PNH C mediates cellular destruction but serum titer is usually within the normal range. In strokes, heart attacks, and burns, C deposition occurs at sites of tissue damage, but serum levels are normal.

The various body compartments may be isolated from one another with respect to transport of C components. Although C is activated in the joint space of patients with rheumatoid arthritis, this activation usually is not reflected in an abnormality in serum C. The blood–brain barrier prevents access of the C proteins to the brain, except in inflammatory states; again, the compartmentalization of these rather large protein components makes it unlikely that the serum titer will be lowered. These data point out that an excess of C is present, which is more than sufficient to carry out its functions related to host defense and clearing dead and dying tissue. To decrease levels substantially usually requires pathologic situations featuring excessive amounts of, for example, immune complexes and chronicity.

The presence of depressed levels of several components of the CP or AP implies that C is being activated (59,60). Many factors can activate the C cascade, but depressed component levels are most commonly associated with the presence of circulating immune complexes, such as in SLE. Proteolytic enzymes released from

dying cells, from phagocytic cells during phagocytosis, and from metabolizing bacteria all may activate the C sequence.

The C cascades may be activated by certain polyanions, such as heparin or heparin protamine complexes, or the surface of uric acid crystals, or C-reactive protein binding to its substrate on the C-polysaccharide of pneumococci. Various exogenous factors, such as the dialysis membrane in an artificial kidney and heart–lung bypass machines, activate C via the AP. Antigen-antibody complexes of many classes, including IgA and IgE, also activate the AP, although, in general, large quantities of complexes are required. Many infectious organisms interact directly with the proteins of the AP and mediate depressed levels of C components.

If a depression in the level of a component is found either by antigenic analysis or by measurement of CH_{50} titer, the first question to ask is whether the finding represents the absence or depression of one or more components of the pathway. Some diseases, such as HAE, activate C4 and C2 but go no farther in the activation sequence. In HAE, C4BP binds to the C4b that is generated to prevent further activation. Other disease processes stop with the activation of C3 or C5. Therefore, it is not surprising to observe lowered titers of several, but not all, components of a pathway. As an example, it has been reported that many patients with membranoproliferative glomerulonephritis have low levels of factor B and low levels of properdin (60). In contrast, most patients with poststreptococcal glomerulonephritis have extremely low levels of properdin but relatively normal levels of factor B. The reasons for this difference in the amount of activation of components of a pathway are unknown. Nevertheless, both situations clearly point to AP activation.

In summary, if a single component is lowered, it is reasonable to question whether the patient has a genetically controlled deficiency. Studies of family members may be in order. If several components of a pathway are lowered, a reasonable supposition is that C is being activated. When hypocomplementemia is associated with a fall in titer of several components, it is often possible to determine whether the classical or AP is the major pathway being activated; this in turn may suggest certain diagnoses and rule out others.

Assays

Antigenic analyses are useful in following patients with ongoing C activation (Table 30.3). For example, monitoring the levels of C3 or C4 by this assay is of value in evaluating disease activity in patients with SLE. In general, a fall in C4 or C3 levels indicates a worsening of the active disease process and an increase with therapy serves as a guide to resolution. Because C3 and C4 activation products are rapidly cleared from the circulation, antigenic analysis of this type provides reasonable information about the functional level of the protein under study.

Method	Use	Comments
CH_{50} or THC	Good screen for complement deficiency or gross activation of classical (CP) pathway	Functional assay—requires appropriate sample handling
AH_{50}	Good screen for complement deficiency or gross activation of alternative pathway	Functional assay—requires appropriate sample handling
Antigenic (ELISA, immunodiffusion, nephelometry)	Standard for C3, C4, factor B, C1-inhibitor, and MBL determinations	Widely available, easy to perform, reliable, inexpensive
Antigenic or hemolytic assay of individual components	Further define a suspected deficiency	Samples usually sent to reference laboratory for complement assay
Activation fragments C3a C5a Bb C1-INH–$C1_2C_2$ C3b-H (neoantigen)	Very low positive with normal complement levels	Expensive, sample collection important; commercial kits often for these assays; more sensitive than ordinary levels
C1-inhibitor function	Clinical picture consistent with HAE but C1-inhibitor levels by antigenic assay are normal or elevated	15% of HAE patients have normal or elevated levels of a nonfunctional protein
Immunofluorescence	Demonstration of complement activation fragments in tissue	$C1_q$, C4, and C3 are most commonly studied in kidney and skin biopsies
Antiglobulin testing	Demonstration of C3 fragments on erythrocytes	Used fragment detected is C3d

AH_{50}, alternative pathway equivalent of THC; CH_{50} or THC, total hemolytic assay for classic pathway; ELISA, enzyme-linked immunosorbent assay; HAE, hereditary angioedema; MBL, mannose-binding lectin.

TABLE 30.3. Assays for Complement Activation in Human Disease

The most commonly used parameter for functional analysis of C activity is the total serum hemolytic C (CH_{50}) or total hemolytic C (THC) titer (Table 30.3). This test, which examines the ability of fresh serum to lyse antibody sensitized sheep erythrocytes, reflects activity of all components, C1 through C9, of the CP. In a genetically controlled total deficiency of any one except C9, the CH_{50} titer approaches zero. A comparable assay, AP CH, measures the functional capability of the AP. Usually, these tests are used on a one-time basis to screen patients for deficiency states. These assays, however, are not particularly sensitive to C activation, generally requiring more than 50% depletion of a component before it will register.

The examination of freshly obtained ethylenediamine-tetraacetic acid (EDTA) plasma (to block C activation in association with clotting, reduced temperatures, or storage) provides a method of determining whether there is ongoing C activation *in vivo* (61,62). The identification of increased levels of cleavage/activation fragments of C3, C1-INH, factor B, and C5 suggests activation of C, even in the absence of lowered C titers (59).

Antisera have been prepared that recognize neoantigens that develop during C activation (Table 30.3). For example, poly-C9 does not exist in native plasma but may be found in patients with complement activation. Antibodies to poly-C9 can be fluoresceinated and incubated with tissue sections from patients with various autoimmune diseases. Antibody to the neoantigens brightly stains glomeruli in patients with glomerulonephritis, suggesting that the C5b-9 lytic complex has formed on the basement membrane of the glomerulus. Similarly, such antibodies stain the dermal–epidermal junction of SLE patients in whom skin lesions are present but are absent where there are no skin lesions. This is in contrast to antisera to C3 that in SLE typically stain the dermal–epidermal junction in both pathologic and nonpathologic areas.

Other antisera monitor specific components or cleavage fragments. For example, in Alzheimer's disease, C1q, C4d, and C3d are deposited in plaques, indicating CP activation. These can be identified with antisera. Other tests are based on enzyme-linked immunosorbent assays (ELISAs), which that can detect activation of the CP or AP in plasma. The ELISA detects the presence of (a) complexes between C1s and C1-INH as evidence of CP activation, (b) complexes between properdin and C3 as evidence of AP activation, and (c) the neoantigen associated with the formation as evidence of formation of membrane attack complex (59). As little as 1% of C activation via the AP can be detected by the ELISA.

Interpretation of Hypocomplementemia

The components of the C sequence are usually considered in groups (59,60): (a) the CP, with its early components C1, C4, and C2; (b) the components of the AP, factor D, factor B, and properdin; (c) C3; and (d) the membrane attack complex, C5 through C9. If the CP is extensively activated as in SLE, utilization of the components C1, C4, C2, and C3 through C9 outstrips synthesis, resulting in a lowering of their titers. If the AP is substantially activated, titers of factor B, properdin, C3, and C5 through C9 (see Chapter 26, Fig. 26.2) will be decreased.

It is therefore useful to study a serum sample for its levels of C4 (a component of the CP) and C3 (a component of both pathways). These proteins can be easily, accurately, and inexpensively measured in serum or plasma. If C4 and C3 are low, as often is the case in patients with lupus, it can be surmised that the classical CP has been activated. In contrast, if C4 is normal in the setting of low C3, as is often the case in membranoproliferative glomerulonephritis, it can be surmised that the AP plays the primary role in C activation. Measurement of factor B levels also can be utilized to confirm such interpretations. In some patients with SLE and glomerulonephritis, a decrease in synthesis of C3 as well as an increase in utilization contribute to the low C3 levels. The reasons for this are still not clear, but C-degradation products may feed back to regulate the rate of synthesis of C proteins. The C proteins themselves turn over rapidly, about 2% per hour or about 50% per day, even in the resting state. Patients who are deficient in plasma regulatory proteins C1-INH, factor H, and factor I excessively turnover complement proteins, resulting in extremely low levels.

Foreign-body Reactions

Foreign materials placed in contact with the bloodstream usually result in activation of AP on the surface of the material [reviewed in (63)]. The two most common clinical situations are kidney dialysis and operations requiring heart–lung bypass. In both cases, by measuring products such as C3a or C5a, C activation can be shown to occur within minutes of exposure. A syndrome of agitation, shortness of breath, and pulmonary edema occurring within the first 30 minutes or so of dialysis was traced to C5a causing neutrophil aggregation and then lodging of these aggregates in lung capillary beds. Subsequently, dialysis membranes have been modified to reduce the intensity of this reaction. In heart–lung bypass, high levels of C5a correlate with longer pump time, ischemic complications, and increased morbidity and mortality. Such reactions are caused by most foreign bodies, but in many situations, blood contact is less because of small surface (intravenous needle or line) or more limited exposure to blood (not in the intravascular space). As exemplified by the dialysis membrane story, surfaces can be designed so as to reduce these reactions.

Ischemia–Reperfusion Injury

Reperfusion of a tissue following transient ischemia leads to complement attack on the still viable but partially damaged zone of tissue that surrounds necrotic tissue [reviewed in (64)]. These situations are common in clinical medicine, occurring to various degrees during myocardial infarctions, strokes, and other arterial thrombotic syndromes. Preservation of the damaged but still viable tissue is of paramount importance in many of these conditions. In animal models, as much as 30% to 50% of ensuing heart-muscle damage can be prevented by blocking C activation. The use of thrombolytics and other means of acutely opening blood vessels provides additional clinical scenarios where C inhibition could be helpful. The mechanism of the C activation in ischemia–reperfusion injury is poorly understood, but the evidence currently favors a CP activation phenomenon versus an inhibitor deficiency condition. Thus, alterations in the surface of ischemic cells may allow natural Abs or (more likely) lectins to bind to the surface and activate the CP. A role for C in the clearance of dead and especially injured (ischemic) tissue may be an important function of C not directly related to infections or immune responses.

This system for clearance of cell debris probably arose to facilitate the healing of cutaneous injury. Rapid removal of damaged tissue likely would limit infectious complications and facilitate wound repair. Of course, this same efficient process might be undesirable in heart or brain tissue. Other conditions that have similarities to these conditions, and where C activation may play a role, include acute respiratory distress syndrome, burns, septic shock, acute pancreatitis, and polytraumatic and multiorgan dysfunction syndromes. As noted, a failure of proper clearance apoptotic cells has been theorized to result in autoimmunity (12,14).

Complement Therapeutics

Currently, no therapy exists that can abrogate undesirable C-mediated cellular and tissue injury in clinical medicine. Two C inhibitors, however, are in clinical trials [reviewed in (65) and (66)]. Ideally, one would like to have an orally administered agent that specifically and temporarily inhibits C activation. Even better would be therapeutics targeting a single pathway or the MAC, depending on what is mediating the tissue damage. Interestingly, both *in vitro* and in experimental animals, intravenous immunoglobulin (IVIG) blocks complement binding to target tissues (discussed in greater detail in the chapter on IVIG).

One compound, sCR1, selectively inhibits the C3 and C5 convertases of all the activation pathways. It accomplishes this task by serving as cofactor for factor-I mediated cleavage of C3b and C4b and accelerates the disassembly of the C3 and C5 convertases. The second agent is a humanized anti-C5 monoclonal antibody (mAb). It binds to C5 and blocks C5 convertase activity of both pathways. Thus, this reagent has no effect on C3 cleavage by the C3 convertases and, therefore, would not inhibit C3b- and C3a-mediated pathologic events. Because many diseases are considered to be mediated by the actions of C3, the mAb to C5 would be of no value. On the other hand, the mAb to C5 would leave C3 reactions intact, and, as a result, the patient would not be as susceptible to infections. Unfortunately, in many clinical situations, we do know the contributions of C3 deposition versus that of MAC formation to a pathologic entity.

There are now more than 70 peer-reviewed publications reporting on the efficacy of sCR1 in inhibiting C activation in disease models in animals (67). Ischemia–reperfusion injury, burn injury, acute respiratory distress syndrome, antibody-mediated autoimmune disease (rheumatic and neurologic), and organ allografts were all ameliorated by sCR1. The data were sufficiently compelling that human trials (phase 1, 2, and 3) have been under way for several years. Reports have indicated no adverse events attributed to the drug and have shown it to be safe and nonimmunogenic and that C inhibition can be achieved. The second agent (anti-C5 mAb) effectively inhibited C5 activation in patients undergoing cardiopulmonary bypass or renal dialysis. There were no adverse effects, and an immune response to the preparation was apparently not observed. This mAb has also been effective, somewhat surprisingly, in several mouse models of immune complex-mediated disease and in several ischemia–reperfusion models. Advantages of this reagent include considerable clinical experience with the use of mAbs, the long half-life of IgG, less expensive production, and lack of immunogenicity. This technology could also be expanded to include a mAb to C2 as well as factor D to block a specific pathway.

Acute, transient C inhibition should be well tolerated, although patients requiring a sustained inhibitor may be at risk for pyogenic infections (sCR1) and *Neisserial* infections (mAb to C5). Potential complications also include exacerbation or development of an autoimmune state.

Xenotransplantation

Hyperacute organ rejection and blood transfusion reactions are two conditions in which sudden, massive, lethal CP activation may occur. Modern blood-banking technology has significantly reduced major transfusion reactions. Similarly, sophisticated tissue typing and other types of pretransplantation testing can limit hyperacute organ rejection. Currently, however, a shortage of transplantable human organs exists. Thus, transplantation of animal organs (i.e., xenotransplantation), is being investigated [reviewed in (68), (69), and (70)]. The major block to such an approach is the ensuing hyperacute rejection of the animal organ mediated by natural Abs and the C system. For example, a pig kidney, if transplanted to primate, survives only a few minutes secondary to a massive attack on the endothelium by natural Abs, which recognize sugars on the pig endothelial cells. These largely IgM Abs activate the CP, producing rapid endothelial damage. Clots soon form at these sites of endothelial damage, causing massive necrosis. To overcome this process, transgenic pigs have been produced that express human membrane C inhibitors. The expression of DAF, CD59, and MCP can ameliorate the hyperacute rejection process, offering hope that eventually xenografting can be a successful alternative approach. The problems of xenosis, especially the concern relative to pig retroviral to human infection, and delayed or vascular graft rejection are issues to be examined. This is reviewed in detail in Chapter 85.

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*Numbers represent Online Mendelian Inheritance in Mam Citations. National Library of Medicine.

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31 LYMPHOCYTIC LYMPHOMAS AND HODGKIN'S DISEASE

Eric H. Westin, M.D., and Dan L. Longo, M.D.

The Hodgkin's and non-Hodgkin's lymphomas are a heterogeneous group of diseases with diverse cytologic, immunologic, and biologic features. These features result in a wide spectrum of clinical presentations and natural histories in treated and untreated patient groups. Information on the biology of the lymphoproliferative disorders continues to increase rapidly as a result of clinical observation, immunologic characterization, cytogenetic analysis, and application of increasingly sophisticated molecular approaches to disease analysis. However, despite our advances in knowledge, the non-Hodgkin's lymphomas in particular are becoming an increasingly important public health problem because of a rapid increase in incidence (5% per year over the last 20 years) for unclear reasons.

The lymphomas are neoplasms of cells of the immune system and generally represent neoplastic clones of cells that in many ways recapitulate specific stages in the normal lymphoid differentiation pathway. In this class of malignancies, however, most cells also acquire genetic abnormalities that are specific to each lymphoma subtype. These genetic changes affect genes of classes that represent both protooncogenes (1) and genes that alter the cell cycle (2) and response of the cell to signals that would normally induce apoptosis (3). In selected cases such as mucosa-associated lymphoid tissue (MALT) lymphomas and lymphomas arising in the immunosuppressed patient, the abnormal immune cell proliferation may not only be due to specific genetic changes within the tumor cell but may also be the result of abnormal immune stimulation or the absence of one or more normal regulatory mechanisms.

A large number of distinct clinicopathologic entities may be considered under the heading of lymphocytic lymphomas and Hodgkin's disease. Table 31.1 lists them according to their cells of origin: B-lymphocyte or T-lymphocyte disorders based on the recent World Health Organization (WHO) adaptation of the Revised European-American Lymphoma Classification scheme (4,5 and 6). The chapter introduces these entities in the context of the normal differentiation of B and T cells and reviews the pathology, clinical features, immunologic features, and treatment issues for the individual diseases that comprise Hodgkin's disease and non-Hodgkin's lymphomas.

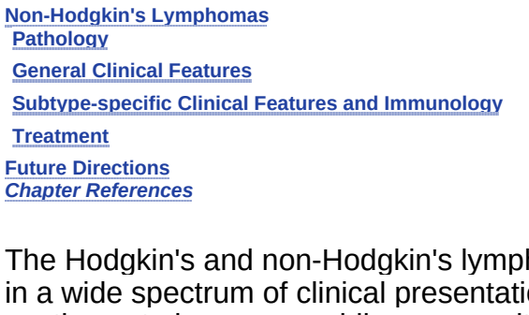

TABLE 31.1. WHO Classification of Lymphoid Neoplasms

ONTOGENY OF B LYMPHOCYTES

A schema for normal B-cell differentiation is depicted in Fig. 31.1 with designation of stages of development that correspond to the WHO classification framework outlined in Table 31.1. The stages of B-cell differentiation have been identified based on data derived from molecular biologic analysis of malignant B cells, an analysis of cell-surface phenotypes using a variety of monoclonal antibodies that detect particular membrane glycoproteins, and certain histochemical staining techniques that determine the presence or absence of intracellular protein products (7,8,9,10,11 and 12).

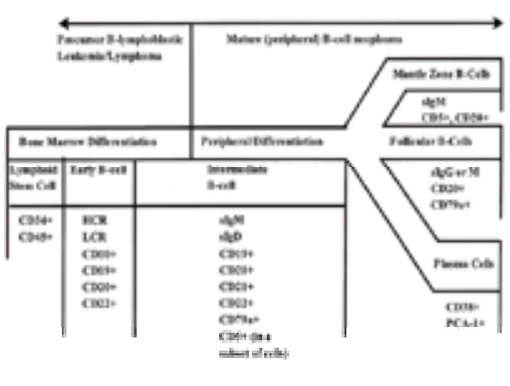

Figure 31.1. B-cell development and lymphoid neoplasms. Normal B-cell differentiation illustrating cells of origin of the B-cell lymphomas. Cell-surface and intracellular markers of B-cell differentiation are given for each discrete stage of development. TdT, terminal deoxynucleotidyl transferase; HCR, heavy-chain rearrangement; LCR, light-chain, k or l, rearrangement; sIg, surface immunoglobulin of indicated subtype (M, D, or G); PCA-1, plasma cell differentiation antigen-1.

Mature B cells usually are identified by the presence of surface immunoglobulin (sIg). The earliest stages of B-cell development can be discerned by detecting the presence of rearranged Ig genes before the expression of their product on the cell surface (10,11 and 12). Ig gene rearrangement is likely the first step in differentiation of cells committed to B-cell differentiation before any expression of B-cell surface markers (11).

As described in detail elsewhere in this book, immunoglobulin molecules are composed of two heavy chains [molecular weight (mol wt), ~50,000] and two light chains (mol wt, ~25,000). There are two types of light chains [k (encoding gene located on chromosome 2) and l (encoding gene located on chromosome 22)] and nine types of heavy chains: four types of g (G_1–G_4), two of a (A_1, A_2), and one each of μ, d, and e (encoding genes all located on chromosome 14). Each heavy and light chain contains a variable region (extensive amino acid sequence variability between immunoglobulin molecules) and a constant region (limited sequence variability). The light and heavy chains are linked by disulfide bonds and aligned so their variable regions are adjacent to one another. This alignment of variable regions forms the antigen-recognition site of the antibody molecule; its unique structural features form a particular set of determinants that can be recognized by other antibodies and are called idiotypes.

Immunoglobulin heavy chains are encoded by four distinct genetic elements: The variable region is encoded by three, the variable (Igh-V), diversity (Igh-D), and joining (Igh-J) elements; the constant region is encoded by one constant (Igh-C) element (11,13). Light chains are devoid of diversity elements and thus comprise three elements, two for the variable region (Igl-V and Igl-J) and one for the constant region (Igl-C).

In the germ line genome (all cells except B cells) the V, D, and J genes are widely separated, and there are numerous forms of each. Once a cell becomes committed to B-cell differentiation, one V and one D gene rearrange to join one J gene, and the intervening genetic material is excised (10,11). The process is termed VDJ joining and results in the production of a unique variable region (11). The newly rearranged VDJ gene is transcribed into a single message along with either an M or D isotype heavy-chain constant gene. On exposure to antigen, another gene rearrangement may occur so that the VDJ gene encoding a particular variable region may be associated with any heavy-chain isotype. The isotype switch usually results from splicing variable region messenger RNA (mRNA) together with constant region mRNA of a different isotype. Similar events occur in light-chain genes (VJ joining) to form its variable region. Because of the mechanics of the gene rearrangements necessary to specify the immunoglobulin variable regions, a particular clone rearranges only one of the two heavy-chain chromosomes and rearranges either its k or its l genes but not both. This process results in allelic exclusion (i.e., a clone expresses only one light-chain isotype). The gene rearrangements result in an altered sequence of DNA on the chromosomes, which in turn produces a molecular signature of a particular clone when the DNA is analyzed by Southern or polymerase chain reaction (PCR) techniques. Using this molecular approach, it has been determined that the overwhelming majority of B-cell malignancies are derived from a single clone, as illustrated by Korsmeyer et al. (14).

Gene rearrangements are the first evidence of commitment to B-cell differentiation. On additional maturation, functional immunoglobulin mRNA is transcribed and translated, leading to detectable cytoplasmic μ heavy chains, a finding that marks the pre–B-cell stage of development (15). When the intracytoplasmic heavy and light chains are assembled and inserted into the plasma membrane, the B cell has achieved an intermediate stage of differentiation. Most B cells go on to express surface IgD as well as IgM, and the resultant cell is the most common circulating B cell. Additional changes can occur on exposure to foreign antigen in the periphery.

Peripheral B cells appear to be divided into two distinct lineages, B-1 cells and B-2 cells. B-1 cells appear first in ontogeny, often express CD5, a pan–T-cell marker on their surface, are distributed in the mantle zones of lymphoid organs, respond to multivalent antigens such as polysaccharides (usually without cognate T-cell help), and do not undergo affinity maturation. Autoantibodies are frequently derived from the B-1 population. B-2 lineage cells are more numerous, do not normally express CD5, and undergo antigen-directed affinity maturation (involving mutations in the antigen-binding site introduced by terminal transferase) and class switch to alternative heavy-chain isotypes in the lymphoid follicle. With further maturation, the cells lose sIgD and sIgM and acquire the sIgG isotype of the antibody they ultimately secrete before terminal differentiation into plasma cells. Plasma cells normally lack sIg.

Monoclonal antibodies that recognize antigens expressed exclusively on B cells have been produced and used to develop models of B-cell differentiation (16). These B-cell–restricted antigens are expressed at certain stages of B-cell differentiation; for example, the CD19 and CD20 antigens appear to span most of B-cell differentiation, with the CD19 antigen appearing earlier. The CD21 antigen is gained and then lost during the middle stages of B-cell development. Maturation to the plasma cell stage coincides with the expression of the plasma cell–associated (PCA-1) antigen (17). Other markers found on certain B cells include Fc receptors, complement receptors, common acute lymphoblastic leukemia antigen (CALLA), CD10, human leukocyte antigen (HLA)-DR antigens, and CD5. These markers are not limited to expression on B cells and may be found on some monocytes, neutrophils, and T lymphocytes. In combination with other B-cell–restricted antigens, they describe a series of discrete stages in B-cell differentiation (Fig. 31.1). In addition, terminal deoxynucleotidyl transferase (TdT) has been found in early B lymphocytes by cytochemical staining techniques.

The role of the cell-surface markers on B cells in their normal physiology is undergoing gradual elucidation. An observation by Fingeroth et al. (18) and Frade et al. (19) implies that certain cell-surface structures may be used by pathogens to target infection, resulting in altered function. They observed that an antibody to the C3d complement receptor on B cells could block B-cell infection by Epstein-Barr virus (EBV). Similar strategies have been used by other viruses such as human immunodeficiency virus (HIV), which targets the CD4 receptor of mature helper T cells.

As depicted in Fig. 31.1 and coupled with the WHO lymphoma classification framework outlined in Table 31.1, most clonal B-cell neoplasms can be linked to at most a small number of discrete stages in B-cell development (16). One may find a degree of heterogeneity among the B-cell acute lymphoblastic leukemia/lymphomas (16,20); however, one does not find diffuse large B-cell non-Hodgkin's lymphomas with the surface phenotype of early B cells, or acute lymphoblastic leukemia cells with the cell-surface phenotype of secretory B cells.

ONTOGENY OF T LYMPHOCYTES

Like B cells, T cells arise from pluripotent marrow stem cells. Unlike B cells, they require a number of adaptations through cell interactions in the thymus, as indicated in Fig. 31.2, which illustrates a number of the steps in T-cell development. T cells have multiple functions, but they specialize, by and large, in the control of the broad spectrum of cell-mediated immunity (21,22). Marrow cells destined to become thymocytes (lymphoid stem cells) express CD71 (transferrin receptor) and CD38 antigens along with CD7. These primitive T-cell precursors account for ~10% of the total thymocyte population and are localized predominantly in the thymic cortex. With further maturation, they lose CD71, acquire CD1, and concurrently express CD4, CD8, and CD38. These cells are functionally immature, constitute 70% to 80% of the thymocyte population, and are nearly exclusively found in the cortex (21,22). The cells then move to the medulla, lose CD1, acquire CD3 (a nonpolymorphic determinant functionally linked to the T-cell antigen receptor), and lose either CD4 or CD8 expression but not both. Such CD3, CD4-positive and CD3, CD8-positive T cells are functionally mature and migrate from the thymus to the periphery. In general, cells expressing the CD4 antigen function as helper or inducer lymphocytes and recognize foreign antigens on cells bearing the class II major histocompatibility complex (MHC) antigens (HLA-DR antigens), and those expressing CD8 function as cytotoxic or suppressor cells and recognize foreign antigens on cells bearing class I MHC antigens (HLA-A, B, C). TdT is present in thymocytes but is not detectable in cells leaving the thymus.

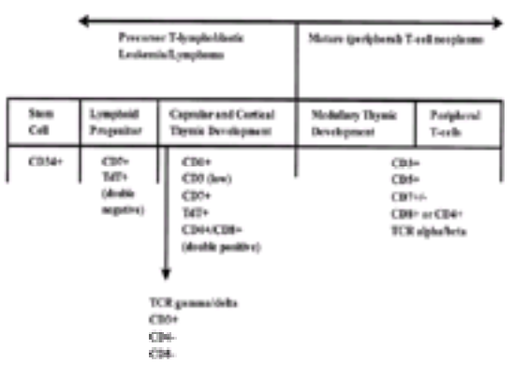

Figure 31.2. Normal T-cell development and lymphoid neoplasms. T-cell differentiation illustrating cells of origin of the T-cell lymphomas. Cell-surface and intracellular markers of T-cell differentiation are given for each discrete stage of development. TdT, terminal deoxynucleotidyl transferase; TCR, T-cell receptor with mature ab or gd receptor expressing T-cells.

Four genes encoding the T-cell antigen receptor have been identified: a, b, g, and d (23,24,25,26 and 27). The antigen receptor is composed of two glycoprotein chains, an ab (the most common) or gd pair, noncovalently associated with the CD3 complex. These paired chains are responsible for antigen recognition. T-cell receptor heterodimers are encoded by rearranged gene segments comprising V, D, J, and C segments, analogous to those encoding the antigen-binding variable domains of immunoglobulins (24,27). Three variable regions corresponding to complementarity-determining regions (CDRs 1–3) have been described, and the V, D, and J gene segments recombine in a developing T-cell to produce a contiguous V(D)J exon, which is spliced together with the C region at the level of RNA. Diversity is further increased by the addition of variable numbers of nucleotides at the V, D, J, and J-C junctions. T-cell receptor gene rearrangements, as with immunoglobulin gene rearrangements, can be used as the signature of a particular T-cell clone and its progeny.

Figure 31.2 depicts T-cell ontogeny and identifies discrete stages of development that correspond with current lymphoma classification (Table 31.1). In a proportion of malignancies of more mature phenotype, for example, mycosis fungoides and T-cell prolymphocytic leukemia (TCL-1 oncogene activation by juxtaposition with a T-cell receptor gene, $CD2^+$, $CD3^+$ and $CD4^+$ or $CD8^+$) and large granular lymphocytic leukemia ($CD3^+CD8^+$ T-cell or $CD56^+$ $CD3^-$ NK cell of origin), the malignant cells can retain a semblance of immune function that may contribute to the clinical picture (28,29,30,31,32 and 33).

In general, T-cell malignancies are considerably less common than B-cell malignancies. A noteworthy exception was found in Japan, where about two thirds of all lymphoproliferative disorders are of T-cell origin, primarily because of the prevalence of human T-cell lymphotropic virus type I (HTLV-I) in the Kagoshima prefecture at

the southern tip of Japan (34). However, given the epidemic increase in HTLV-I infection worldwide, frequency of this subset of T-cell malignancies may increase rapidly in affected populations over the next 10 years along with other HTLV-I–associated diseases (35).

HODGKIN'S DISEASE

First described in 1830 by Thomas Hodgkin (36), ~8,000 new cases are diagnosed every year in the United States with classic and lymphocyte-predominant Hodgkin's disease. Unlike the non-Hodgkin's lymphomas, the incidence of Hodgkin's disease has remained relatively constant over the last 20 years at 2.9 per 100,000 population. The etiology of Hodgkin's disease remains largely unknown, although epidemiologic and clinical features of classic Hodgkin's disease suggested an infectious etiology, with the EBV among others being a potential candidate (37,38,39 and 40). The disease is nearly 100-fold more common in identical twins of affected individuals than in the general population, implying some genetic contribution. There are no strong predisposing factors, although the disease appears to occur with increased incidence in woodworkers, persons who have had tonsillectomy and appendectomy, and those with certain HLA genotypes. In children, it appears that the incidence of Hodgkin's disease is increased in groups with a higher level of education (39,41). Patients with Hodgkin's disease have cellular immune defects (42), at least some of which may precede the development of the disease (43). However, the relation of this finding to disease development is unclear.

Pathology

The malignant cell in Hodgkin's disease is the Reed-Sternberg cell (44,45). It is a large cell with two or more nuclei, each of which has a single nucleolus (thus the “owl's eyes” appearance). Unlike most malignancies, this characteristic malignant cell represents only a small fraction of the cells that compose the masses palpated by the physician. Lymph nodes involved with Hodgkin's disease contain predominantly normal lymphocytes, plasma cells, eosinophils, and fibrous stroma, with only a small percentage of the cells being the malignant Reed-Sternberg cell. Although Reed-Sternberg cells must be seen to make the diagnosis of Hodgkin's disease, their presence is not pathognomonic (44,46). They also may be seen in occasional patients with infectious mononucleosis and breast cancer.

In the current WHO classification of Hodgkin's lymphoma (Table 31.1) (4), two major subtypes of Hodgkin's disease are recognized: nodular lymphocyte predominant and classic Hodgkin's disease are considered distinct entities. In both major groups, the malignant cell has been shown to be a B cell. In classic Hodgkin's disease, the direct precursor cell may be the germinal center B cell with markers including CD30 and 40 with $CD20^{+/-}$. In the lymphocyte-predominant form of Hodgkin's disease, the malignant cells express CD19, CD20, and CD22 (46,47 and 48). The Reed-Sternberg cell variants seen in this entity also are called popcorn cells because of the nuclear contour (46). Within classic Hodgkin's disease, four subtypes are currently recognized: nodular sclerosis, lymphocyte-rich classic Hodgkin's disease, mixed cellularity Hodgkin's disease, and lymphocyte depletion Hodgkin's disease (Table 31.1). In general, the natural history of lymphocyte-predominant Hodgkin's disease is more indolent than that of classic Hodgkin's. However, with ongoing advances in treatment, it is not clear that histologic type is an important prognostic factor (49,50).

Clinical Features

Hodgkin's disease is predominantly a disease of lymph nodes with direct contiguous spread from one lymph node group to another (51,52). When only one site is involved, overwhelmingly it is the left supraclavicular nodes (53). Most patients have an asymptomatic swelling in the neck. Approximately 10% of patients first notice inguinal or axillary adenopathy. The adenopathy is most often painless, unless the growth of the node is rapid. Up to 20% of patients notice pain in the enlarged lymph node on ingesting alcoholic beverages, perhaps because of alcohol-induced eosinophil degranulation. Constitutional symptoms of weight loss (10% of body weight), fevers, and night sweats occur in ~40% of patients. A classic clinical sign in Hodgkin's disease is the Pel-Ebstein fever, cyclic bouts of high fever, each lasting a week or two, separated by afebrile periods of similar duration. When fever occurs, it usually peaks in the evening and falls precipitously in the early morning hours, resulting in drenching night sweats. However, continuous fever is not infrequent. These symptoms, called B symptoms, are more common in patients with advanced stages of disease. Pruritus also is a common complaint of patients with Hodgkin's disease, but it is not a B symptom.

As noted, the spread of Hodgkin's disease is to contiguous lymph node–bearing groups (52). Thus when the left cervical region is involved, the abdominal nodes are rarely involved without concomitant mediastinal disease. Another predictable feature of the disease is that the liver is never involved unless the spleen also is involved. Within the abdominal cavity, the spleen is the most common site of disease. Thus pattern of spread is predictable, and in general, the prognosis is related to the number of sites of disease. Because of the need to standardize the therapeutic approach to patients with Hodgkin's disease, the patterns of spread of the disease were used to formulate the staging classification shown in Table 31.2 (54). This staging system, known as the Ann Arbor classification, has been the basis for treatment decisions for more than 30 years. The increased use of computed tomography (CT) and a greater appreciation of the effect of tumor burden on the outcome of Hodgkin's disease have led to minor revisions of this system in the form of the Cotswolds modifications (55). The revised staging classification recommends that CT be included as the procedure for evaluating intrathoracic and infradiaphragmatic lymph nodes, that the criteria for clinical involvement of the spleen and liver be modified to include focal defects, that liver-function abnormalities be ignored, and that the suffix “X” be used to designate bulky disease. In addition, stage III is subdivided to reflect upper versus lower abdominal lymph node involvement, and patients with residual radiographic abnormalities are given the designation CR_u for unconfirmed complete response, although the value of this latter change is questionable (56,57). Treatment is based on stage of disease. Thus once a histologic diagnosis is made, the stage of disease must be assessed.

Stage	Criteria
I	Involvement of a single lymph node region, or a single structure such as the spleen
II	Involvement of two or more lymph node regions or structures on the same side of the diaphragm
III	Involvement of lymph node regions or structures on both sides of the diaphragm with subdivisions including III₁ Involvement limited to spleen, splenic hilar nodes, celiac nodes and portal nodes III₂ Involvement including paraaortic, mesenteric, and iliac nodes in addition to upper abdominal nodes
IV	Extranodal involvement beyond E including any involvement of liver or bone marrow
Staging Modifiers	
A	Absence of constitutional symptoms of fevers, night sweats, or weight loss
B	Unexplained fever (>38°C), night sweats, or loss of 10% of body weight within 6 mo
X	Bulky disease (nodal mass >10 cm in diameter or a mediastinal mass >1/3 the transverse chest diameter at T_5–T_6)
E	Localized solitary extralymphatic tissue involvement (excluding bone marrow and liver) or extralymphatic direct extension from an involved nodal site or a single discrete geminal site to a regional lymph node site (e.g., IE)

[a] Modified Ann Arbor staging system for Hodgkin's disease.

TABLE 31.2. Staging Classification for Hodgkin's Disease[a]

A number of nonspecific laboratory abnormalities are found in patients with Hodgkin's disease, including an elevated white blood cell count, an elevated erythrocyte sedimentation rate due to an increased fibrinogen level, and elevation of a number of acute-phase reactants including ceruloplasmin (58). However, the most important information for staging patients comes from the sequential use of physical examination, chest roentgenography, CT scanning, lymphangiography, and in selected cases, exploratory laparotomy with splenectomy and multiple lymph node sampling in addition to liver and bone marrow biopsies. CT scans can be thought of as complementary to lymphangiograms, although use of lymphangiography has steadily decreased. Lymphangiography is more sensitive than CT in detecting retroperitoneal adenopathy because it can detect nodes with distorted architecture as well as enlarged nodes. Conversely, CT can visualize celiac, splenic, portal, and mesenteric nodes, which are not readily visualized with lymphangiography (59,60). Thoracic CT is frequently uninformative if plain chest radiographs appear normal (61).

Ninety percent of patients with Hodgkin's disease have clinical stage I or II disease after physical examination and chest radiography. The rest have advanced stages of disease. One third of the patients with early-stage disease are found to have more advanced disease after lymphangiography. Among the 60% of patients still thought to have clinical early-stage disease after lymphangiography, one third are found to have advanced disease on exploratory laparotomy. The probability of finding intraabdominal disease at laparotomy is 50% for patients with B symptoms and 25% for those without B symptoms. When sequential staging evaluation was performed on patients with Hodgkin's disease, 40% had early-stage, and 60% had advanced-stage disease. One of the most difficult areas in the staging of patients with Hodgkin's disease relates to the need to evaluate intraabdominal disease in patients with supradiaphragmatic adenopathy and negative findings on a lymphangiogram. However, this is becoming less critical to outcome, as lower-dose combined modality therapy has come into increasingly frequent use in patients with early-stage Hodgkin's disease.

It is discouraging that noninvasive measures are so insensitive in evaluating involvement of the spleen, the most common intraabdominal site for Hodgkin's disease. Because the spleen has no afferent lymphatics, spleen involvement implies hematogenous spread of disease. Only about two thirds of palpably enlarged spleens are histologically involved with Hodgkin's disease, and one third of normal-size spleens contain Hodgkin's disease, yet removal of the spleen is associated with an increased risk of infection.

The need for staging laparotomy is based on the desire to use radiation therapy alone as the form of treatment. If chemotherapy is used either alone or as a component of treatment, the need to pathologically define intraabdominal disease is removed. It was hoped that one could pick patients with an exceedingly low risk of

having intraabdominal disease on the basis of clinical features. One retrospective study identified women with clinical stage I disease, women younger than 27 years with only two or three sites of disease, clinical stage I patients with only mediastinal disease, and clinical stage I men with either lymphocyte predominance or follicular histologic forms of Hodgkin's disease as patients with a low risk of intraabdominal involvement (62). However, when these criteria were used to treat patients with radiation therapy alone without surgical staging, disease-free survival was compromised. Thus shortcuts in staging are not recommended if radiation therapy alone is to be used as the treatment. However, patients with stage IA disease above the level of the clavicles are at very low risk of having disease below the diaphragm (63). Radiation therapy alone can be safely applied to these patients without compromising treatment outcome.

Immunologic Features

As recently reviewed by Stein and Hummel (48,64) as well as by Chan (47), the cellular origin of the malignant cell in classic Hodgkin's disease and lymphocyte-predominant Hodgkin's disease has been shown with increasing consistency to be of B-cell origin. Reed-Sternberg cells in classic Hodgkin's disease frequently express B-cell markers that include CD30, CD40, and in some cases, CD20. When CD20 is expressed, the Reed-Sternberg cells contain clonally rearranged immunoglobulin genes, clearly placing them in the B-cell lineage depicted in Fig. 31.1. The popcorn Reed-Sternberg cells in lymphocyte-predominant Hodgkin's disease are more clearly B cell in origin, expressing CD20 uniformly but also expressing the epithelial membrane antigen (EMA), which is not known to be expressed at any stage in normal B-cell development.

Although monoclonal antibody panels have failed to prove a single cell type of origin in all cases of Hodgkin's disease, it is now possible in nearly all cases to use certain antibodies to distinguish Hodgkin's disease from other nonneoplastic sources of adenopathy (65,66 and 67). Hodgkin's disease cells nearly always express CD30 (Ki-1, Hefi-1; originally made against the L428 cell line) (68,69) and CD15.

Several investigators also have examined the spectrum of cytokines released by Hodgkin's disease–derived cell lines (70,71). Indeed, the occurrence of B symptoms, sclerosis, and eosinophilia may be characteristic of a tumor containing cytokine-producing cells. Interleukin 6 (IL-6) (72) and IL-9 (73) secretion has been demonstrated in both cultured and primary Hodgkin's tissues. In addition, the transcripts and corresponding proteins for granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1a (but not -b), IL-3, IL-5, IL-6, IL-8, tumor necrosis factor (TNF)-a and -b, and transforming growth factor (TGF)-b have been detected in cytoplasmic RNA and culture supernatant obtained from two Hodgkin's disease–derived cell lines (71,74).

It has been known since Dorothy Reed's initial observation in 1902 that the immune system in patients with Hodgkin's disease is not normal (45). She found that patients with Hodgkin's disease were anergic to tuberculin on skin testing. Many studies that attempted to shed light on the meaning and mechanism of the immune defects have now been reported. These are mentioned here but are also summarized in greater depth in the review by Poppema et al. (42). Hodgkin's disease patients have poor delayed-type hypersensitivity responses to new and recall antigens (75). Lymphocyte proliferative responses to mitogens and auto-MHC and allo-MHC antigens are depressed (76,77). Some evidence from a study of twins suggests that the immune defect may precede the development of Hodgkin's disease (43). The degree of decreased T-cell response loosely parallels the stage of disease. Impaired responsiveness of T cells to IL-2 has been observed, and T cells from Hodgkin's disease patients appear to be defective in their production of IL-2 in response to mitogenic stimuli as well (78,79). It is interesting that these defects persist after the successful treatment of Hodgkin's disease (80,81). It is unclear if this impaired T-cell function is related to the pathogenesis of Hodgkin's disease, although a number of investigators have proposed hypotheses, most of which are not testable. However, despite a growing literature on immune defects in Hodgkin's disease, for the vast majority of patients, the T-cell defects in Hodgkin's disease appear to have no permanent or serious sequelae.

Treatment

Although the treatment of choice for patients with true early-stage Hodgkin's disease (stages I and II) was radiation therapy, which results in long-term disease-free survival in £80% of patients (82), increasing knowledge of late effects from radiation (83) and a variety of effective alternative approaches have lead to the current situation; there is no single standard approach to treatment in stage I and II patients (49). If radiation therapy is used as a single-modality approach to early Hodgkin's disease, then precise radiation techniques must be used to obtain optimal results. These techniques include pretreatment simulation, the use of megavoltage photon beams, the contouring of large radiation fields to the patient's anatomy with shielding of the heart and lungs, the administration of a tumoricidal dose in equal amounts from anterior and posterior ports, and verification that the diseased area is encompassed within the field (84). Most of these patients will require staging laparotomy with its attendant acute surgical risks (15% serious morbidity) and life-long increased risk of bacterial infection, particularly from pneumococci.

Studies have increasingly shown combination chemotherapy to yield at least equivalent outcomes in early-stage Hodgkin's disease. This, in addition to providing curative therapy, obviates the need for staging laparotomy and splenectomy. Radiation therapy alone is reserved with increasing frequency to patients with supraclavicular stage IA Hodgkin's disease only. An early study from the National Cancer Institute (63) compared chemotherapy directly using MOPP (nitrogen mustard, vincristine, procarbazine, and prednisone) versus radiation therapy in stage I to IIA Hodgkin's disease. Disease-free survival from this trial favored chemotherapy alone, with a projected 92% 10-year survival compared with 76% for radiation therapy alone. Currently this, and other regimens such as ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) (85,86), have been increasingly incorporated either as single-modality therapy in stage I and II Hodgkin's disease or combined with radiation therapy in cases with either massive mediastinal disease or persistent disease after completion of chemotherapy (86,87). ABVD in particular is an attractive regimen in early-stage Hodgkin's disease because of a lower incidence of gonadal damage (88), although pulmonary toxicity from bleomycin remains a significant problem with or without the combination of radiation therapy to the chest as a part of treatment. Overall, the increased long-term risks associated with combined-modality therapy are substantial, and generally, the advantage of combined-modality therapy in disease-free survival is mitigated by the increased late toxicity of this approach, with the exception of selected patients. As such, treatments combining limited chemotherapy with lower-dose radiation therapy should be considered with caution as a routine treatment approach until long-term follow-up is available documenting a reduced rate of late fatal complications.

The mediastinum is involved in ~70% of patients with Hodgkin's disease, and massively involved in ~25%. Residual mediastinal abnormalities persist in the majority of patients with massive mediastinal disease after treatment; the larger the original mass, the higher the likelihood of abnormality (56,89). Such persistent radiologic abnormalities do not necessarily represent persistent Hodgkin's disease. Gallium and positron emission tomography (PET) scans should show no uptake at the completion of therapy. Radiographic abnormalities that are stable and gallium or PET negative after radiotherapy or chemotherapy should be managed conservatively. An enlarging mass in the anterior mediastinum during the first 6 months after treatment can represent a regenerating thymus and pose a diagnostic problem (90). Gallium and PET scanning cannot distinguish recurrent Hodgkin's disease from regenerating thymus. Needle biopsy is necessary to make the diagnosis; the presence of CD4/CD8 double-positive thymocytes diagnoses normal thymus.

Combination chemotherapy alone is the treatment of choice for patients with advanced stage IIIA to IVB Hodgkin's disease. Any number of regimens appear to be roughly equivalent in their efficacy, inducing complete responses in 70% to 90% of patients with advanced-stage disease. These regimens ultimately result in cures in 65% to 75% of all patients with advanced-stage disease. The question of whether alternating or hybrid eight-drug regimens are superior to standard four-drug regimens remains controversial. Studies addressing this question have yielded conflicting results, but in general, rates of complete remission and relapse, as well as disease-free and overall survival rates are similar for the four- and eight-drug regimens. Currently, ABVD remains the most frequently chosen standard for treatment of advanced Hodgkin's disease, with some data to support its superiority over MOPP in treatment outcome (91) and in late complications from therapy (92).

Recent efforts to improve treatment outcomes have involved the development of new combination chemotherapy programs. Stanford V, a regimen using short-course chemotherapy with radiation to previous sites of disease, has completed a pilot trial indicating potential promise (93) and is currently undergoing testing against ABVD in Intergroup trials in advanced-stage Hodgkin's disease. Another regimen, BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone) also has completed trials in Germany and shows great promise in direct comparison with ABVD (94). Autologous bone marrow transplant and, in selected circumstances, allogeneic transplant also is a promising option in patients with either refractory or relapsed Hodgkin's disease (95,96 and 97), although it is important to note that patients relapsing after a long remission (more than 12 months) often retain chemotherapy sensitivity to standard regimens, although remission duration is considerably shortened.

NON-HODGKIN'S LYMPHOMAS

The non-Hodgkin's lymphomas are a heterogeneous group of diseases ranging from aggressive and rapidly fatal tumors to slow-growing indolent diseases, some of which may need essentially no therapy for many years (20). Although the etiology is unknown and can only be partly accounted for by the increased average age of the population in addition to occupational and environmental risk factors (98,99), the incidence of this group of neoplasms has continued to increase at a rate approaching 5% per year for the last 20 years. These lymphomas are neoplasms of the immune system (Fig. 31.1 and Fig. 31.2 along with Table 31.1). A wealth of information continues to evolve from clinical observation, immunologic phenotyping, cytogenetic analysis, and the application of recombinant DNA technology, leading to improved understanding of this group of diseases and their classification, behavior, and treatment.

Despite progress, the etiology is known in fewer than 1% of the 50,000 new cases of lymphoma diagnosed every year. For example, adult T-cell leukemia (ATL), endemic to Japan and the Caribbean and sporadically seen in the United States, is caused by HTLV-I (100,101,102,103 and 104). Many primary lymphomas of the central nervous system (CNS), aggressive lymphomas accompanying immunosuppressive therapy, and African Burkitt's lymphoma are likely caused by the EBV (105,106,107 and 108). A number of other conditions have been demonstrated to predispose individuals to lymphoproliferative disorders (Table 31.3). Most of these

conditions are associated with some form of immunodeficiency or hyperreactivity as a common denominator.

Immunosuppressive therapy to prevent heart or kidney transplant rejection
Wiscott-Aldrich syndrome
Ataxia-telangiectasia syndrome
Klinefelter's syndrome
Lymphomatoid granulomatosis
Chediak-Higashi syndrome
Common variable immunodeficiency disease
Sjogren's syndrome
Rheumatoid arthritis
Systemic lupus erythematosus
Acquired immunodeficiency syndrome
Acquired hypogammaglobulinemia
Swiss-type agammaglobulinemia
Exposure to ionizing radiation
Prolonged phenytoin (Dilantin) therapy

TABLE 31.3. Conditions Predisposing Patients To Non-Hodgkin's Lymphoma

EBV B-cell transformation may be a partial explanation for the lymphomas arising in immunodeficient patients, but there is little evidence suggesting that similar mechanisms can account for most of the lymphomas that occur in patients without apparent predisposing illnesses. It has been said that chronic antigenic stimulation may play a role in the development of lymphomas, on the basis of animal models demonstrating an increased incidence of lymphomas in graft-versus-host disease. Much of the neoplasia in these models appears to be related to the activation of endogenous retroviruses. However, chronic antigenic stimulation is difficult to document in most human lymphoproliferative diseases outside of the marginal zone subtypes.

Cytogenetic abnormalities are common in the non-Hodgkin's lymphomas (Table 31.4). Although the modal chromosome number is near diploid in most cases, banding techniques have detected a variety of structural rearrangements, inversions, and deletions, which have been reviewed extensively (109,110). For the vast majority of rearrangements, the antigen-receptor genes are one component of the translocation, and a normally silent oncogene is the other partner. The translocation thus activates expression of a normally silent transforming gene, and it appears that this occurs at an early developmental stage when the antigen-receptor genes are normally rearranged. It is increasingly clear that these acquired translocations play a critical role in lymphomagenesis, altering genes critical in programmed cell death as well as transcriptional control, control of the cell cycle, and development (110). The single most common abnormality involves a translocation of the bcl-2 gene in the t(14;18)(q32;q21). This translocation is seen in ~85% of patients with follicular lymphoma (111), in £30% of patients with diffuse large B-cell lymphoma, and at lower frequencies in other lymphomas as well. Variant translocations involving bcl-2 between chromosomes 2 and 22 with 18 also are seen (112).

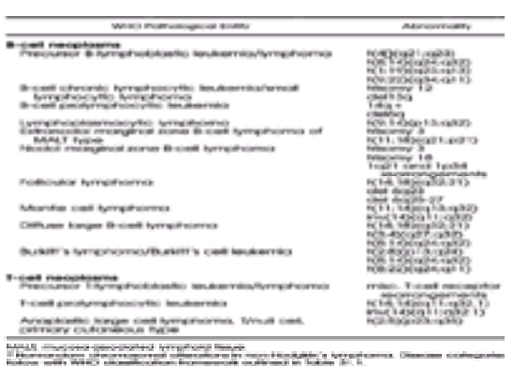

TABLE 31.4. Nonrandom Chromosomal Abnormalities in Non-Hodgkin's Lymphoma and Lymphoid Leukemias[a]

The translocations found in Burkitt's lymphoma are instructive; the distal segment of the long arm of chromosome 8 translocates to chromosome 14 (113,114) in 75% of the cases and to either chromosome 2 (115) or 22 (116) in the remaining 25%. These chromosomes (14, 2, and 22) contain the loci for immunoglobulin heavy chains and k or l light chains, respectively. These translocations are of particular interest because of the apparent integral role played by the cellular oncogene c-*myc* on chromosome 8 (117,118). In Burkitt's lymphoma cell lines with the t(8:14) translocation, the c-*myc*oncogene located on band q24 of chromosome 8 is translocated to the heavy-chain locus on chromosome 14. In the variant translocations t(8:22) and t(2:8), the c-*myc*oncogene stays on chromosome 8, and gene sequences from either the k or l chain loci translocate to 8. These rearrangements can result in the altered transcription of c-*myc* so that it is expressed constitutively at high levels in contrast to the transcriptionally silent normal c-*myc* oncogene on the uninvolved chromosome 8 (119). These translocations also are associated with mutations in conserved coding sequences (120).

Pathology

Between 1832, when Thomas Hodgkin (36) first reported seven patients with tumors of the “absorbent glands,” and 1956, when Henry Rappaport (121) devised his classification of the malignant lymphomas, the diagnosis of lymphoma was difficult, and many disease entities were not classifiable. However, with the development and popularization of the Rappaport scheme, an organized approach to patients with lymphoma became possible. The Rappaport classification grouped the lymphomas based on their pattern of growth (nodular or diffuse) and the degree of differentiation of the predominant malignant cell (well-differentiated lymphocytic, poorly differentiated lymphocytic, histiocytic, or mixed). This classification had several salutary features. It was more reproducible than the previous scheme (122), it was easily learned, and it provided clinicians with prognostic information on the likely clinical course of the patient (123).

In general, patients with diffuse well-differentiated lymphocytic (DWDL), nodular poorly differentiated lymphocytic (NPDL), or nodular mixed lymphocytic/histiocytic (NML) lymphomas followed an indolent course. Those with nodular histiocytic (NHL), diffuse mixed (DML), or diffuse histiocytic (DHL) lymphomas tended to have an aggressive clinical course (123). Clinicians found that they could plan a therapeutic strategy based on the histologic diagnosis. However, the Rappaport scheme was flawed; it did not encompass certain rare cases. The DHL group in particular was heterogeneous and the term histiocytic was in most cases a misnomer (“histiocytes” are usually transformed lymphocytes). With this, a bewildering variety of classifications was proposed in an attempt to improve on the perceived deficiencies.

The continuing controversy over these classification schemes and the difficulty of comparing treatment results from trials using diverse terminology led to the large-scale lymphoma-classification project sponsored by the National Cancer Institute (NCI) (124) and development of a “Working Formulation.” However, ongoing use of the Kiel classification scheme in Europe (125), originally proposed by Lennert et al., continued to lead to confusion in comparison of studies across national boundaries. A subsequent international collaboration resulted first in the Revised European-American Lymphoma Classification scheme (6), and with ongoing work, adaptation of a modified version of this scheme into the recently published WHO classification of hematopoietic and lymphoid tissue malignancies that is shown in Table 31.1 (4,5). This classification amalgamates morphologic, immunologic, genetic, and clinical information to define discrete clinical entities. Based on our growing knowledge of normal B-cell development and the genetics of transformation in lymphoid malignancies, this classification method should lead to increasingly effective and consistent classification of the non-Hodgkin's lymphomas in both retrospective analysis of previous trials and prospective analysis of ongoing clinical treatment trials. It also will need to be gradually modified over time as knowledge based on increasingly sophisticated molecular techniques such as adaptation of microarray technology to analysis of global expression patterns reveals further information permitting subgrouping of lymphomas in clinically and pathologically relevant fashions. This has been demonstrated in the diffuse large B-cell lymphomas (126) and will, without question, be applied to other subgroups of non-Hodgkin's lymphomas in the next several years.

General Clinical Features

Unlike Hodgkin's disease, the clinical patterns of the non-Hodgkin's lymphomas are heterogeneous. The more indolent lymphomas are virtually always associated with asymptomatic lymphadenopathy. Nearly half of patients with indolent lymphoma have liver and bone marrow involvement, and more than 85% of patients have advanced stages of disease. B symptoms are unusual in patients with indolent histologic subtypes. The adenopathy can wax and wane without treatment. The natural history of the more indolent of the non-Hodgkin's lymphomas is quite variable, but in general, the median survival time is 8 to 10 years (127,128). It appears that the natural history becomes progressively shorter as the large-cell component of the follicular lymphoma increases. In addition, the more indolent small cell lymphomas

have a tendency over time to undergo histologic progression from a follicular to a diffuse pattern of growth, and from small cleaved cells to large cells as the predominant malignant cell (129). The actuarial risk appears to be 5% to 10% per year and is not increased by administering therapy during the follicular stage. This tendency to progression is so marked that the overwhelming majority (94%) of patients with indolent lymphoma who die with lymphoma have converted to aggressive histologic subtypes such as diffuse large B-cell lymphoma (130). This histologic progression from indolent to aggressive histology is nearly always a morphologic change in the same malignant B-cell clone (131,132 and 133), although exceptions to this have been noted (133,134,135 and 136). The altered morphology reflects accumulation of genetic damage in genes that affect cell growth (137). Histologic transformation is always accompanied by a change in the natural history of the disease from an indolent to more aggressive behavior (129).

The aggressive subgroups of the non-Hodgkin's lymphomas have a much higher propensity to originate in or to involve extranodal sites such as the bowel, skin, brain, and testes than do the indolent lymphomas. The most common aggressive lymphoma is the diffuse large B-cell lymphoma. The aggressive lymphomas are more likely to produce B symptoms (Table 31.2) and are considered aggressive in natural history because the median survival of such patients untreated is ~8 months (138). The aggressive lymphomas are prone to widespread dissemination and are more likely to involve Waldeyer's ring and epitrochlear nodes than Hodgkin's disease or the indolent lymphomas (139). Liver involvement in more aggressive non-Hodgkin's lymphomas is rare. Despite this, there is a subgroup of patients that present with liver involvement as their primary site of disease in the absence of significant lymphadenopathy (140). Perhaps because of their rapid growth pattern, the aggressive subgroups have been overall more responsive to combination chemotherapy treatment programs than the clinically indolent lymphomas.

Because of the diversity of behavior in individual patients, it is difficult to distinguish the various types of non-Hodgkin's lymphoma on clinical grounds alone. The diagnosis relies on the pathologist's interpretation of biopsy material and, with increasing frequency, the immunologic and genetic characteristics of the lymphoma. The optimal management of individual patients is determined by careful diagnostic studies and assessing the extent of disease by the staging evaluation that is used for patients with Hodgkin's disease with certain modifications. Because only a minority (20%) of patients with non-Hodgkin's lymphomas have localized disease, there are fewer therapeutic implications to the staging of patients. Virtually all patients need treatment with chemotherapy at some point and the major value of the staging evaluation is to define the sites that must be reexamined after therapy to document complete response. Staging laparotomy as performed previously in Hodgkin's disease patients has no role in the routine management of patients with non-Hodgkin's lymphomas; however, because many patients have abdominal signs and symptoms, diagnostic surgical procedures are fairly common. Abdominal and chest CT, in some instances lymphangiography, and bilateral bone marrow biopsies and aspirations are routinely performed. Beyond that, specialized tests are used to pursue an individual patient's symptoms.

Although the Ann Arbor staging system (Table 31.2) is used in most subtypes of non-Hodgkin's lymphomas, it applies poorly to these lymphomas. The difference is largely because Hodgkin's disease spreads contiguously until late in its course, whereas non-Hodgkin's lymphomas disseminate hematogenously early in their course. In fact, with the sensitivity of the fluorescence-activated cell sorter, it has been clear for many years that most patients with non-Hodgkin's lymphomas of all stages have circulating malignant lymphoma cells (141,142,143 and 144). Nevertheless, there are clinical predictors of prognosis. In the clinically indolent lymphomas, B symptoms, advanced age, gender, and tumor bulk are generally considered to have a significant impact on survival (145,146,147,148 and 149). Surrogate measures of tumor bulk such as number of sites of disease, degree of marrow involvement, and lymph node size also are found to predict survival in some series. Other potentially useful staging/prognostic testing may include the serum b_2-microglobulin level, which was reported by investigators at the M.D. Anderson Cancer Center to be the single best prognostic factor and was found to be independent of Ann Arbor stage (150). Ascertainment of TNF-a, and its receptor levels may also provide prognostic information (151).

For the clinically aggressive lymphomas, advanced stage, B symptoms, large gastrointestinal or mediastinal masses, bone marrow involvement, liver involvement, and elevated lactic acid dehydrogenase or b_2-microglobulin levels in the serum are particularly poor prognostic signs (139,152,153,154 and 155). Poor performance status negatively affects the outcome of all lymphomas. Many of these risk factors have been combined into the now standard International Prognostic Index (156). This incorporates features listed in Table 31.5 into a simple scoring system, which divides patients into relevant prognostic groups based on a summed score of one for each category present in a given patient. However, the relevance of this to patients with follicular lymphoma remains somewhat controversial (157,158 and 159), despite application by many research centers.

Factor	Adverse Prognosis
Age	>60
Ann Arbor stage	III or IV
Serum LDH	> Normal
Number of extranodal sites of involvement	≥2
Performance status	>ECOG 2 or equivalent

LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group.
From The International Non-Hodgkin's Lymphoma Prognostic Factors Project. A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med 1993;329:987–994.

TABLE 31.5. International Prognostic Index for Non-Hodgkin's Lymphoma

In the individual patient, the prognostic index must be combined with information regarding the WHO subtype of lymphoma present to then guide treatment recommendations based on the composite assessment of prognosis.

Subtype-specific Clinical Features and Immunology

Immunologic phenotyping of the lymphomas has in large measure driven our increasing understanding of the heterogeneity of the non-Hodgkin's lymphomas that was not originally predicted by morphologic studies (Fig. 31.1 and Fig. 31.2). The WHO classification framework has become the new international standard for lymphoma subtyping (4,5). Given the variable clinical and immunologic behavior of individual lymphoma variants within the WHO classification of non-Hodgkin's lymphomas (Table 31.1), it is best to consider each subtype separately to more accurately assess the spectrum of immunologic findings and clinical behavior expected within a given subclass. According to the WHO classification, lymphoid malignancies are divided into B-cell disorders, T-cell disorders, and Hodgkin's disease, which has already been discussed. Individual entities within the non-Hodgkin's lymphomas are considered next.

PRECURSOR B-LYMPHOBLASTIC LEUKEMIA/LYMPHOMA (PRECURSOR B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA)

Seventy-five percent of the cases of acute lymphoblastic leukemia occur in children younger than 16 years, and the remaining 25% are roughly evenly distributed throughout the remaining age groups. Eighty percent of childhood leukemias but only 20% of adult leukemias are lymphoblastic in origin (160,161). Of the lymphoblastic lymphomas, only 20% are of B-cell origin. Children with acute leukemia often have the symptoms and signs of bone marrow failure, fever, pallor, petechiae, and easy bruising; adults usually have nonspecific symptoms of malaise, lethargy, fatigue, anorexia, and weight loss (162,163). Hepatosplenomegaly and lymphadenopathy are seen in most adults and children. Tumor cells characteristically express TdT, CD19, CD22, and CD79a and are HLA-DR^+ while not expressing surface or cytoplasmic immunoglobulin (164,165,166,167,168 and 169). Of these, CD79a is the most reliable marker to differentiate the lymphoblastic B- versus T-cell origin of the malignancy. Cytogenetic abnormalities are variable, with presence of the Philadelphia translocation t(9;22) and t(12;22) being of clear prognostic significance (170,171,172,173,174,175 and 176). Dose-intensive therapy offers a high rate of cure, approaching 80% in some studies (163,177).

B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA/SMALL LYMPHOCYTE LYMPHOMA

This malignancy derives from a mature B-cell population (Fig. 31.1) expressing CD5 and usually CD23, with weaker expression of CD20 and CD22 along with surface immunoglobulin. In patients with more than 4,000 circulating malignant cells per µL, the diagnosis is chronic lymphocytic leukemia. In patients with lymphadenopathy and less than 4,000 circulating malignant cells per µL, the diagnosis is small lymphocytic lymphoma. Genetically, trisomy 12 is frequently seen (110). Clinically, the disease increases with age and accounts for 6.7% of cases based on the non-Hodgkin's Lymphoma Classification Project analysis (178,179). Immunologically, the disease is associated with hypogammaglobulinemia. Patients frequently are first seen with or have complications related to infection. Overall, the disease is characterized by an indolent course, with gradual progression over many years depending on stage, and may be associated with autoimmune phenomena including hemolytic anemia and thrombocytopenia (180,181). Although the malignant cells express CD5, about half of the cases express mutated immunoglobulin genes, suggesting that the cells had passed through a lymphoid follicle. Cases with mutated immunoglobulin genes have a more indolent disease than do cases with unmutated immunoglobulin genes (182,183). Staging follows one of two systems in widespread use, that of Rai or the Binet system (184,185). Both are based on assessing of the amount of marrow compromise. No therapy appears curative for patients with systemic disease although the very small subset of patients with localized small lymphocytic lymphoma may be cured with radiotherapy alone. Systemic treatment often is withheld until symptoms demand treatment; alkylating agents

with or without glucocorticoids or single-agent fludarabine are the most widely used agents. Treatment in early stages does not influence survival. Interestingly, ~5% of patients will undergo transformation of their lymphoma/chronic leukemia to a more aggressive phenotype termed Richter syndrome, in which the immunoglobulin gene rearrangements may be identical to or divergent from that of the original indolent leukemia/lymphoma (135,186).

B-CELL PROLYMPHOCYTIC LEUKEMIA

Occasionally, patients with high white blood cell counts have a somewhat larger cell seen on peripheral smear compared with those in chronic lymphocytic leukemia (187). Whereas surface marker studies are similar to that of chronic lymphocytic leukemia, only half of patients will have tumors expressing CD5 and more often express CD22 and generally have strong rather than weak surface immunoglobulin expression (188). Genetically, $14q^{+}$ and $6q^{-}$ defects along with other chromosomal abnormalities and abnormalities in p53 are frequently seen (189,190 and 191). Overall, the clinical course is shorter, with therapy less effective than in chronic lymphocytic leukemia; median survival is only 3 years.

LYMPHOPLASMACYTIC LYMPHOMA

Lymphoplasmacytic lymphoma occurs infrequently, representing only 1% on non-Hodgkin's lymphoma (179). Patients often have the clinical syndrome of Waldenström macroglobulinemia (192,193). Although no consistent cytogenetic abnormality is seen, a t(9;14)(p11;q32) translocation in seen in a subset of patients though t(11;18)(q21;q21) translocations are seen as well (194). The immunophenotype of cells on lymph node or bone marrow biopsy reveals B cells that are CD20 and cytoplasmic immunoglobulin positive and do not express CD3, CD5, CD10, or CD23. Patients have variable symptoms, the most consistent of which are related to macroglobulinemia with hyperviscosity from the monoclonal IgM protein present in the circulation. The course is clinically indolent, with a variety of therapies showing efficacy in the absence of apparent cure, with median survival of 65 months (193,195).

SPLENIC MARGINAL ZONE B-CELL LYMPHOMA (±VILLOUS LYMPHOCYTES)

B-cell lymphomas of this subgroup may have their origin in the marginal zone of B-cell development outside the follicular mantle zone. Cells express surface immunoglobulin (IgM and IgD) along with CD19, CD20, and CD79a (Fig. 31.1) (196,197 and 198). Cells are typically negative for CD5, CD10, and CD23 expression. Chromosome abnormalities are seen involving chromosomes 1, 3, 7, and 8, with complex karyotypes present (199,200). A subset may have specific rearrangements of the *bcl*-1 locus (201). Among the non-Hodgkin's lymphomas, this is a rare variant accounting for only 0.8% of all lymphoma cases (179). Clinically, patients usually have splenomegaly in the absence of lymphadenopathy and may have circulating cells that must be distinguished from other B-cell leukemias with splenomegaly (202,203). Overall the clinical course appears to be indolent (200), although splenectomy is frequently required for either treatment or diagnosis.

HAIRY CELL LEUKEMIA

This is a rare clinical/pathologic malignancy of B-cells that produces splenomegaly and pancytopenia. It typically responds to treatment with 2-chlorodeoxyadenosine with long-term hematologic remission in the majority of patients (204,205 and 206). Morphologically, cells have a characteristic fuzzy or “hairy” cell-surface appearance, leading to the name (207). The malignant cells express CD11c, CD19, CD20, CD22, and CD25 in increased amounts and are found to have tartrate-resistant alkaline phosphatase by immunohistochemistry (208,209). Serum soluble CD25 is a reliable tumor marker (210) with elevations preceding relapse by a median of 8 months (211).

PLASMA CELL MYELOMA/PLASMACYTOMA

For completeness, this is reviewed only briefly here but is covered extensively elsewhere in this text. Histologically, tumors of this subgroup, seen either as isolated masses (plasmacytomas) or as an infiltrating bone marrow process (myeloma), resemble immature plasma cells (212,213). Tumor cells are generally surface immunoglobulin negative and may secrete either IgG, IgA, or light chains alone. In rare cases, IgD or IgE may be the primary immunoglobulin secreted (214,215 and 216). Cells are usually CD19, CD20, and CD22 negative. Clonal rearrangement of the immunoglobulin loci is seen (217). The most frequent genetic changes appear to be related to abnormal immunoglobulin class switch recombinations (218). Aneuploidy is a common cytogenetic finding although translocations also are seen and may have prognostic significance (219,220,221,222,223 and 224). Alterations in *ras* and *Rb* proteins have been found in subsets of patients. Patients may have a concomitant infection with human herpesvirus 8 (HHV-8) (225,226). Patients have abnormalities including paraproteinemia, anemia, thrombocytopenia, renal failure, and hypercalcemia along with isolated or diffuse bony pain symptoms (227,228). Clinical course is variable depending on the presence of negative prognostic findings such as hypercalcemia and renal failure at presentation (229,230). Although cure remains elusive in the majority, patients respond to a variety of aggressive and nonaggressive therapeutic approaches (157,231,232,233 and 234).

EXTRANODAL MARGINAL ZONE B-CELL LYMPHOMA OF MALT TYPE

Extranodal marginal zone lymphomas of MALT type arise most frequently in patients with prolonged lymphoid reactive proliferations (235). The gastric mucosa is the most common site due to infection with *Helicobacter pylori*, and other sites are related to autoimmune disease such as Sjögren syndrome (lacrimal glands) or Hashimoto thyroiditis (thyroid) (236,237 and 238). Lymphomas of this group account for 7.6% of all non-Hodgkin's lymphomas (179). Histologically, these appear uniformly to be proliferations of small to medium-sized lymphocytes. As with the other marginal zone lymphomas, cells typically express pan B-cell antigens including CD19, CD20, and CD79a along with surface immunoglobulin and lack expression of CD5, CD10, and CD23 (239,240 and 241). In cases in which confusion may exist as to the malignant nature of the lymphoid infiltrate, the demonstration of k or l light-chain restriction becomes critical. Genetically, abnormalities with trisomy 3 and the t(11;18)(q21;p21) translocation are seen (242) with less frequent trisomies involving chromosomes 7, 12, and 18. With the most frequent gastric presentation, patients have nonspecific upper gastrointestinal symptoms of pain and/or reflux. Clinically, the disease follows an indolent course (243,244). Nearly all patients have *H. pylori* infection, and its eradication leads to cure in 50% to 65% of cases (240,243,245,246,247 and 248). Surgery, once a critical aspect of treatment, now has a secondary role. Interestingly, eradication of infection with *Borrelia burgdorferi* in patients with marginal zone lymphomas involving the skin has been shown to have similar clinical efficacy, indicating a common theme in pathogenesis and treatment of many of the patients with this general class of lymphoma (249). Patients then require frequent follow-up, and in patients not responding to this treatment, surgery, radiation, and/or chemotherapy may be required, particularly in patients with histologic evidence of transformation to a more clinically aggressive histologic subtype, which occurs in ~5% of cases.

NODAL MARGINAL ZONE B-CELL LYMPHOMA (±MONOCYTOID B CELLS)

B-cell lymphomas of this subgroup (250), as with their splenic counterpart, are thought to have their origin in the marginal zone of B-cell development outside the follicular mantle zone (251,252). Cells express surface immunoglobulin (IgM and IgD) along with CD19, CD20, CD79a, and CD11c (Fig. 31.1) (251,253,254). Cells are typically negative for CD5, CD10, CD23, and CD25 expression and can be differentiated from hairy cell leukemia by the absence of tartrate-resistant acid phosphatase expression. Chromosomal abnormalities with trisomy 3 and 18 are frequent (255). Clinically, this is a relatively rare entity, accounting for only 1.8% of the non-Hodgkin's lymphomas (179). Patients have isolated lymphadenopathy that, like the MALT lymphomas, may be associated with Sjögren syndrome and other autoimmune diseases (256,257,258 and 259). These lymphomas appear to follow an indolent clinical course similar to that for advanced follicular lymphomas, although, because of their rarity, extensive studies of treatment and outcome have not yet been done (260,261).

FOLLICULAR LYMPHOMA

Follicular lymphoma histologically has a nodular or follicular growth pattern without the zones of maturation seen in normal lymphoid follicles. In the WHO classification framework, this group is subdivided into three grades based on the presence of increasing numbers of large cells in which grades I and II are thought to follow an indolent natural history, and grade III (formerly termed follicular large cell lymphoma) follows a more aggressive course. The majority of patients have gradually increasing lymphadenopathy at one or more sites but with frequent involvement of extranodal sites including bone marrow. It is unusual for patients to have B symptoms. After initial diagnosis by lymph node biopsy and staging, up to three fourths of patients will be stage IV (Table 31.2), with only 10% to 15% of patients being stage I (179,262). Although a high percentage of stage I patients appear curable, at this point, cure in patients with more advanced stages has not been unequivocally proven. The majority of patients respond to a spectrum of treatment options discussed in the next section, whereas otherwise following a clinically indolent although relapsing and progressive course measured in years, with average survival of 8 to 10 years from diagnosis (263).

Genetically, the hallmark of follicular lymphoma is the t(14;18)(q32;q21) translocation involving the *bcl*-2 gene, which is centrally involved in control of apoptotic cell death pathways (264,265). The immunophenotype of this group of lymphomas tends to reflect the cell of origin in the B-cell lineage, with surface IgM positivity in addition to expression of CD19, CD20, CD22, and in many cases, CD10, but not CD5 (Fig. 31.1).

MANTLE CELL LYMPHOMA

Patients with mantle cell lymphoma account for £6% of all patients with non-Hodgkin's lymphoma (179). Histologically, neoplastic cell expansion in the mantle zone surrounding lymph node germinal centers occurs, with the cells representing a range of small to medium-sized lymphocytes. Cells of this lymphoma subtype express

not only B-cell markers including CD19, CD20, and CD22 but also CD5. Cells are negative for CD10 expression and usually for CD23 expression as well (266,267 and 268). Cytogenetically, the majority of these lymphomas have the characteristic t(11;14)(q13;q32) translocation (Table 31.4), which deregulates expression of the *bcl*-1 (cyclin D1) gene on chromosome 11 by juxtaposition with the immunoglobulin heavy chain gene on chromosome 14 (269,270 and 271). Because of heterogeneity in the breakpoints, PCR analysis is helpful clinically in only a subset of patients (272), but antibodies that detect increased cyclin D1 expression or PCR analysis of cyclin D1 mRNA levels are very helpful in distinguishing this lymphoma subtype (273,274,275 and 276). Clinically, patients are seen with mantle cell lymphoma at an average age of 60 years, with generalized lymphadenopathy, splenomegaly, and bone marrow involvement being frequent, as is the presence of circulating lymphoma cells (277). At present, no clinical treatment regimens are considered curative, with particular difficulty obtaining clearing of the bone marrow in these patients with or without inclusion of anthracyclines in the treatment regimen (261,268,278). Overall survival is therefore short, with median survival of 3 to 4 years (179,279,280).

DIFFUSE LARGE B-CELL LYMPHOMA

This lymphoma, which is more likely localized than are the other non-Hodgkin's lymphomas, generally is seen with lymphadenopathy. Median age of onset is 64 years, based on analysis done as part of the Non-Hodgkin's Lymphoma Classification Project (179). Patients have a full range of Ann Arbor stages, although involvement of at least one extranodal site is frequent, occurring in £70% of patients. Overall clinical behavior is aggressive in untreated patients, with up to one third of patients having B symptoms and masses more than 10 cm at the time of presentation, with half of patients having an elevated lactate dehydrogenase (LDH) and performance scores on the Eastern Cooperative Oncology Group (ECOG) scale of ³2 (156,281). However, as discussed later, this lymphoma is quite responsive to chemotherapy, with a significant number (40%-50%) of patients cured (282).

Cells from this class of lymphoma generally express an immunophenotype that is $CD20^{+}$ and $CD3^{-}$. Acquired cytogenetic abnormalities are frequent (listed in Table 31.4) and include translocations involving the *bcl*-2, *bcl*-6, and c-*myc* genes (109,110,283,284,285,286,287,288 and 289). Morphologically, the predominant cell is large, with the nucleus at least twice the size of a small lymphocyte, with prominent nucleoli and a basophilic cytoplasm. The diagnosis can be complicated in a subset of patients with the variant T-cell–rich B-cell lymphoma because of the presence on infiltrating normal T lymphocytes (290), which do not otherwise alter prognosis.

MEDIASTINAL LARGE B-CELL LYMPHOMA

As a subset of large cell lymphoma, primary mediastinal large B-cell lymphomas (291,292) account for 13% of these lymphomas (293) and ~2% of all non-Hodgkin's lymphomas (179). Cells express B-cell markers of CD20, CD22, and CD79a, although they do not express either surface or cytoplasmic immunoglobulin (294,295,296 and 297). No characteristic chromosome translocations have been described, in contrast to their diffuse large B-cell lymphoma counterparts, although alterations in 9p and amplification of the *rel* protooncogene have been described (298,299). The disease is rare in patients older than 65 years and typically is seen with respiratory symptoms associated with a large anterior mediastinal mass (300,301,302 and 303). Bone marrow, visceral organ, and lymph node involvement is uncommon. Clinically, the course is aggressive in untreated patients, but this lymphoma has response and cure rates at least as good as and probably better than those for diffuse large B-cell lymphoma, although more aggressive chemotherapeutic regimens beyond CHOP may be required (301,303,304 and 305).

PRIMARY EFFUSION LYMPHOMA

First described in patients infected with HIV and EBV, this form of lymphoma occurs in effusions without associated nodal or extranodal masses (306) and may occur in HIV-negative patients (307). Immunophenotypically, the malignant cell is of B-cell origin. All cases show infection with HHV-8 (307,308 and 309). Rapid progression characterizes the clinical course, with median survival of ~6 months (306) despite treatment.

BURKITT'S LYMPHOMA/BURKITT'S LEUKEMIA

Burkitt's lymphoma was first described by Dennis Burkitt in 1961 (310) as a disease of African children with tumors of facial bones. Endemic regions include equatorial Africa and New Guinea (311,312). In Western countries, Burkitt's lymphoma is the most common childhood lymphoma (35%–45%), although overall incidence as a part of the spectrum of non-Hodgkin's lymphomas is very low (10 of 1,400 cases in a recent review) (179). Histologically, cells grow in a diffuse pattern of medium-sized cells compared with other lymphomas, and in the nonendemic form, patients may have a variety of clinical manifestations (313). The malignant cell is a mature B cell (Fig. 31.1), expressing surface IgM as well as CD19, CD20, CD22, CD79a, and usually CD10. Cells are typically CD5 and CD23 as well as TdT negative (314,315). Chromosomal translocations involving 8q24 (the site of the c-*myc* gene) are universal. Partners in the reciprocal translocations found in Burkitt's lymphoma include all immunoglobulin chain loci. The most frequent translocation is t(8;14)(q24;q32), involving the immunoglobulin heavy-chain gene, whereas k (2p11) and l (22q11) light-chain gene translocations occur in a smaller percentage of cases (113,114,115 and 116). Precise break points vary in endemic (African) versus sporadic Burkitt's lymphoma, with truncation of the first coding exon in sporadic cases. Point mutations also are found in c-*myc* coding sequences similar to those found in the viral transforming *myc* gene seen in the MC29 retrovirus (120,314,316,317). EBV infection is characteristic of endemic Burkitt's lymphoma, although it also occurs with lower frequency in sporadic cases (313,314,318). Inactivation of p53 and deletions in chromosome 6q also are frequent (319).

Because of an extremely high growth fraction within the tumor (nearly 100% based on Ki-67 immunohistochemistry), patients have rapidly progressive masses requiring emergency treatment if life-threatening complications are to be avoided. Patients have a rapid response to chemotherapy with high probability of cure in early-stage and even advanced-disease patients (320,321,322,323 and 324). Staging currently is based on one of two Burkitt's lymphoma-specific staging systems, the St. Jude staging system (325) or the Uganda Cancer Institute staging system (326).

PRECURSOR T-LYMPHOBLASTIC LYMPHOMA/LEUKEMIA (PRECURSOR T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA)

This entity is seen predominantly in adolescent and young adult patients and composes £40% of childhood lymphomas and 15% of acute lymphoblastic leukemias (327). On morphologic grounds, the cells are indistinguishable from their B-cell acute leukemia counterparts. Clinically after a rapidly fatal untreated course, patients can nevertheless achieve remission and cure with aggressive therapy. The transformed cells are generally CD3 and CD7 positive and express TdT and are either CD4/8 double positive or negative, indicating a precursor T-cell origin (Fig. 31.2) (328,329 and 330).

T-CELL PROLYMPHOCYTIC LEUKEMIA

This is a rapidly progressive T-cell lymphoproliferative disorder seen with a high white blood cell count, diffuse adenopathy, and skin lesions. It is characterized by inversions and translocations involving chromosome 14 (Table 31.4), with activation of the *TCL*-1 locus, which is critically involved in genesis of this disorder based on transgenic mouse studies (331,332 and 333). Cells express CD2 and CD3, with the majority expressing CD4 as well, although a subset will express CD8. Poor response to treatment leads to a median survival of only 6 months.

T-CELL GRANULAR LYMPHOCYTIC LEUKEMIA

Clinically, this is a somewhat more chronic leukemia compared with its NK-cell leukemia counterpart, with a spectrum of immune abnormalities including recurrent infections, hepatosplenomegaly, and pancytopenia. Cells have a T-cell phenotype, being $CD3^{+}/CD8^{+}$ (334).

AGGRESSIVE NK-CELL LEUKEMIA

Clinically, this is a rapidly progressive leukemia compared with its T-cell chronic granular lymphocytic counterpart, but with a similar spectrum of immune abnormalities including recurrent infections, hepatosplenomegaly, and pancytopenia. Cells have an NK-cell phenotype, being $CD56^{+}/CD3^{-}$ (334).

ADULT T-CELL LYMPHOMA/LEUKEMIA (HTLV-I^{+})

On epidemiologic and clinical grounds, adult T-cell leukemia was first recognized as a distinct clinical entity in Japan (335,336). Subsequent work identified the first pathogenic human retrovirus, HTLV-I, as the cause, based on initial isolation of this virus coupled with subsequent epidemiologic studies worldwide (337,338,339 and 340). Clinically, this is an aggressive lymphoma (340,341) seen endemically in Japan and the Caribbean. Pathologically, lymph nodes show an infiltrate of T cells of variable size and morphology. However, immunophenotypically, the cells express CD2, CD3, and CD4, but not CD7 or CD8, and have lower than typical CD3/TCR, based on stage of differentiation (Fig. 31.2). The malignant cell is clonal based on TCR-b gene rearrangement and contains a clonal integrated HTLV-I genome.

EXTRANODAL NATURAL KILLER/T-CELL LYMPHOMA, NASAL TYPE

Clinically similar to peripheral T-cell lymphoma, not otherwise specified, diagnosis of this named entity relies on the finding of NK cell–specific antigen CD56 expression in the lymphoma cells that also contain clonal EBV genomes (342,343 and 344). Clinical presentation is generally with an aggressive, destructive, midline facial tumor,

which has a poor prognosis if dissemination has occurred (345,346).

ENTEROPATHY-TYPE T-CELL LYMPHOMA

This lymphoma generally develops late in life in patients with a long-standing history of celiac disease. It is included in the peripheral T-cell lymphoma group with presentation that is predominantly gastrointestinal. Clinically, this entity has or results in intestinal perforation and has a very poor prognosis because of poor response to therapy. Phenotypically, the lymphoma cell is $CD3^+$ but $CD4^-$ and generally $CD8^-$ (347,348).

HEPATOSPLENIC gd T-CELL LYMPHOMA

This is a rare form of T-cell lymphoma involving the TCR-gd rather than TCR-ab subset of T-cells (Fig. 31.2). It affects a young patient group with or without bone marrow involvement and is characterized by an aggressive clinical course with involvement of extranodal sites in the absence of significant lymphadenopathy (349,350). Immunophenotypically cells are CD2 and CD3 positive but do not express CD4, CD5, or CD8 (350).

SUBCUTANEOUS PANNICULITIS-LIKE T-CELL LYMPHOMA

Clinically distinct from other peripheral T-cell neoplasms, this T-cell lymphoma subgroup is characterized by systemic symptoms of fever and fatigue along with subcutaneous nodules, particularly involving the legs, in the absence of nodal and visceral organ involvement. Patients have an aggressive clinical course with poor response to therapy and thus poor overall prognosis (351). Immunophenotypically, cells show a T-cell phenotype ($CD3^+$ and $CD8^+$) along with expression of NK-cell antigens such as CD56 (351,352).

MYCOSIS FUNGOIDES/SÉZARY SYNDROME

Mycosis fungoides/Sézary syndrome accounts for 65% of lymphoma patients with primarily cutaneous manifestations (353,354). The tumor cells are $CD3^+$, and CD4, CD8, and $CD30^-$. Clinical behavior of mycosis fungoides is indolent, with a chronic course in which multiple therapies directed at cutaneous manifestations of the disease are effective but not curative (354). Sézary syndrome, as a leukemic cell variant of mycosis fungoides, is defined as a triad of erythroderma and lymphadenopathy along with circulating lymphoma cells and follows a more aggressive course. Like follicular B-cell lymphoma, cutaneous T-cell lymphoma also can undergo histologic transformation to a more aggressive large cell lymphoma (355,356).

ANAPLASTIC LARGE CELL LYMPHOMA, T/NULL CELL, PRIMARY CUTANEOUS TYPE

This entity, seen with predominantly cutaneous nodules (as opposed to nodal involvement characterizing anaplastic large cell lymphoma, T/null cell, primary systemic type) otherwise has similar histologic and immunophenotypic findings, with the malignant cell showing CD3 and CD30 expression. However, clinically, this is a distinct entity with poorer overall response to therapy and a poor prognosis (357,358 and 359).

PERIPHERAL T-CELL LYMPHOMA, NOT OTHERWISE CHARACTERIZED

On clinical grounds, with presentation as generalized lymphadenopathy in the majority of cases, this form of peripheral T-cell lymphoma cannot be distinguished from its aggressive B-cell counterparts (360). Histologically, nodal architecture is effaced, with lymphocytes of varying sizes. Based on analysis of 1,400 patients (179), this form of peripheral T-cell lymphoma occurs in 3.7% of patients with lymphoma. The only clinical association with a T-cell versus B-cell phenotype is presence of the hemophagocytic syndrome in selected cases of peripheral T-cell lymphoma (361). Distinction relies on immunophenotypic analysis with, in most cases, expression of one or more pan T-cell antigens including CD2, CD3, CD5, and CD7. In up to two thirds of cases, expression of one or more of these surface antigens is lost, and the tumor cells have a phenotype unlike that of any normal T cell. Genetic heterogeneity also is frequent, although clonality can be proven with TCR gene rearrangements, and up to two thirds of patients have a translocation involving the TCR gene (110). When treated like diffuse large B-cell lymphoma, outcome in peripheral T-cell lymphoma is often poorer.

ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA

Previously, patients with this T-cell lymphoma were thought to have an abnormal immune reaction to an unknown stimulus, with diffuse adenopathy, skin rash, fevers, and the laboratory finding of hypergammaglobulinemia. With the understanding that this was indeed a lymphoma, the name has been changed, with the clinical syndrome termed AILD (angioimmunoblastic T-cell lymphoma with dysproteinemia) (362). Immunophenotypically, the transformed cells are CD3 and $CD4^+$ without uniform cytogenetic abnormalities, although trisomy 3 and inversions within chromosome 14 have been detected (363,364), along with the EBV genome (365). Early in the disease, interferon may produce responses; however, advanced disease requires aggressive multiagent chemotherapy (366).

ANAPLASTIC LARGE CELL LYMPHOMA, T/NULL CELL, PRIMARY SYSTEMIC TYPE

Diagnosis of this entity relies on the finding of CD30 expression in the lymphoma cells (367,368). Genetically, this is a distinct subgroup within the peripheral T-cell lymphomas, with chromosome rearrangements involving the anaplastic lymphoma kinase (ALK) gene product in translocations such as t(2;5)(p23;q35) characteristic of this subtype (369,370). There is a bimodal age incidence, with treatment in the pediatric and adult settings leading to a high probability of cure (368,371,372), making this the single most curable entity of the non-Hodgkin's lymphomas.

Treatment

Given the spectrum of entities in the non-Hodgkin's lymphomas with the large differences in their biology and clinical behavior, it is unlikely that any single therapeutic modality or approach will ever have universal utility. Consistent with this, therapeutic approaches at present depend on the specific lymphoma subtype in question. Patients with clinically indolent lymphomas such as small lymphocytic lymphoma/chronic lymphocytic leukemia may require little or no treatment initially, given that randomized clinical trials have shown no improvement in survival with treatment of early-stage disease (373). From this as well as other studies, although purine analogs such as fludarabine and 2-chlorodeoxyadenosine produce higher response rates, there is no difference in overall survival and no apparent cures when compared with watchful waiting and institution of oral chlorambucil with disease progression (373,374). Similar principles are applicable to the treatment of lymphoplasmacytic lymphoma, although newer purine analogs have again showed early promise compared with or as salvage for failure with chlorambucil alone (375,376,377 and 378). In a clinical trials setting in young patients, stem cell transplantation with either auto- or allografting may also produce complete remissions, although cure remains to be proven (379).

As with small lymphocytic lymphoma/chronic lymphocytic leukemia, the indolent follicular lymphomas are considered incurable outside of the unusual setting of patients with limited, stage I or II (Table 31.2) disease, in which radiotherapy or chemotherapy combined with radiation may allow cure (380). However, application of increasingly intensive therapy for remission induction and maintenance, including use of CHOP-like regimens with or without interferon for maintenance (381,382) and use of high-dose radiolabeled anti-CD20 antibodies in the transplant setting (383), have led to prolonged remission even in relapsed and otherwise treatment-refractory patients. New agents such as anti-CD20 (rituximab; Rituxan) alone (384), and purine analogs such as fludarabine, along with anthracyclines such as mitoxantrone and taxanes such as paclitaxol also hold promise for improvement of therapy in the future (385,386). Molecular complete remissions may be achieved in two thirds of patients with advanced-stage follicular lymphoma treated with the FND (fludarabine, mitoxantrone, dexamethasone) regimen alone or incorporating rituximab into each monthly cycle (386). In the grade III (follicular large cell lymphoma), aggressive therapy may, in a significant proportion of patients, offer a chance for cure (387).

Although the approach to treatment of the diffuse large B-cell lymphomas has been directed at cure, considerable evolution in our knowledge of effectiveness of treatment regimens has occurred in the last several years. Multiple iterations of combination chemotherapy regimens have been developed, leading to progressively complex and in some cases, more toxic regimens. Each generation of therapy, developed in the setting of single-arm clinical trials, appeared to give improved outcomes when compared with historic controls. However, with publication of the direct comparison of the early CHOP regimen with m-BACOD, MACOP-B, and ProMACE-CytaBOM, it was asserted that no regimen offered a clear advantage over another in cure rate (although the cure rates were less than half the level reported initially). Thus many consider the CHOP regimen to be the treatment standard because of ease of administration and excellent patient tolerance (388,389 and 390). Contrasting with this, patients with the primary mediastinal variant of large B-cell lymphoma may benefit from more aggressive therapy (293,391). In patients with relapse, high-dose chemotherapy with autologous stem cell support offers a further chance of long-term disease-free survival and cure in chemotherapy-sensitive patients (392,393 and 394). At present, similar approaches are used in the treatment of the peripheral T-cell lymphomas, although, in the case of HTLV-I^+ disease, no standard treatment approach appears effective with patients exhibiting a rapidly progressive course. In contrast, treatment of anaplastic T-cell lymphomas with CHOP or CHOP-like regimens has proven extremely effective.

In mantle cell lymphoma, although cure remains elusive, limited data suggest improvement in survival when anthracycline-containing regimens such as CHOP are

used, although this remains controversial (261,278,395). Certainly, in older patients and patients with poor performance status, use of chlorambucil alone or use of CHOP-like regimens without the anthracycline remain reasonable options. CHOP plus rituximab appears to achieve a higher response rate, but follow-up is still short (396). Only limited work has examined the potential role of high-dose therapy with stem cell support, which, although showing improved complete remission rates, does not appear to lead to cure (397,398). Therefore, outside of the clinical trial setting, dose-intensive therapy with stem cell support cannot be routinely recommended for this patient group.

Extranodal marginal zone B-cell lymphoma of MALT type presents a unique therapeutic problem in oncology, in which conventional approaches using surgery, radiation, and chemotherapy are no longer considered the treatments of choice. In patients with *H. pylori* infection, initial treatment of choice should be treatment with appropriate antibiotics to eradicate the infection (240,243,245,246,247 and 248). Eradication of infection with *B. burgdorferi* in patients with marginal zone lymphomas involving the skin may have similar clinical efficacy (249). Patients then require frequent follow-up, and in patients not responding to this treatment, surgery, radiation, and/or chemotherapy may be required, particularly in patients with histologic evidence of transformation to a more clinically aggressive histologic subtype.

Evolution of treatment of Burkitt's lymphoma, unlike that for diffuse large-cell lymphoma, has continued to show progress. With increasing intensity of therapy and incorporation of multiple agents into therapy, patients with Burkitt's lymphoma now have one of the highest probabilities of cure in even advanced-disease patients (320,321,322,323 and 324) with one of several treatment regimens. This is in contrast to hairy cell leukemia, in which introduction of new purine analogs, particularly 2-chlorodeoxyadenosine, has led to a high probability of cure with a single treatment course (204,205 and 206). Thus, while defying many tenets underlying the application of modern chemotherapy to malignant disease, hairy cell leukemia also highlights the potential importance of introduction of new agents into treatment and the manner in which these agents can radically alter prognosis and outcome in properly selected patient groups.

As with Burkitt's lymphoma, success in treatment of lymphoblastic lymphomas/leukemias has continued to improve. This is due in large measure to the application of acute lymphoblastic leukemia approaches to the therapy of this related lymphoma. Successful regimens combine principles such as use of multiple agents in combination and sequence, including high-dose methotrexate for CNS prophylaxis and concepts such as induction, consolidation, and reinduction with maintenance therapy that has been a part of acute lymphocytic leukemia therapy for decades. Regimens such as the LMT81 protocol and BFM 86 and 90, although complex, result in event-free survival rates that approach 75% to 80%, even in patients with advanced-stage disease (320,399,400 and 401). From these studies, patients that remain in remission for more than 3 years are probably cured.

Thus chemotherapeutic approaches to the treatment of non-Hodgkin's lymphomas continue to undergo evolution. From an immunologic perspective, immune-based therapy has only recently been shown to have promise for incorporation into specific treatment recommendations. Despite continuing controversy, interferon does appear to improve durability of remission in patients with otherwise poor-prognosis follicular lymphomas who are treated with an anthracycline-based chemotherapeutic regimen (402). Interferon in conjunction with high-dose therapy also may have a role in the treatment of plasma cell myeloma/plasmacytoma (403). In the CD20-positive lymphomas, CHOP plus rituximab in a strategy combining conventional chemotherapy with immune-directed therapy also has shown promise in improving overall response rate and potential reduction in relapse rate in the clinically indolent lymphomas (396). Finally, with our rapidly improving abilities to identify tumor antigens, vaccines may play an increasing role in lymphoma therapy although trials are only now beginning in earnest (404,405,406 and 407).

FUTURE DIRECTIONS

It is clear that the understanding of the biology as well as treatment of both Hodgkin's disease and the non-Hodgkin's lymphoma is undergoing a period of rapid evolution. In the case of Hodgkin's disease, work in the clinical arena must now concentrate on reducing long-term treatment-related complications from successful therapy. Whether this will involve increasing use of chemotherapy alone in early-stage disease or combined-modality, limited-dose radiation and chemotherapy to achieve the best long-term results will need to be established from long-term follow-up of recently completed as well as ongoing trials.

In the non-Hodgkin's lymphomas, evolution of both lymphoid biology and therapeutic approaches is likely to continue to be rapid. This is manifested in its simplest form by the rapid evolution in the classification of non-Hodgkin's lymphomas in the last 10 years, with establishment of the recent WHO framework, which, by definition, is a work in progress rather that a fixed scheme (4,408). As increasingly sophisticated molecular approaches are applied to the characterization of both normal lymphoid development and the lymphomas, this is likely to undergo further rapid evolution by application of new technologies to assess gene expression within the heterogeneous large B-cell lymphoma subgroup (126). Elucidation of the etiology of specific lymphoma subtypes also is progressing rapidly. Understanding the role of HHV-8 in multiple myeloma may provide new insights into both the pathogenesis and treatment or prevention of this plasma cell disorder. With the marginal zone lymphomas, further work to identify additional antigenic stimuli of infectious agents such as the recently identified *B. burgdorferi* infection in patients with this lymphoma involving the skin (249) is likely to change both our understanding of pathogenesis and treatment of this group of lymphomas. Progress in “traditional” treatment, although complicated by the broad spectrum of diseases within the non-Hodgkin's lymphomas, also is likely to undergo rapid evolution. Progress in subtypes such as the diffuse large B-cell lymphomas will require the introduction of new agents if significant progress is to be made, whereas in other cases such as the Burkitt's lymphomas, refinements of established regimens may permit significant progress. In addition, the role of immune cell–directed or modulating agents including antibody-based therapies such as anti-CD20 and IL-2 fusion toxins (409), interferon, and DNA- or protein-based vaccines will need to be increasingly incorporated in experimental treatment programs. Clearly, much has been achieved in our understanding of Hodgkin's and non-Hodgkin's lymphomas and their treatment in the last 10 years. However, the next decade should bring even more exciting changes in this understanding.

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32 MULTIPLE MYELOMA

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Multiple myeloma (MM) is a malignant disease affecting the most mature cell of the B-cell lineage (i.e., the plasma cell). MM has attracted much attention from both immunologists and oncologists because it represented the first model of a clearly clonal disorder, the monoclonal immunoglobulins (Ig) produced by the malignant plasma cells being an invaluable clonal marker; allowed elucidation of the structure of antibodies; and permitted investigators to gain new insights into the understanding of tumor progression and response to therapy and, more recently, to dissect the role of lymphokine-mediated regulatory circuits in the growth of plasma cells. This fundamental progress has had, however, no discernible effects on the prognosis of MM, which is still a badly disabling malignancy with patients' mean survival time being 20 to 30 months. Present efforts are evaluating the efficacy of high-dose chemotherapy regimens followed by hematopoietic stem cell grafting, with some promising results.

HISTORY

The main clinical and pathologic features of MM were described nearly 150 years ago (85). The term *multiple myeloma* was coined by Rustizky (295) to emphasize the multiple focal bone lesions. In Europe, MM is still called Kahler disease because of the definite description of the disease by Kahler (191). Wright (374) noted that the tumor cells in MM belong to the plasma cell lineage. Finally, the serum protein abnormality was recognized more than 60 years ago (268). The value of L-phenylalanine mustard in the treatment of MM was recognized in 1958 (50).

EPIDEMIOLOGY

Although the incidence and mortality rates of MM have been increasing until recently (107), the disease remains a relatively rare cancer, accounting for 1% of all malignancies in whites and 2% in blacks in the United States (289) as well as in other countries (73,264,346). The average annual incidence rates per 100,000 are 4.7 in men and 3.2 in women for whites. The median age at diagnosis is ~70 years (289).

Apart from exposure to ionizing radiation (220,235,316), no convincing risk factor has been established. Farming occupations have been linked to the occurrence of MM in some studies (53,61,65,145,371). Prolonged exposure to benzene is not a risk factor (42). Special interest focused on the possible role of long-term antigenic stimulation leading to plasma cell dysregulation; the results of most studies, however, were inconclusive (53,88,96,155,205,224,372).

Familial occurrence of MM is well established (54,163,176,228,317); an environmental agent may be implicated, as MM has occurred among spouses (62,193,211). Genetic anticipation may occur in successive generations as observed in other lymphoid malignancies (164).

PATHOGENESIS

Current ideas on the differentiation of normal B cells suggest that they proliferate on antigenic stimulation in the primary and secondary follicles of the lymph nodes, the spleen, and Peyer patches and give rise to rapidly dividing plasmablasts, which circulate and home in bone marrow, where they mature to plasma cells (181,231). Interleukin (IL)-6 plays a major role in the late maturation of B cells (200,352). To what extent this scheme applies to the biology of MM is largely unknown. The phenotype of the vast majority of plasma cells in most patients appears similar to that of their normal counterpart (337), featuring CD38, CD10, CD138 (109), the HM1-24-antigen (252) and plasma cell-associated 1 antigen (PCA-1) molecules (166,180), and, unexpectedly, some antigens associated with other hematopoietic lineages such as myeloid, monocytic, and erythroid markers (127,162,336). A notable exception is the frequent expression of adhesive molecules such as CD11b, CD44, and CD56 (neural cell-adhesion molecules, N-CAM) (3,115,166,219,336,351), the latter mediating homotypic adhesion between cells. Most plasma cells and cell lines express the Fas-Fas ligand (Fas-L) molecules; whereas Fas-L is functional and may protect cells from cytotoxic T cells, Fas triggering does not provoke apoptosis, and mutations in the death domain of Fas have been noted in some patients (214,359). Loss of CD56 occurs in plasma cell leukemias, and expression of CD28 (as noted in a subset of normal plasma cells) may indicate aggressive myeloma (291). Secretion of matrix-degrading enzymes as well as angiogenic mediators may be associated with the progression of disease (349). In patients with inactive or slowly progressive disease, plasma cells are mostly nondividing, whereas in rapidly progressive disease, a variable number of actively proliferating plasmablasts are found. Some of them may give rise to colonies growing in soft agar, which define a myeloma stem cell assay that is highly correlated with the severity of the disease (165). The phenotypic and functional characterization of such stem cells is still elusive. Indeed, one may wonder whether in most cases, the bulk of the nondividing, end-stage, mature plasma cells do not preclude the identification of the important cells whose renewal constantly replenishes the mature pool of tumor cells. Evidence has been provided that some bone marrow atypical B cells ($CD10^+$, $HLA\text{-}DR^+$, surface and cytoplasmic Ig^-) (70) and blood precursors (43) may differentiate *in vitro* in the presence of lymphokines (IL-3 and IL-6) to plasma cells. Of note, cells with a phenotype between that of mature B cells and plasma cells coexist in myeloma bone marrow with typical plasma cells (198,221,303).

Some observations indicate that the clonal disease process also may include immature pre-B cells and mature B cells. Idiotypes are shared by the μ chain synthesized by pre-B cells and the serum monoclonal Ig (206). In different studies, different proportions of blood B cells displayed surface Ig idiotypically related to the serum monoclonal Ig (1,24,223,238,271,308). Genetic studies of these monoclonal populations of B cells yielded contradictory results (37,82,86,186,217); however, several studies unequivocally showed that a very small subset of IgM-producing B cells (1 in 10^4 or 10^5) share with malignant plasma cells the same mutated variable segment (17,45,91). It seems likely that if such B cells do exist, they account for a minority of blood B cells in any case. The significance of myeloma-related pre-B or B cells is unknown because functional studies could not be performed. The establishment of cultured lines derived from these peculiar B cells may help to resolve these issues (160,258).

A major advance in the biology of MM came from studies indicating that the growth of abnormal plasma cells was under the control of several lymphokines. IL-6 plays a key role in the proliferation of plasma cells (352). Initially it was shown that plasma cells produced IL-6 and expressed the surface receptor for this lymphokine and that antibodies to IL-6 inhibited cell proliferation (194). Whereas the latter finding was confirmed in several studies (9,201,245), the nature of the cells producing IL-6 is more debatable. Production of IL-6 by malignant cells was demonstrated only in some patients and in a subset of autonomously growing plasma cell lines (170,255,273). With *in situ* hybridization and immunocytochemistry, IL-6 messenger RNA (mRNA) and protein were detected in all patients with MM or benign monoclonal gammopathy (304). However, bone marrow nonplasmacytic cells (stromal cells, fibroblasts, macrophages) produce huge amounts of IL-6, suggesting that the proliferation of plasma cells was paracrine (273). The hypothesis of autocrine growth, however, holds true for certain plasma cell lines (218). It seems conceivable that the heterogeneity of plasma cell populations on the basis of phenotypes and kinetics in individual patients or from case to case accounts for some of these apparent discrepancies. The major role of IL-6 in the growth of MM also is supported by the antitumor effects of anti-IL-6 antibodies *in vivo* (202). Overproduction of this pleiotropic lymphokine also is responsible for fever in rare patients with fulminant disease and an increase in C-reactive protein (CRP) levels in more than one third of patients with MM (29,227). IL-6 both triggers plasma cell division and enhances plasma cell survival by upregulating *bcl*-XL and *Mcl*-1 (75,280). IL-6 signaling uses both Jak-stat 1,3 and ras–mitogen-activated protein (MAP) kinase pathways (75,250).

Lymphokines using the gp130 transducing chain of the IL-6 receptor (oncostatin M, IL-11, leukemia inhibitory factor, ciliary neurotropic factor) also trigger plasma cell proliferation (248,380). Oncostatin M production may be constitutive or its receptor upregulated by IL-10 (369), which is produced by plasma cells in half of the patients with myeloma (203). Evaluation of the level of cytokine or cytokine receptors in myeloma has no accepted clinical significance (305,330,370).

IL-6 is not the sole lymphokine triggering plasma cell growth. Hematopoietic cytokines [granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-5, granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor (TNF), interferon-a (IFN-a)] also may modulate plasma cell proliferation, mostly because they synergize with IL-6 or induce IL-6 production by plasma cells (9,43,189,190,379). These cytokines are now believed to mediate the characteristic increased osteoclastic bone resorption and inhibition of bone formation in MM (31,242). IL-6, TNF, and IL-1 appear to be the most critical factors (30,71,94,195,375). The recent identification of the osteoclast-differentiating factor may shed new light on bone disease in MM.

Some animal models also suggest that IL-6 may be involved in the development of plasma cell tumors. Mouse plasmacytoma growth is IL-6 dependent, and transfection of the IL-6 gene in tumor cells leads to an autocrine growth and increased tumorogenicity (339,360). Furthermore, mice homozygous for a null IL-6 gene are refractory to induction of plasma cell tumors (168). In mice transgenic for the IL-6 gene fused with the Ig heavy-chain enhancer, a massive polyclonal plasmacytosis develops in the C57BL/6 strain (331), and transplantable plasma cell tumors develop in the myeloma-prone BALB/c strain (332). The latter observation suggests that additional factors besides IL-6 are needed for tumor generation. Mouse plasmacytomas invariably carry a rearranged c-*myc* gene (92). Cytogenetic studies in human MM point to a frequent involvement of chromosomes 1 and 14 (14q32 abnormalities), with several and less frequent numeric or structural changes of chromosomes 3, 5, 7, 11, 17, and 18 (78,108,123,152,247,348). The t(11;14), t(8;14), and t(14;18) translocations, which are recurrent in other hematologic malignancies, have been observed in a limited number of patients (246,355). In view of the mouse model, particular attention had been paid to the c-*myc* oncogene. Its expression is frequently high, but identifiable gene rearrangements are rare (149,262,311). Expression of the *bcl*-2 oncogene is a feature of both normal and malignant plasma cells (269). Recent sophisticated techniques using fluorescent *in situ* hybridization, spectral karyotypes, and comparative genomic hybridization demonstrate that chromosomal anomalies exist in the malignant cells of most, if not all, patients with MM (69,84,285,377). Chromosomal translocations involve the heavy-chain locus and the site of the isotype switch region, indicating that the translocation might occur in cells maturing in germinal centers.

Various non-Ig partner chromosomes have been identified, most commonly chromosomes 11q13 and 4p16, and less commonly, chromosomes 8q24, 16q23, and 6p25. Genes expressed because of the translocation code for cyclin D1, the receptor for basic fibroblastic growth factor receptor, c-*myc*, c-*maf* transcription factor, and IFN regulatory factor 4 (79,80 and 81,143,376). Abnormalities of chromosome 13, including monoallelic deletion, carry a poor prognosis and may be associated with transition between benign monoclonal gammopathy and myeloma (15,77). The presence of p53 gene deletion predicts short survival (116). Structural oncogenic modifications of the Ki-*ras* and N-*ras* genes were noted in approximately one third of patients (225,244).

Because the differentiation and maturation of normal B cells are tightly regulated, and because there is frequently a latent period before plasma cell proliferation becomes aggressive, several studies aimed to define immunoregulatory circuits. There is a decrease in T-cell number and an imbalance between T-cell subsets, with evidence of abnormal helper function (216,234,239,272,312). A monoclonal T-cell population (367) and an abnormal distribution of Va and Vb genes expressed by peripheral T cells (185) were noted in a few patients. Three kinds of suppressive cells belonging to the monocytic (57), B- (230), and T-cell (265) lineages were identified. Cytotoxic T cells against autologous plasma cells were noted (261). The actual role of these cell populations in the biology of MM remains speculative. It seems likely that they are responsible for the humoral immune deficiency that is a hallmark of MM. The possibility that monoclonal populations of CD4 and CD8 T cells are specific for idiotopes of myeloma protein may have therapeutic significance (59,281). Efforts are being made to stimulate the immune response to autologous Ig idiotypes by immunization with myeloma protein-loaded dendritic cells (287). In addition, myeloma cells may express other tumor-associated molecules such as MUC1, an underglycosylated epithelial mucin (23), or MAGE-type proteins (350). Transfection of malignant plasma cells with the T-cell costimulatory CD80 molecule allowed the expansion of T cells cytotoxic for autologous plasma cells (336).

In MM, the bone marrow microenvironment is important in supporting the growth of plasma cells. Adhesion of plasma cells to stroma triggers IL-6 secretion (347). These data prompted investigators to search for human herpesvirus 8 (HHV-8) infection because this herpesvirus harbors a viral gene that is homologous to human IL-6. HHV-8 genes were detected by gene amplification and *in situ* hybridization in the adherent nonmalignant cell population from long-term culture of bone marrow from most patients with MM and one fourth of patients with benign monoclonal gammopathy (99). However, no antibodies to HHV-8 are present in patients' sera, and detection of HHV-8 was not reproduced in several laboratories (256,335). The role of HHV-8 in MM pathogenesis therefore remains controversial.

Most symptoms in patients with MM are related to the high tumor cell burden. Determination of the Ig secretion rate by plasma cells, the monoclonal Ig serum level, and the catabolic rate allow a precise estimation of the tumor mass (120,173,297,301,333). Patients become symptomatic when the number of plasma cell tumors reaches an average doubling time of 6 months. The disease is fatal when the number of cells is 1 to 2 × 10^{12} (i.e., 1- to 2-kg tumor mass). In progressive MM, the doubling time of the tumor follows a gompertzian growth curve; back-calculation indicates that the subclinical phase of the disease lasts 1 to 5 years before diagnosis. However, such calculation may be hazardous because it fails to account for latent or smoldering MM or even so-called benign monoclonal gammopathies.

In more than 99% of patients, malignant plasma cells retain the property of secreting Ig molecules; the detection of monoclonal Ig (or light chains) in serum and urine is a frequent mode of diagnosis. However, 80% of patients have an excess of light-chain synthesis and secretion, leading to proteinuria and/or the presence of free light chain in serum, especially when light chains form oligomers (101,172). In 10% of patients, only light chains are secreted; however, in a fair percentage of these patients, heavy chains are synthesized by plasma cells, as shown by immunofluorescence studies (275). In the rare patients in whom no abnormal Ig or Ig chains are detectable in serum and urine, immunofluorescent studies of malignant plasma cells evidence Ig chains (178,275,290). Biosynthetic studies performed in these nonsecretory MMs as well as in light-chain MMs suggest that the Ig chains produced are structurally abnormal and rapidly degraded (276).

The incidence of the secretion of a particular Ig isotype in MM is roughly in accordance with the serum level and with the distribution of plasma cells secreting a given isotype in normal bone marrow. IgG MM is therefore the most common variant (159); it displays a 66% k/l light-chain ratio. Tumor growth is slower than that in IgA, IgD, or light-chain MM. Self-aggregation of IgG3 molecules may induce a hyperviscosity state (72,328). The latter complication occurs more frequently in IgA MM because IgA molecules are uniquely prone to polymerize with a molecular size up to 900,000 d (207). IgD MM represents 1% to 2% of all cases and exhibits special features. More than 90% of patients display IgD with l light chains (147), indicating that the malignant cells may arise from rare germinal center cells with a similar phenotype including a high rate of mutations (10). The serum level of monoclonal IgD is often low, and light-chain proteinuria is constant. Extraosseous localizations (lymph nodes, liver, spleen, nervous system) are frequent (174,184,279), and the prognosis is especially poor (172). IgE MM comprises less than 0.01% of all cases and often is seen with aggressive plasma cell leukemia (124,138,251,294). Of note, ~1% of patients with otherwise typical MM display a serum monoclonal IgM (64,366); in these cases the prognosis and course of the disease are those of MM.

The presence of two or more monoclonal Igs (the most common combination being IgG and IgA) in serum is not very rare (209,278). There is evidence that the cells synthesizing these Igs are clonally related (142,175,266,274,319). In certain lymphoid malignancies such as acute lymphoblastic leukemia or chronic lymphocytic leukemia, a bias in the use of the diverse V_H and V_L families has been noted (177,199,315). No such bias seems to exist in MM; however, individual genes of the V_L1 and V_H3 families may be overrepresented, whereas others such as the V_H4-34 gene, which is often used in chronic lymphoid disorders or autoimmune diseases, are absent (288). Different mutational patterns have been observed in myeloma (no clonal heterogeneity) and benign monoclonal gammopathy (296).

The determination of the antibody activity of monoclonal Ig had been extensively worked out with the hope of gaining insight into the triggering mechanism of the disease (310). Some indirect evidence does exist that antigen may play a role in the development of the malignant clone (309). A patient with acquired immune deficiency developed a myeloma characterized by a monoclonal IgG directed to a viral protein (254). However, most identified antibody activities belong in the groups of natural antibodies, autoantibodies, or antibodies against common pathogens (the most usual is against streptolysin), all of which belong to the repertoire expressed in normal serum IgG (46,97,110,274,381). Some antibody activities may be correlated with unusual clinical symptoms. Patients whose IgG or IgA is directed against IgG cryoprecipitate can develop circulatory impairment (Raynaud phenomenon), skin necrosis of cold-exposed areas, or skin (vascular purpura) and kidney (glomerulonephritis) vasculitis (58). In 5% of patients, although no antibody activity is demonstrable, the monoclonal Ig is also a cryoglobulin. Antibody activity to lipoproteins can induce hyperlipidemia (197); that to transferrin can cause hemochromatosis (90); antibody activity to fibrin monomers or von Willebrand factor can precipitate hemorrhages (90,378); that to platelets can lead to thrombocytopenia (111); and antibody activity to insulin can provoke hypoglycemia (286). A discoloration of hair and nails was observed in a patient whose IgG reacted with flavin (130).

Systemic symptoms may be related to the deposition of Ig chains or fragments in tissues that compromise their function (see Chapter 34 on amyloidosis). Light-chain (and rarely light- and heavy-chain) deposition disease is characterized by deposits of amorphous, nonfibrillar material that is stained by antisera to light chains (66,148). Deposits of k chains occur twice as often as those of l chains. Renal involvement is constant, with light-chain deposits in glomerular and tubular basement membranes sometimes with nodular glomerulosclerosis. Immunofluorescent studies of kidney biopsy specimens allow an easy diagnosis of this complication. Widespread deposition of light chains in heart, liver, vessel walls, and nerves is common and variably affects organ function (146,284,313,345). Biosynthetic studies of the secretion of Ig chains as well as chemical study of the deposits indicate frequent structural abnormalities of the light chains, which may be truncated or polymerized or may feature an unusually high state of glycosylation (67,270,277,340). Of note, the abnormal light chains may not be detectable in serum or urine because of a small tumor mass, increased catabolism, or deposition in tissues. A mouse experimental model indicates that discrete mutation of a *Vk 4* gene led to widespread tissue deposition (196).

CLINICAL AND LABORATORY DIAGNOSIS

The diagnosis of MM may be suspected in a number of clinical settings. Bone pain, nonspecific symptoms such as weakness or weight loss, and laboratory abnormalities (electrophoretic spike, proteinuria, increased sedimentation rate) are common presenting signs. A complication of MM such as bone fracture or tumor, hypercalcemia, renal failure, recurrent infectious process, amyloidosis, and neurologic injury also frequently leads to diagnosis. The detection of serum monoclonal Ig by routine serum electrophoresis in otherwise asymptomatic individuals is increasingly frequent and legitimates an extensive workup. The diagnosis of MM relies on the study of serum and urinary Ig, the search for abnormal bone marrow plasmacytosis, and skeletal lesions.

Identification of Monoclonal Immunoglobulin

Serum electrophoresis detects the clonal Ig as a narrow spike of homogeneously migrating Ig molecules. Immunoelectrophoresis or immunofixation identifies the isotype of light and heavy chains. Special care must be taken not to overlook a small amount of free light chains. Neither normal total protein or electrophoretic fraction levels nor quantitative estimates of the various Ig isotypes eliminate the presence of a monoclonal Ig. Aside from the monoclonal Ig, these studies in most cases show a decrease of polyclonal Ig isotypes. Such hypogammaglobulinemia of recent onset in adults justifies a search for MM. Note that a quantitative nephelometric estimate of the monoclonal Ig is often unreliable and that the value of densitometric tracing of serum electrophoresis is more accurate for the follow-up of the disease.

Sulfosalicylic acid precipitation must be used to detect urinary light chains as a routine, because other tests, and especially the classic heat test, are insensitive to detect a small concentration of light chains. As for serum study, immunoelectrophoresis and immunofixation are needed to identify urinary monoclonal light chains. Study of a 100-fold concentrated urine sample is required before acceptance of the diagnosis of nonsecretory MM. All these tests detect abnormal clonal Ig or light chains in more than 99% of patients with MM.

Hematologic Features

The diagnosis of MM depends on the presence of an increased number of plasma cells, often with atypical features. The cytoplasmic maturity contrasts with the degree of nuclear differentiation (118,154,323). Plasma cells account usually for more than 10% of bone marrow cells. Plasmablasts with a high labeling index are indicative of a poor prognosis (157). In early MM, the malignant infiltrate is often nodular, and several aspirates or a bone marrow biopsy may be useful to ascertain the degree of marrow involvement.

Anemia is present in roughly two thirds of patients with MM, whereas leukopenia and thrombocytopenia are found in less than 15%. A leukoerythroblastic picture indicates massive bone marrow involvement. A small number of plasma cells is detectable in many patients; a leukemic dissemination (plasma cells 2×10^9/L) may be a presenting feature but tends to occur more often at the late stages of the disease. Rouleaux formation is common and related to the presence of the monoclonal Ig, as is the rapid cell sedimentation.

Skeletal Findings

The typical changes of MM are multiple, punched-out, purely osteolytic lesions occurring in hematopoietically active areas (208,362). Frequently, diffuse osteoporosis coexists with these lesions and may be the sole abnormal finding. Rarely, the lesions are osteoblastic (125,215,293), and these patients may have unusual symptoms, especially a peripheral neuropathy (241).

Fewer than 10% of patients harbor a single osteolytic lesion, raising the possibility of a solitary plasmacytoma. This distinction with MM is of great importance because true solitary lesions may be cured with local radiotherapy. The disappearance of a monoclonal Ig, when present, is good evidence for this possibility. However, most patients with ap-parent localized plasmacytomas exhibit new focal lesions or develop MM, sometimes years after treatment of the initial lytic lesion (25,76,105,204,236).

The importance of establishing the occurrence of bone lesions in a patient suspected of having MM may legitimate a bone survey with computed tomographic scanning (167,324) or magnetic resonance imaging (MRI) (140,167). The latter is of considerable value in detecting bone marrow abnormalities and may have a therapeutic impact in patients with asymptomatic, stage I disease (112,233). In contrast, bone scintigraphy does not detect myeloma lesions (27,139,361). These studies are useful to delineate the extent of compressive tumors, especially in patients with neurologic symptoms.

COMPLICATIONS

Patients with MM are exposed to multiple complications related to tumor formation, immune humoral deficiency, and toxicity of light (and rarely heavy) chains.

Neurologic Symptoms

Spinal cord and nerve root compression occurs in nearly 10% of patients with MM (55,320). The malignant plasma cell tumor often originates from a rib or vertebra, invades the spinal canal, and leads to extradural compression. Radicular pain is a frequent early sign. Sensory and motor loss and paraplegia are late symptoms. Emergency evaluation includes spinal cord radiographic studies and MRI, which are useful to assess the extent of tumor and to plan the treatment.

Patients with aggressive MM may have myelomatous meningitis with infiltration of the meninges and presence of plasma cells in the cerebrospinal fluid (232,365).

In fewer than 1% of patients, a sensorimotor polyneuropathy develops. The onset is progressive, and pain and dysesthesias predominate. Patients with peripheral neuropathy exhibit some distinct features: young age, monoclonal Ig with l light chains, osteosclerotic bone lesions (102,104,117), endocrine abnormalities (diabetes, hypertrichosis, gynecomastia), and skin thickening with hyperpigmentation. This constellation of symptoms had been described as the POEMS (polyneuropathy, organomegaly, endocrinopathy, M protein, skin changes) syndrome in Japan (334). Although the pathogenesis of this complication is unknown, it is directly related to the plasma cell tumor because improvement follows treatment, especially irradiation of a solitary plasmacytoma.

Renal Impairment

The most common renal injury is the distinctive cast nephropathy associated with tubulointerstitial inflammation. It is caused by light-chain excretion and occurs in approximately half of patients with MM. The nephrotoxicity of light chains is quite different from patient to patient (169); there is no correlation with the level of light-chain excretion. Studies of the physicochemical characteristics of light chains such as their isoelectric point provide no satisfactory explanation for their toxicity. Animal models of renal toxicity correlate well with clinical findings (325). Development of acute renal failure is often precipitated by dehydration, infection, hypercalcemia, or the fluid restriction needed for radiographic examination with iodinated contrast medium, which is contraindicated in these patients (87). Chronic and acute renal failure due to cast nephropathy may be reversible, at least in part, after rehydration, treatment of the hypercalcemia or infection, and chemotherapy (44,87). Hemodialysis must be considered when the improvement is slow and even when renal failure appears irreversible (60,93,98,187). In selected patients with an excellent response to chemotherapy, successful renal transplantation has been performed (56,93,106,327,363).

The second cause of renal disease is amyloidosis (Chapter 34).

Light (and heavy) chain deposition disease is increasingly recognized, as discussed in the preceding section; it legitimates performance of a renal biopsy when cast nephropathy is not clearly the cause of renal dysfunction. Light-chain proteinuria also has been associated with defects in renal tubular reabsorption, including the adult Fanconi syndrome (103,126,299).

Miscellaneous Complications

The characteristic increased bone resorption in MM patients often leads to hypercalcemia, which is a presenting symptom in one third of symptomatic patients. Hypercalcemia develops in a majority of patients during the course of the disease. It may exacerbate kidney damage, as anorexia, vomiting, polyuria, and drowsiness or coma are usual symptoms that cause dehydration. Some monoclonal Ig may bind calcium ions with increased total Ca^{2+} but normal ionized calcium (182,222,326).

The humoral deficiency of MM favors the development of bacterial infections. Pneumonia due to *Streptococcus pneumoniae* remains the predominant infectious process, although gram-negative organisms, especially *Pseudomonas*, are increasingly isolated in these patients (240).

The hyperviscosity syndrome is actually rare in MM. It occurs mainly in patients with IgG3 or IgA monoclonal Ig; its manifestations (bleeding, impaired vision, ischemic

symptoms) are relieved by plasmapheresis.

Dissemination of plasma cell tumors outside the bones is not rare but occurs mainly in the late stage of MM. Hepatic and splenic infiltrations are usually asymptomatic and may be found at pathologic examination early in the disease. Extraosseous myelomas (or extramedullary plasmacytomas) feature a distinctive natural course, ending in MM in less than 20% of patients (26,204). Most extramedullary plasmacytomas arise in the upper respiratory tract, especially in the nasopharynx and oral cavity (141,192); other visceral localizations include the lungs, lymph nodes, spleen, skin, kidneys, and stomach (2,74,114,192,373). Prognosis of these localized tumors is fairly good, and local treatment yields a cure in 80% of patients. Extraosseous myelomas may arise in different anatomic sites simultaneously; recurrence may occur at various sites or as medullary plasma cell spreading.

PROGNOSIS AND TREATMENT

MM remains a universally fatal malignancy, and limited advances in its treatment have been made since the introduction, in the early 1960s, of alkylating agents (4,40). Median survival time from diagnosis, which was ~6 months before the use of these drugs (257), thereafter reached a median duration of ~3 years (8). Recently additional drug therapies and therapeutic strategies, including bisphosphonates and high-dose cytotoxic regimens supported by hematopoietic stem cell transplantation, have provided some improvement in survival and quality of life of patients with MM (52,243).

Prognostic Factors

The survival time of individual patients with MM is variable, ranging from few months to more than 10 years (5,210). Various prognostic criteria have been proposed to distinguish between patients who might require different therapeutic strategies.

The most useful clinical staging system, proposed by Durie and Salmon in 1975 (118), is based on a combination of data that correlate with the myeloma tumor cell mass (298). The serum and urine monoclonal Ig concentrations, levels of hemoglobin and serum calcium, and the extent of lytic bone lesions allow patients with low, intermediate, and high tumor mass (stages I, II, and III) to be distinguished (118). Patients with relatively normal renal function are designed as A, whereas those with a serum creatinine level more than 20 mg/L are designed as B, to take into account the poor survival rate associated with renal failure. The variability of survival within a given stage led to the development of other prognostic factors, the most powerful of which is the serum b_2-microglobulin (b_2-M) level, which depends on both tumor mass and renal function. Prospective studies have shown that b_2-M levels at diagnosis are the best predictive factor of survival duration; patients with a low value (b_2-M, less than 4 mg/L) have a two- to three-fold increase in life expectancy compared with those with a high level (28,122,158).

The percentage of dividing plasma cells can be determined *in vitro* by incorporation of tritiated thymidine or by a more convenient immunofluorescence technique using monoclonal antibodies that recognize DNA precursor molecules, such as bromodeoxyuridine or nuclear Ki-67 proliferative antigen (158,161). A plasma cell–labeling index (PCLI) of 1% implies a poor prognosis, and in combination with a serum b_2-M level of 4 mg/L and plasmablastic morphology of myeloma cells, the PCLI allows a subclassification of patients into low-, intermediate-, and high-risk groups (158,283). Indirect measures of myeloma cell proliferation include levels of serum cytokines that can stimulate it, such as IL-6 and its agonist, serum soluble IL-6 receptor (29,283). In addition, the serum CRP level can be used as a surrogate marker of serum IL-6 activity, and its combination with the serum b_2-M level may be a useful staging system (32).

A high serum level of lactic dehydrogenase identifies a small group of patients who have aggressive myelomas with features of extraosseous disease (20). Many other prognostic factors have been identified, but the applicability of very few has been demonstrated (283). In contrast, the prognostic importance of cytogenetic abnormalities is now well documented, particularly of abnormalities involving the 11q chromosome, monosomy and deletions of chromosome 13, which are associated with an adverse outcome and with significantly decreased survival time (267,342).

Various means have been proposed to assess bone status in patients with MM, and serum levels of bone markers, such as peptides derived from type I collagen, were reported to be of prognostic value (95). Response to treatment is a major determinant of prognosis and survival (5,210), but drug sensitivity remains difficult to predict. In particular, studying myeloma cells from newly diagnosed patients for the membrane-associated P-glycoprotein linked to multidrug resistance is of limited interest because it is expressed only in 6% of cases (100). Last, the patient's age is an important consideration, as complications of the disease and treatments are life threatening in older patients (137).

Initiation of Therapy, Monoclonal Gammopathy of Unknown Significance

Patients with overt myeloma require specific treatment to relieve discomfort and to prevent complications by reducing myeloma tumor load. Virtually all patients with an asymptomatic myeloma, who are increasingly diagnosed by chance, will require chemotherapy eventually to prevent or control complications. Patients with early disease progression should be treated soon after diagnosis, but there is presently no evidence that prompt treatment provides a survival benefit to others whose disease may remain stable for many years (171). In addition, inappropriate chemotherapy, particularly with alkylating agents, exposes patients to the risk of acute leukemia, the rate of which may reach 17% at 4 years (41,63).

In asymptomatic patients, the presence of any lytic bone lesion on skeletal survey is usually associated with early progression within a year (6). MRI of the thoracic and lumber spine reveals myelomatous lesions in ~40% of patients with normal bone radiographs. In patients with an abnormal MRI pattern, median time to progression is on the order of 2 years, whereas treatment may be delayed safely for many years when the MRI pattern is normal (233,364). The other prognostic factors identifying patients with early disease progression include serum myeloma protein more than 30 g/L, Bence Jones protein excretion (50 mg/day), IgA type, hemoglobin level less than 12 g/dL, and bone marrow plasmacytosis (25%) (6,128,233,364).

Very slowly progressive malignant plasma cell proliferation often cannot be differentiated from monoclonal gammopathy of unknown significance (MGUS), until the patient eventually develops a clinically distinct MM or related disorder. MGUS is usually considered when the serum monoclonal Ig concentration is less than 30 g/L, plasma cells account for less than 5% of bone marrow cells, and monoclonal Ig in urine is absent or less than 1 g/day. These patients must not have bone lesions (including spinal MRI, if performed), anemia, hypercalcemia, and renal failure. MGUS is diagnosed in 63% of patients with monoclonal Ig (212). Its incidence, which is greater among blacks than whites (89), increases with age and is ~3% in white patients older than 70 years (16,89,179,212). Long-term follow-up indicates that 26% of these patients will develop MM or related disorders 2 to 29 years (median, 10 years) after detection of the monoclonal Ig (212). There is presently no reliable marker for differentiating patients with a benign monoclonal Ig from those with incipient MM (48,212,353).

Conventional Chemotherapy

Since its introduction in the 1960s (4,40), the intermittent oral administration of melphalan (L-phenylalanine mustard) and prednisone remains a therapy of reference. Melphalan and prednisone are usually given for 4 days, and courses are repeated at 4- to 6-week intervals. This chemotherapy results in subjective and objective improvement in a majority of patients. Objective changes include a significant decrease in the monoclonal protein concentration, hematologic improvement, and stable bone lesions. A good response is usually considered when there is a 50% or greater reduction in the concentration of the monoclonal Ig (83), as occurs in ~50% to 60% of patients (329). The disappearance of the monoclonal Ig is noted in less than 3% of patients (329), and cure is exceedingly rare (354). In patients who respond, the disease is usually stabilized within the first year of therapy. At any time, the tumor mass size tends to plateau, and continuation, intensification, or diversification of the therapy induces no further cytoreduction (119). When continued, chemotherapy delays the time of relapse but does not offer a significant advantage in survival time (34). Accordingly, suspension of treatment during the plateau phase is advisable. After a mean period of ~20 months, there is usually a relapse, and the overall median survival time of patients does not exceed 3 years (51).

Other alkylating agents, such as cyclophosphamide and, to a lesser extent, carmustine, are effective drugs in MM (237). Patients resistant to one drug may still respond to the other, prompting the introduction, in the early 1970s, of the combined use of these agents (129). In subsequent studies, cycle-specific drugs were tested, and the addition of vincristine was found to improve remission rate and survival duration (300). Thereafter, the potential advantage to using drugs with differing modes of action has promoted regimens that associate vincristine, melphalan, cyclophosphamide (Endoxan), and prednisone, alternated monthly with vincristine, carmustine, prednisone, and doxorubicin (121).

More recently, interest has been demonstrated in the administration of high-dose glucocorticoids, such as dexamethasone, either alone or in combination with vincristine and doxorubicin to relapsing patients or those with refractory disease (18,68,302). As initial treatment, vincristine, doxorubicin, and dexamethasone are not more beneficial to the survival time than are other multidrug regimens (7,8,302).

Randomized studies have compared melphalan/prednisone with combination chemotherapy regimens. In virtually all trials, combination chemotherapy was superior in terms of incidence of remission, but conflicting results have been reported with regard to survival (51,150,156,338). A recent overview of more than 6,600 patients from 27 trials found no significant difference in the mortality rate overall, in poor-risk patients, or in any subgroup, between the two therapeutic options (338). However, current opinion is that patients with symptomatic MM should be treated with combination chemotherapy rather than with melphalan/prednisone in an attempt to relieve

their discomfort more rapidly and more frequently (8,51).

High-dose Therapy

The demonstration that dose escalation of intravenous melphalan can produce high response rates with apparent complete remissions has provided evidence for a dose–response effect of alkylating agents in MM (229,307). To reduce the duration of the drug-induced myelosuppression with its high risks of morbidity and mortality (307), high-dose melphalan has been combined with stem cell transplantation, which, in addition, has allowed the use of more intensive high-dose regimens, even including total body irradiation (19,131,151,183). Allogeneic, syngeneic, or autologous grafts with blood or bone marrow as the source of hematopoietic stem cells have been used to support various marrow-ablative regimens (19,35,131,144,151,183).

In the past 10 years, the concept of dose intensity with hematopoietic stem cell support has modified the treatment of young patients with MM (243). Transplantation from an allogeneic donor may have the advantage over autotransplantation of a potential "graft versus myeloma" effect (226,341,356), but the procedure still has a high rate of related mortality, even when considered in patients younger than 50 years with human leukocyte antigen (HLA)-identical siblings (36,47). Many more patients are candidates for autologous transplantation, which is now usually performed with peripheral blood stem cells (134). Peripheral blood stem cells have many advantages over marrow stem cells, including an easier collection procedure that avoids general anesthesia, the potential to treat a larger number of patients, lower hazard of reinfusion of malignant cells, and shortened duration of marrow aplasia (132).

Phase II studies (133,358) and pair-mate analyses (21) have suggested that high-dose therapy (HDT) with autologous stem cell support is superior to conventional chemotherapy in newly diagnosed patients with MM. This has been confirmed by a randomized clinical trial that demonstrated benefits of HDT as compared with conventional chemotherapy in terms of higher response rate, longer event-free survival, and longer overall survival (11,12). HDT with autotransplantation may allow a median overall survival exceeding 5 years, whether performed early, as first-line therapy, or late, as rescue treatment for refractory or relapsed disease after conventional chemotherapy (135). Early HDT may be preferred because it is associated with a longer time without any chemotherapy (135). In any case, early autologous stem cell collection is critical for the relatively safe conduct of transplants (132,153).

Controversies remain with regard to the upper age limit at which HDT is of greater benefit than conventional chemotherapy (136,63,318). Melphalan with or without total body irradiation is the most common HDT regimen, but no regimen has been proven superior to others. The benefit of further intensification with tandem HDT and transplantations is still controversial (14,22). Attempts at further improvement also have included reduction of tumor cells in reinfused grafts, for example, with positive selection of $CD34^+$ stem cells (306,343,357).

Whatever the HDT protocol, it is unlikely that any patients will be cured after HDT and autotransplantation, emphasizing the need for additional strategies (13,243). Immunologic approaches, such as immunization against idiotypic determinants of the monoclonal Ig, are under investigation to stimulate autologous immunity to myeloma cells and to treat the residual disease, often minimal, that frequently persists after autografting (260,368). Present studies of allogeneic transplantation also aim to reinforce immunologic antitumor effects while decreasing treatment-related mortality rates through, for example, nonmyeloablative conditioning regimens (322).

Bisphosphonates

Bisphosphonates, which are derived from the natural compound pyrophosphate, are potent inhibitors of bone resorption: they reduce osteoclast development, enhance apoptosis of these cells, and reduce production of most bone-resorbing cytokines (292). Currently biphosphonates are the treatment of choice for hypercalcemia in patients with MM, as in other malignant diseases (52). Bisphosphonates also prevent the skeletal complications of MM bone disease (33), especially the second-generation agent pamidronate, which may reduce by half the risk of a new skeletal event (38). Long-term monthly cycles of intravenous pamidronate are of continued benefit, and a survival advantage over chemotherapy alone has been reported for patients receiving salvage chemotherapy and pamidronate (39). Third-generation bisphosphonates, such as zoledronate and ibandronate, which are much more potent than pamidronate, hold the promise of further reducing the skeletal morbidity in patients with MM (33). In addition, other therapeutic agents might derive from the recent characterization of the osteoprotegerin ligand, which appears to play a key role in osteoclast activation in MM (344).

Other Agents

Thalidomide appears to have an emerging role in the treatment of MM. Used alone, in incremental daily doses beginning at 200 mg, it can induce responses, sometimes marked and durable, in ~30% of patients who relapsed, including those after HDT (321). Major toxicities are neurologic (mainly somnolence) and gastrointestinal (constipation). The duration of response, optimal dose, use in combination with chemotherapy, and use in newly diagnosed patients are being investigated. The mechanism of action of thalidomide in MM is currently unclear but may include antiangiogenesis (282,321).

IFN-a may be used in patients with MM either in combination with chemotherapy during the induction phase or as maintenance treatment, but its actual benefit remains controversial. Indeed, IFN-a may prolong relapse-free survival, but the magnitude of the gain does not exceed a few months and does not translate into a clear prolongation of overall survival (213). Benefit appears as more marginal, as it is achieved at the cost of reduced quality of life from frequent adverse effects.

Recent advances in understanding resistance mechanisms to chemotherapy are resulting in the investigation of novel therapies for MM, including P-glycoprotein inhibitors, which increase intracellular drug transport in tumor cells (100). Therapeutic strategies based on the pathophysiologic role of cytokines in myeloma cell growth, such as approaches blocking IL-6 signal transduction, also are being investigated (249).

Supportive Care

In addition to bisphosphonates, prevention for skeletal complications such as fractures of long bones or vertebral collapses and treatment of bone pain may include cimentoplasty, local irradiation, and, more rarely, surgical fixation. Local radiotherapy is the treatment of choice for solitary plasmocytoma (113). Spinal cord compression also should be treated with radiation therapy, combined with high-dose glucocorticoids; surgical decompression is rarely necessary (68,213).

About 50% of MM patients with symptomatic anemia may benefit from recombinant erythropoietin therapy (usually 150 U/kg subcutaneously 3 times weekly) (213,259). In addition to supportive care, most patients with acute renal failure should promptly receive chemotherapy such as vinblastine and dexamethasone (VAD) or high-dose steroids alone (49,213). The benefit of combining plasmapheresis is controversial (188). For patients with irreversible renal failure, hemodialysis or peritoneal dialysis must be initiated (314). When hyperviscosity is suspected, the decision to perform plasmapheresis depends on the symptoms and changes in the ocular fundus, regardless of the serum viscosity level (150).

Many infections occur in the first months after the initiation of chemotherapy, during which antibiotic prophylaxis may be useful (253). Intravenously administered gamma globulin also may be useful but is inconvenient and costly. Patients may receive pneumococcal and influenza vaccination despite a suboptimal antibody response.

Finally, patients with MM need continuing emotional support, with emphasis on the potential benefits of therapy and the prolonged survival of some patients.

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33 MACROGLOBULINEMIA

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The term macroglobulinemia usually refers to the presence of a serum monoclonal immunoglobulin M (IgM) protein and is not used to describe the polyclonal elevations of IgM associated with infectious or inflammatory conditions. Diseases associated with monoclonal macroglobulinemia include (a) primary or Waldenström's macroglobulinemia (WM), a lymphoplasmacytic lymphoma with distinct clinical features; (b) IgM monoclonal gammopathy of undetermined significance (MGUS), which refers to detection of a monoclonal paraprotein (usually less than 3 g/dL) without any other identifiable disease; and (c) secondary macroglobulinemia, the presence of a monoclonal IgM in association with other malignant neoplasms, including other non-Hodgkin's lymphomas, chronic lymphocytic leukemia, or rarely infectious or inflammatory conditions (1). The distinction between WM and IgM MGUS is somewhat arbitrary, with MGUS progressing to WM in 10% to 30% of patients by 30 years. The distinct clinical presentation of isolated peripheral neuropathy and a serum monoclonal IgM with anti–myelin-associated glycoprotein (MAG) properties is difficult to classify, but may represent a spectrum of the differentiation of MGUS to WM. This chapter describes in detail the epidemiology, clinical and immunologic features, treatment, prognosis, and pathobiology of WM, with mention of MGUS and secondary macroglobulinemia where appropriate.

HISTORICAL FEATURES

In 1944, Jan Waldenström (2) described two patients with a unique clinical syndrome that included oronasal bleeding, mild lymphadenopathy, profound anemia, high serum viscosity, hypoalbuminemia, low fibrinogen, and marked hyperglobulinemia with a globulin fraction that contained “a very large molecule” with a molecular weight of ~1,000,000 (3). In contrast to myeloma, none of these patients had bone marrow plasmacytosis or skeletal lesions. Because these “giant molecules” most closely resembled human antibodies, Waldenström initially suggested this disorder resulted from an immune reaction to an infectious disease of unknown origin, or that inoculation with a virus led to transformation of a human protein into a virus protein.

As serum electrophoresis was performed with increasing frequency, numerous patients were identified with a moderate increase in macroglobulin, but who were otherwise symptomless (4). In 1958, Kappeler et al. (5) maintained that there were two different types of macroglobulinemia, one benign and the other progressive and malignant. By the late 1950s, Waldenström no longer supported his original “immune reaction” hypothesis and proposed that just as myeloma was an unlimited proliferation of one plasma cell clone, macroglobulinemia was a neoplastic disease resulting from unlimited proliferation of lymphocytoid cells “of a special type” (4). This disorder of malignant lymphocytes was termed primary macroglobulinemia or WM, and the Ig designated IgM. In the most recent World Health Organization (WHO) classification of hematologic malignancies and the revised European-American classification of lymphoid neoplasms, WM is classified as a mature B-cell neoplasm and referred to as lymphoplasmacytic or lymphoplasmacytoid lymphoma (6,7).

Skoog and Adams in 1959 (8) and Schwab and Fahey in 1960 (9) suggested that many patients with WM and clinical symptoms attributed to increased serum macroglobulins and elevated serum viscosity might benefit from intensive plasmapheresis. Two patients with WM, severe retinopathy, and congestive heart failure improved markedly and remained asymptomatic with continued plasmapheresis (9). In 1961, Bayrd (10) from the Mayo Clinic reported the first objective responses to the alkylating agent chlorambucil, which remains a mainstay of therapy today. The prognosis of WM has changed little in the last 50 years.

IMMUNOLOGIC HIGHLIGHTS

Like their counterparts in the normal population of Igs, monoclonal Igs have antigen-binding properties, including autoantigen binding (11). Whereas 10% to 15% of patients with WM have autoimmune manifestations such as peripheral neuropathy, Coomb's-positive autoimmune hemolytic anemia (cold agglutinin disease), thrombocytopenia, gastric ulcer with parietal cell autoantibodies, and IgM-cardiolipin syndrome, most patients with WM and an IgM exhibiting autoantibody activity have no clinical manifestations of autoimmune disease (12,13 and 14). The binding patterns of the monoclonal rheumatoid factor (RF) detected in ~15% of patients with WM differ significantly from those of patients with rheumatoid arthritis, perhaps explaining the clinical silence of RF in WM (15,16). In contrast, Varticovski et al. (17) found binding and idiotypic characteristics of the monoclonal IgM in a patient with WM and thrombocytopenia similar to those of a human monoclonal IgM secreted by a hybridoma established from the peripheral blood lymphocytes of a patient with systemic lupus erythematosus and immune thrombocytopenia, and suggested a common origin for autoantibodies in autoimmune diseases and monoclonal gammopathies. Further support for this includes studies of relatives of patients with WM, especially those families with more than one member with WM, which have demonstrated diverse immunologic abnormalities including autoantibodies and abnormal Ig levels, raising the possibility of a common susceptibility gene for WM and autoimmune disorders (18,19).

Immunophenotyping and the high rates of somatic mutation in the Ig heavy-chain (IgH) complementarity-determining regions (CDRs) of tumor cells from patients with WM imply derivation from memory B cells that have undergone antigenic stimulation (20,21). Case reports of cross-reactivity of monoclonal IgM with canary droppings in a bird breeder with WM and of the lipopolysaccharides of *Campylobacter jejuni* in two patients with distinct neurologic manifestations and WM, also suggest a role for antigenic stimulation as the initiating factor in at least a subset of patients with WM (22,23).

Both humoral and cellular immunity are impaired in a subset of patients with WM (24,25,26,27 and 28). The incidence of infectious complications in patients with WM is twice that seen in a control population, but considerably less than in multiple myeloma (25). Hypogammaglobulinemia is present in approximately one third to one half of patients with WM, but levels are less markedly depressed than in multiple myeloma (24,25,28). Most patients with WM exhibit significant impairment of antibody response after immunization with typhoid, diphtheria, and mumps antigens (25). The ratio of CD4 to CD8 T cells is significantly reduced in many patients with WM, because of a decrease in the CD4 subset (26,27). Patients with the highest number of circulating tumor cells had the lowest CD4-to-CD8 ratio, and those with IgM MGUS usually had a normal ratio. More detailed studies of the immune system in patients with WM and IgM MGUS may eventually contribute to our understanding of the mechanism of transformation to more advanced disease. In addition to underlying immune defects in patients with WM, the widespread use of the purine analogs, fludarabine and 2-chlorodeoxyadenosine (2-CdA) to treat this disease, have contributed to the severe immunodeficiency and opportunistic infections seen in some patients (29,30 and 31).

EPIDEMIOLOGY

Currently there are ~1,500 cases of WM reported in the United States each year. The etiology of WM is unknown, but epidemiologic studies have implicated both genetic and environmental factors in a small subset of patients. To clarify the epidemiologic pattern of WM, Groves et al. (32,33) recently evaluated 624 cases of WM reported to 11 population-based cancer registries over a 7-year period. WM is twice as common in men as in women, and rates increase significantly with age, with 0.1 cases per 1 million person-years at risk for those aged 45 years or younger and 16.4 to 36.3 cases per 1 million person-years at risk for those 75 years or older. The rate among black men is half that of white men, in contrast to multiple myeloma, which occurs twice as frequently among blacks.

Genetics

Familial reports of WM are rare, but suggest a genetic component in a small subset of patients, although exposure to a common stimulus cannot be completely ruled out in these cases. After the first two reports of familial WM in 1962, Seligmann (34) studied the sera of 216 close relatives of 65 patients with WM. In seven of the 65 families, there was an asymptomatic monoclonal IgM identified in at least one relative; in 45 of the 65 families, abnormal Ig levels without an “M-component” were noted; and there was an increased incidence of anti-g-globulins in family members younger than 60 compared with normal controls. A more recent case–control study of 65 patients with WM in the Baltimore area showed that cases were more likely than were controls to have a first-degree relative with a history of pneumonia,

diphtheria, rheumatic fever, and diabetes mellitus (18). In addition, laboratory tests of 109 first-degree relatives of these patients demonstrated that close to 40% had diverse immunologic abnormalities.

Blattner et al. (19) reported WM in a father and three offspring, all of whom had the identical human leukocyte antigen (HLA) haplotype A2, B8, and DRw3. Interestingly, multiple family members also had autoantibodies including antithyroid microsomal antibody, antithyroglobulin antibody, and antimyocardial antibody. Whereas the findings in this family favor a chromosomal linkage between the HLA complex and a susceptibility gene for WM and autoimmune disorders, other reported families do not share a common haplotype (35). Additional studies are needed to determine whether the consistent finding of familial immunologic dysfunction in patients with WM is etiologic in the development of WM, or whether both the immunologic dysfunction and WM are secondary to an as-yet-unidentified genetic defect or environmental stimulus.

Ögmundsdóttir et al. (36,37) followed up an Icelandic family with macroglobulinemia and B-cell malignancies for more than 20 years. Of a sibship of seven, one had WM; one, malignant lymphoma; and two, benign monoclonal gammopathy. Increased serum IgM levels were subsequently detected in nine additional family members in three generations. Additional studies of 35 or more family members have shown a significant increase in Ig production after minimal stimulation to pokeweed antigen compared with controls, but no difference in serum levels of interleukin (IL)-4 or alterations in natural killer (NK) cell function, known regulators of B-cell function (36). More recently, these authors have shown that family members with increased Ig production have enhanced B-cell survival, which is associated with increased expression of the programmed cell death–repressor gene, *bcl-2* (37). It is not known whether Bcl-2 overexpression is a primary or secondary phenomenon.

Environmental

Environmental factors are not yet known to play a significant role in the etiology of WM. One case-control study found no association between WM and a variety of occupational exposures including benzene, petroleum, lead, asbestos, and radiation and no significant difference between cases and controls in tobacco or alcohol use (18). Three cases of WM in shoe repairers in Sheffield, England, a catchment area that included only ~20 shoe repairers, suggests an association with leather or rubber dust, alcohol-based dyes, or organic solvents (38). One case of WM in a bird breeder with monoclonal IgM antibody activity to canary droppings also indicates a possible role for antigenic stimulation in some cases, similar to that seen between *Helicobacter pylori* and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (22).

An association between WM and the hepatitis C virus (HCV) has been confirmed in several small studies (39,40,41 and 42). Most show a high rate of HCV antibodies only in the subset of WM with a monoclonal IgM with cryoglobulinemic activity (40,41). However, a report from southern Italy showed an extremely high prevalence of HCV in patients with a variety of B-cell lymphoproliferative disorders (22.4%) and an extraordinarily high rate in WM (61%) (42). Recent reports also suggest a possible association between WM and the hepatitis G virus (HGV) (43,44 and 45). De Rosa et al. (42) proposed that HCV and HGV may be causative in a subset of WM either by producing an early hit in a sequence of oncogenic events or indirectly by causing expansion of lymphoid clones that may subsequently undergo mutational events. Additionally, HCV triggers autoimmunity, and the association between non-Hodgkin's lymphoma (NHL) and autoimmune disorders also suggests this as a possible mechanism (46).

Recently human herpesvirus-8 (HHV-8) DNA has been isolated from the marrow and dendritic cells of some patients with multiple myeloma and WM, although this finding has been questioned by others (47,48 and 49). Interestingly, HHV-8 contains a biologically active homolog to IL-6, an important growth factor for plasma cells (50,51 and 52). In some cases, HHV-8 may play a causative role in the transformation of MGUS to a malignant lymphoproliferative disorder and the propagation of malignant cells (47).

CLINICAL PRESENTATION

Many patients are asymptomatic and are diagnosed during evaluation of an incidental laboratory abnormality such as mild anemia, an elevated serum protein, or hypoalbuminemia. Initially it can be difficult to distinguish between asymptomatic WM and an IgM MGUS (53).

Signs and Symptoms

Dimopoulos et al. (28,54) in a recent review categorized the clinical manifestations of WM according to whether they were related to direct tumor infiltration, to complications of circulating IgM protein, or to deposition of the IgM protein in various tissues. Patients with extensive marrow involvement often have weakness, anorexia, dyspnea, or fatigue secondary to anemia. Patients may report fever, night sweats, or weight loss, most commonly in the late stages of the disease. Abdominal pain or bloating may occur secondary to hepatosplenomegaly (55). Localized bone destruction and severe bone pain occur in fewer than 2% of patients with WM (28,53). There are also rare reports of pulmonary infiltrates with dyspnea or cough, involvement of the stomach or intestine seen as diarrhea and malabsorption, and skin manifestations including urticaria, rash, and paraneoplastic pemphigus (56,57,58,59,60,61 and 62).

When IgM levels greater than 3 to 4 g/dL are present, patients may experience signs and symptoms of hyperviscosity syndrome, most commonly bleeding and ocular, neurologic, and cardiovascular manifestations (54). Hyperviscosity occurs because of the increased concentration of the large, pentameric IgM molecule. Easy bleeding of mucous membranes, most commonly the gums and nose, complaints of blurred or decreased vision, headaches, vertigo, drowsiness, hearing loss, confusion, altered mental status, or seizures should prompt evaluation of serum viscosity. The increased plasma volume associated with macroglobulinemia may lead to congestive heart failure, especially in older patients receiving red cell (RBC) transfusions for anemia.

Approximately 5% of patients with WM will experience symptoms or complications of cryoglobulinemia including arthralgias, acrocyanosis, Raynaud phenomenon, palpable purpura, renal insufficiency, or peripheral neuropathy (54,63,64 and 65). The cryoprecipitate may activate complement and create an immune complex vasculitis resulting in ischemia of skin, nerve, and renal tissues (28). Ten percent of patients with WM have cold agglutinin disease (28). The IgM will react with RBC antigens at temperatures less than 37°C and result in episodic or chronic hemolytic anemia.

Peripheral neuropathy is reported in 5% to 45% of patients with macroglobulinemia and can antedate hematologic symptoms by years (66,67). As discussed in detail later in this chapter, most cases of neuropathy are believed to result from the monoclonal IgM protein binding to a component of myelin or the axolemma (67,68 and 69). Nerve-conduction studies are consistent with a demyelinating neuropathy in most patients (69). Typically, the neuropathy affects the lower extremities first and is slowly progressive, symmetric, and predominantly sensory (67). Patients commonly experience foot numbness, painful paresthesias, imbalance, gait ataxia, and occasionally tremor (68). Neurologic symptoms are more heterogeneous in the subset of patients in whom IgM does not react with any known nerve antigen or has non-MAG specificity (67,68,69 and 70). Pure motor neuropathy has been described (68). Muscle weakness also may result from a humorally mediated immune myopathy (71).

Amyloidosis is a rare complication of macroglobulinemia (72,73). Deposition of polymerized light-chain fragments can result in cardiomyopathy, renal insufficiency, liver dysfunction, autonomic neuropathy with hypotension, impotence and bladder dysfunction, and sensorimotor peripheral neuropathy (72,73).

Physical Findings

Physical examination may reveal lymphadenopathy and hepatosplenomegaly. Findings associated with hyperviscosity syndrome include altered mental status, decreased visual acuity, retinal exudates or hemorrhage, and sausage-linked retinal veins. Neurologic examination in those afflicted with peripheral neuropathy most often reveals sensory loss, hyporeflexia, gait ataxia, and occasionally muscle weakness and tremor.

Laboratory Tests

Serum protein electrophoresis (SPEP) and serum immunofixation are critical for the diagnosis and follow-up of WM (74,75 and 76). An SPEP should be repeated approximately every one to two cycles in patients receiving treatment and every 2 to 4 months during periods of observation. Alternatively, disease activity and effectiveness of therapy can be assessed with serial quantitative IgM levels. IgG and IgA levels are often decreased in patients with WM (53). Urine protein electrophoresis (UPEP) detects the presence of Bence Jones proteins in the majority of patients with WM; however, levels more than 1 g/dL are uncommon (28,53,77). Serum viscosity should be checked in all patients with an IgM monoclonal protein. Elevated serum viscosity occurs most commonly when IgM levels are greater than 4 g/dL, and symptoms of hyperviscosity are rare if viscosity is less than 4 cp (centipoises) (74). Serum should be evaluated for the presence of cryoglobulins in patients with a history of cold sensitivity. Type I cryoglobulins are detected in 10% to 20% of patients with WM, but less than 5% are clinically evident (53,54).

Many patients have a normochromic, normocytic anemia, with the peripheral blood smear showing rouleaux formation. Macroglobulinemia can result in an expanded plasma volume and consequently a factitiously low hemoglobin. Direct measurements of red cell volume may be warranted in patients who are at high risk for transfusion complications because of hypervolemia (78). Episodic or chronic hemolytic anemia can occur when the IgM protein acts as a cold-agglutinin (54). Thrombocytopenia occurs occasionally. All patients with WM have an elevated erythrocyte sedimentation rate. Hyponatremia may result from an inability to excrete water and has been attributed to tumor production of a substance functionally similar to antidiuretic hormone (ADH) in a subset of patients with WM (79).

Hypercalcemia and renal insufficiency are rare. The most common abnormalities of coagulation are a prolonged bleeding time and thrombin time attributed to platelet coating by macroglobulins and binding of Ig to fibrin, respectively (1,80,81).

Pathology

Current classifications of lymphoid malignancies categorize WM as a mature B-cell neoplasm, specifically a lymphoplasmacytic or lymphoplasmacytoid lymphoma (6,7). Morphologically, the malignant cells have eccentric nuclei with paranuclear clearing similar to that of plasma cells, but the nuclear outline is often slightly irregular, and the nuclear chromatin is diffusely arranged (82). Dutcher bodies, intracytoplasmic inclusions due to cytoplasmic Ig, are a classic histologic finding in WM (82). Lymph node and bone marrow biopsies often show a pleomorphic infiltrate composed of small lymphocytes, lymphoplasmacytic lymphocytes, and plasma cells (83). Marrow involvement can have a diffuse, nodular, or nodular/interstitial appearance, and the growth pattern in lymph nodes is often interfollicular (6,83). Increased numbers of mast cells and basophils are seen in affected tissues.

Because other mature B-cell neoplasms including chronic lymphocytic leukemia (CLL), marginal cell, mantle cell, and follicle center cell lymphomas occasionally show maturation to plasmacytoid or plasma cells and contain cytoplasmic Ig, immunophenotyping is often used to differentiate lymphoplasmacytic lymphomas from other B-cell neoplasms (6). Lymphoplasmacytic lymphomas strongly express the B-cell–associated antigens CD19, CD20, CD22, CD79a, and surface IgM, but lack or weakly express IgD, CD5, CD10, CD23, and CD38 (24,84,85 and 86). The immunophenotype may vary because of the pleomorphic cells of WM, with small lymphocytes being strongly positive for surface Ig and negative for CD38, and plasma cells being negative for surface Ig and strongly positive for CD38 (54).

Treatment and Prognosis

WM is incurable with standard therapies. Goals of therapy are to palliate or prevent symptoms and to prolong survival. Therapy often is deferred in asymptomatic patients. Patients observed off therapy must agree to close follow-up, including a history and physical examination, IgM level, and complete blood count (CBC) every 3 to 6 months. The goal is to intervene when the disease appears to be progressing, but before complications of anemia, splenomegaly, or hyperviscosity occur. Although prospective randomized trials comparing immediate with deferred treatment have not been done in WM, there is no evidence in other low-grade lymphoproliferative disorders that immediate treatment of asymptomatic patients prolongs survival (13).

PLASMAPHERESIS

Emergency therapy is needed for signs and symptoms of hyperviscosity. Plasmapheresis rapidly reduces IgM levels and serum viscosity and usually results in marked improvement in symptoms including mental status changes, visual disturbances, and bleeding (9,87). Techniques used to remove IgM paraproteins include centrifugal plasma exchange and membrane plasma filtration. Plasma exchange results in nonselective removal of plasma and requires volume replacement with albumin or fresh frozen plasma. Intensive plasma exchange can result in a transient decrease in platelets, complements, coagulation factors, and gammaglobulins (87,88). However, plasmapheresis stimulates rapid antibody rebound with levels reaching 50% to 80% of preplasmapheresis levels in 48 hours and peaking after 1 to 2 weeks, much faster than the 30 days that would be predicted based on normal synthesis and catabolism rates of IgG (89). Membrane plasma exchange, which selectively removes macromolecules and does not require replacement fluid, was introduced in 1978 and is used commonly in Europe and Japan, but not in the United States (87).

Because the response to plasma exchange is transient, as a single modality it must be repeated every 1 to 4 weeks to maintain an acceptable serum viscosity (90). Consequently, cytotoxic agents are generally given concurrently, and plasmapheresis discontinued after a few weeks or months, if the disease responds. Interestingly, some patients resistant to cytotoxic therapy whose predominant symptoms are related to hyperviscosity without symptomatic organomegaly, lymphadenopathy, or pancytopenia are successfully maintained for months or years on intermittent plasma exchange (90,91). There also are several reports of plasma exchange slowing progression of macroglobulinemia-associated neuropathy (68,87,92).

CHEMOTHERAPY

Historically, first-line treatment for WM has been oral alkylating agents, primarily chlorambucil. IgM levels decrease slowly, and several months of chlorambucil treatment may be required before a documented objective response occurs (93). Daily (0.1 mg/kg/day) and intermittent (3 mg/kg daily for 1 week, every 6 weeks) schedules have equivalent response and survival rates (28,55,93). In a recent randomized trial comparing daily with intermittent therapy, the M-protein or hemoglobin improved in 88% of patients given daily doses and in 68% of patients given intermittent doses, with the onset of response for hemoglobin being 5 to 7 months, and for IgM improvement, 18 to 22 months (93). Complete responses are unusual. Treatment is generally continued for 3 to 6 months after stabilization of IgM levels and restarted when symptoms develop or IgM levels increase above 3 to 4 g/dL. There is no evidence that maintenance therapy improves survival rates, and prolonged exposure to alkylating agents increases the risk of myelodysplasia or secondary leukemia (94,95).

The addition of steroids to chlorambucil does not improve survival or response rates, but may be useful in treating autoimmune hemolytic anemia, cryoglobulinemia, or cold-agglutinin disease associated with WM. Chemotherapy regimens combining alkylating agents with vinca alkaloids, anthracyclines, or nitrosoureas have occasionally shown higher response rates than single-agent alkylators, but none has shown an improvement in survival (28,96,97,98,99 and 100).

More recently, the purine analogs fludarabine and 2-CdA have been tested in WM with encouraging results. Response rates ranged from 33% to 85% in previously untreated patients (29,101,102,103 and 104). Median time to response was ~6 months, with median response durations of 2 to 3 years (29,101). IgM levels can continue to decrease for several months after discontinuing therapy (30). The overall response rate to 2-CdA using a 7-day continuous infusion schedule appears similar to that of a 5-day daily bolus regimen; however, time to response with the continuous infusion was significantly shorter in a nonrandomized comparison (29,103,104). Evaluation of the combination of alkylating agents and purine analogs is under way in many indolent lymphoproliferative disorders, including WM. Weber et al. (105) reported an 89% response rate in 19 previously untreated patients with WM treated with 2-CdA and oral cyclophosphamide.

Both 2-CdA and fludarabine also have been effective in treating disease refractory to alkylating agents, again with reported response rates ranging from 22% to 78% (102,106,107,108,109 and 110). Higher response rates were recorded among patients relapsing off treatment (78%) than in patients who were resistant to first- or second-line treatment, with the worst response rates in those patients who had resistant disease of more than 1-year duration (22%–57%) (109). A randomized multicenter trial compared six cycles of fludarabine with six cycles of CAP (cyclophosphamide, doxorubicin, prednisone) in 92 patients with WM resistant to first-line therapy or in first relapse after alkylating agents (111). Thirteen (28%) patients responded to fludarabine compared with five (11%) patients with CAP (p = .019).

The primary side effects of 2-CdA and fludarabine are myelosuppression and immunosuppression. Myelosuppression is usually moderate, but repetitive courses of these agents can produce cumulative and delayed pancytopenia. Dramatic decreases in $CD4^+$ and $CD8^+$ lymphocytes occur after a single cycle, significantly increasing the risk of opportunistic infections (29,30 and 31). CD8 counts generally recover in 3 to 12 months, but CD4 counts may remain profoundly depressed for more than 2 years after treatment (30). The use of fewer courses of nucleoside analogs may result in less myelosuppression and immunosuppression without significantly decreasing the chance of response (103,112).

The therapeutic potential of high-dose radiochemotherapy and stem cell rescue in multiple myeloma and other low-grade lymphoproliferative disorders has led to clinical evaluation of this approach in a limited number of younger patients with WM. Preliminary results of autologous stem cell transplant in 18 patients showed responses in all patients, but persistent paraproteinemia in the majority. However, as with other indolent B-cell neoplasms, remissions appear to be more durable than with standard therapies (113,114 and 115). Martino et al. (116) described a 34-year-old woman and 39-year-old man with aggressive, refractory WM, who remain in complete remission 3 and 9 years after HLA-identical allogeneic transplants.

BIOLOGIC THERAPY

Use of the anti-CD20 monoclonal antibody, rituximab, is also under study for patients with WM. CD20, a pan-B-cell antigen that does not internalize or shed, is expressed on nearly all lymphoplasmacytoid lymphomas. The mechanism of action of rituximab is not completely understood, but evidence suggests it acts through stimulating both antibody-dependent cytotoxicity and cell-mediated cytotoxicity, as well as perhaps having a direct effect on apoptosis (117). Small series evaluating rituximab in relapsed or refractory WM reported response rates of 23% to 75% (117,118,119 and 120). Most had failed to respond to both purine analogs and alkylators. Responses lasted a median of 6 to 9 months. Treatment with rituximab does not induce cytopenias or cellular immune dysfunction, a distinct advantage over chemotherapy. Studies are ongoing to test the efficacy and synergy of rituximab in combination with chemotherapy.

Another biologic agent, interferon, also has shown some activity in WM. Rotoli et al. (121) reported a partial response in 33% and a minor response in 16% of 36 patients with WM treated with a-interferon, 3 million units (MU) per day for 1 month and then 3 times a week, with most responses occurring after 4 to 6 months of treatment. Doses of a-interferon as low as 1 MU 3 times a week have resulted in decreased IgM levels in 28% of patients and an improvement in hemoglobin in 42% (122). Higher doses are poorly tolerated and not used routinely in indolent B-cell neoplasms. IgM-associated neuropathy also occasionally responds to a-interferon (123).

SPLENECTOMY

Although splenectomy is not usually reported as a treatment for WM, a case report and review of the literature published in 1995 described 14 patients with massive splenomegaly refractory to chemotherapy who underwent splenectomy (124). Three had no response, but in 11 patients, splenectomy resulted in a major decrease or even complete disappearance of the IgM paraprotein. One response lasted 13 years, and another is ongoing at 12 years. The authors hypothesized that the spleen may be a favored site of growth of IgM-producing malignant B cells. Observations in humans and mice, including a permanent decrease in IgM levels and an impaired IgM response to pneumococcal vaccine after splenectomy in humans, and low levels of IgM but normal levels of IgG and IgA in neonatally splenectomized mice, suggest the spleen may provide a special environment for differentiation or homing of normal IgM-producing B lymphocytes (124,125,126,127 and 128).

Prognosis

Most series reported median survivals of ~5 years, with longer survivals for responders (median, 7.7 years) than for nonresponders (median, 2 to 3 years) (28,129). Approximately 20% of patients survive more than 10 years (77,130). Transformation to aggressive lymphomas occurs in a small percentage of patients and is usually fatal (131,132). Approximately 20% of patients with WM die of unrelated causes (28,129).

Because of small patient numbers, few trials have been able to define prognostic factors accurately for patients with WM. In multivariate analyses, age 60 years or older, anemia, male gender, low neutrophil count, weight loss, cryoglobulinemia, elevated serum b_2 microglobulin (sb_2M), and low IgM levels have all been identified as independent poor prognostic variables (110,129,133). Investigators in the Southwest Oncology Group and Eastern Cooperative Oncology Group identified low sb_2M and high IgM levels as favorable predictors of overall survival (OS) and progression free survival (PFS) in 234 patients with WM participating in a clinical trial of fludarabine (111). Three risk groups were identified. A low-risk group with sb_2M less than 4 g/L had a median PFS of 61 months (median OS not yet reached), an intermediate-risk group with sb_2M 4 g/L or greater and IgM 4 g/dL or greater with a median OS of 42 months and PFS 31 months, and a high-risk group with a sb_2M 4 g/L or greater and IgM less than 4 g/dL with OS 26 months and PFS 7 months. The basis for the observation of a relatively favorable prognosis with high IgM is not understood, although the authors hypothesized that this may be associated with more differentiated disease. A second prognostic model based on age, albumin, and total number of cytopenias has also been proposed (134). This model also successfully identified three risk groups with 5-year survival rates of 86%, 61%, and 26%, depending on the number of poor prognostic features.

PATHOBIOLOGY AND IMMUNOPATHOLOGY

The malignant cells of WM are characterized by a postgerminal center phenotype ($CD19^+$, $CD20^+$, $CD5^-$, $CD10^-$, $CD23^-$, IgD^-) and therefore are believed to arise from marginal zone memory B-cells (86). Analysis of the DNA sequences of the IgH CDRs of tumor cells from patients with WM supports this theory (20,21). Somatic hypermutations, which are essential for the affinity maturation of antibody, occur most frequently in IgH CDRs during the generation of memory B cells (21). High rates of somatic mutation were found in most cases of WM, implying derivation from B cells that have undergone antigenic stimulation (20). In normal memory B cells, antigenic stimulation is often accompanied by antibody class switching to IgG. Some investigators proposed WM cells are B cells that carry somatic Ig mutations, but have lost the ability to undergo class switching (20,21,54). Alternatively, the malignant cells of WM may derive from the more recently described subset of IgM-only expressing memory B cells (135,136,137 and 138).

Il-6 has been implicated in the differentiation process of both IgM MGUS and WM (52). Levy et al. (52,139) showed that neutralizing anti–IL-6 antibodies abrogated spontaneous B-cell differentiation and IgM secretion in patients with IgM MGUS, but not those with WM, possibly indicating a two-step pathogenesis for this disease. All *trans*-retinoic acid (RA), a compound known to interfere with IL-6–dependent growth of plasmacytoma cell lines, inhibited *in vitro* differentiation of purified B cells in four of five patients with MGUS, but only in one of five patients with WM (139). RA appeared to inhibit IL-6 secretion by B cells and downregulated the IL-6 receptor on B cells. The absence of RA-mediated regulation of B-cell differentiation in WM, in contrast to MGUS, may represent a step toward malignancy in WM (139). Understanding the mechanism of autonomous IL-6 secretion in WM may have therapeutic implications (139).

Approximately 30% of patients with WM have abnormal karyotypes in tumor cells. However, there is no “common” chromosome change, as described in other lymphomas such as follicular center cell (t14;18), Burkitt (t8;14), or mantle cell (t11;14). Described abnormalities include trisomies or deletions of chromosomes 3, 6, 10, 11, 12, 15, 18, 20, and 21, with some patients having complex karyotypes (84,85,140,141,142 and 143). In other subtypes of lymphoma, overexpression of genes such as *bcl-2* on chromosome 18, *myc* on chromosome 8, and *PAX*-5 on chromosome 9 juxtaposed to the strong promoter elements of IgH on chromosome 14 are thought to contribute to lymphomatogenesis (54,144). Isolated cases with these abnormalities also have been described in WM, but do not explain the pathogenesis of most cases of WM.

In addition to trying to determine how and why WM develops, work continues on understanding the pathologic consequences and mechanisms of injury of monoclonal macroglobulinemia, both circulating IgM and tissue IgM. As discussed briefly in the introduction, the diverse antibody properties of monoclonal IgM account for many of the unique clinical and laboratory features of WM and have been studied in detail. Interestingly, the spectrum of activities of IgM monoclonal components does not reflect the frequency with which various specificities are represented in the total pool of antibody-forming cells (145). This provides further evidence that the monoclonal Ig-producing cells are memory B cells participating in an immune response when they undergo monoclonal proliferation. Given this, it is striking that so many of the observed activities of the monoclonal IgMs fall into the autoantibody class (145). Possible explanations for this increased repertoire of autoantibodies include cross-reactivity of antibodies produced in response to an exogenous antigenic stimulus to self-antigens, or alternatively, that memory B cells with autoantibody activity are more prone to malignant transformation (146).

The first reports of antibody activity of a monoclonal IgM occurred in 1957 with the association of cold-agglutinin disease and high titers of monoclonal IgM (145,147,148 and 149). The protein is usually IgMk, and its most common target is the Ia antigen on RBCs (28,63,64). Rosse et al. (65) suggested that conformational changes occur in RBC membranes at low temperatures, exposing epitopes not accessible to IgM at 37°C. The RBCs may fix complement and agglutinate in colder areas of the circulation, producing Raynaud syndrome, acrocyanosis, livedo reticularis, and episodic or chronic hemolytic anemia (28). The cold agglutinin dissociates in warmer parts of the body, but C3d complement remains on the RBCs, resulting in a positive Coomb's test.

The most studied complication of tissue deposition of monoclonal IgM is that of polyneuropathy seen in 10% or more of patients with WM. Multiple pathogenetic mechanisms have been proposed for these neuropathies, accounting for the diversity of clinical findings. Dimopoulos et al. (54) divided the neuropathies of WM into five subsets: (a) demyelinating polyneuropathy with IgM anti-MAG antibodies; (b) demyelinating polyneuropathies with monoclonal IgM reacting with gangliosides, but not MAG; (c) polyneuropathies with monoclonal IgM nonreactive with known peripheral nerve antigens; (d) cryoglobulinemic neuropathy; and (e) amyloid neuropathy.

In at least half the cases of polyneuropathy in WM, a monoclonal IgMk targeted against the minor carbohydrate epitopes of MAG is identified (150,151). The anti-MAG IgM paraprotein co-reacts with a glycolipid on peripheral nerves, sulfoglucuronyl glycosphingolipid (152). Because MAG is widespread in both central nervous system (CNS) and peripheral nerves, whereas sulfoglucuronyl glycosphingolipid is present only on peripheral nerves, the latter may be the more likely target, given the lack of CNS manifestations in patients with peripheral neuropathy and anti-MAG IgM. Direct immunohistochemical study of peripheral nerve biopsy specimens shows deposition of M protein on myelin sheaths (153). Monaco et al. (154) used direct and indirect immunofluorescence and immunoperoxidase methods to demonstrate colocalization of monoclonal proteins and complement components C1q, C3d, and C5 along myelin sheaths of peripheral nerve fibers, suggesting that demyelination in this disorder may be complement mediated. Terminal complement complex also was detected in myelin sheaths, specifically at sites of separation of myelin lamellae, suggesting that the terminal complex contributes to myelin damage. Inflammatory cells can be seen infiltrating the nerves in some cases of IgM-associated neuropathy. Immunohistochemical staining of these cells shows equal distributions of CD4- and CD8-positive T cells, but no B cells, indicating a possible role for cell-mediated immunologic mechanisms (155).

A number of non-MAG nerve-related antigens also have been implicated in immune-mediated peripheral neuropathy (68). Identified antigens include the gangliosides, complex glycosphingolipids (GM1, GM2, GD1a, GD1b, GT1b, GQ1b, and LM1), sulfatide, and chondroitin sulfate C (66,67,68,69,70,151,152,156,157 and 158). Patients with anti-GD1b and anti–chondroitin sulfate C have a predominantly motor impairment with axonal degeneration (69,159). Patients with anti–chondroitin sulfate C antibodies also often have epidermolysis because of cross-reactivity in the skin (159).

Monoclonal IgMs with antibody activity against gangliosides with disialosyl moieties are associated with chronic ataxic neuropathy and occasionally ophthalmoplegia and RBC cold-agglutinin activity (23,70,160). Willison et al. (160) prepared a human hybridoma secreting an antidisialosyl monoclonal IgM from peripheral blood B cells of an affected patient, and injected the affinity-purified antibody intraperitoneally into mice. Electrophysiologic recordings demonstrated impairment of nerve excitability, confirming the pathophysiologic effects of the antibody. Interestingly, these same authors showed that purified antidisialosyl paraproteins from two affected patients bound not only a wide array of peripheral nerve structures, but also lipopolysaccharides of *Campylobacter jejuni* isolates in all of three cases of Miller Fisher syndrome, a variant of Guillain-Barré syndrome characterized by acute ataxia, areflexia, and ophthalmoplegia (23). Based on these data, Jacobs et al. (23) suggested the following possible pathophysiologic sequence: (a) individuals are infected with *C. jejuni* or other pathogens bearing disialylated gangliosidelike epitopes, (b) a low-level low-affinity IgM response is mounted with insufficient autopathogenic potential to cause an acute postinfectious neuropathy such as Miller Fisher syndrome, (c) transformation from a state of antigen-dependent B-cell proliferation to autonomous B-cell proliferation with the formation of monoclonal IgM, and (d) increase in serum

monoclonal antibody levels to a level at which binding kinetics activate proinflammatory pathways in target membranes. Further clinical evidence to support this hypothesis of prolonged antigenic stimulation as the etiology of some cases of WM includes a description of a canary breeder who developed pulmonary infiltrates, initially thought to be consistent with “breeder's disease,” a hypersensitivity pneumonitis (22). Lack of improvement with steroids and avoidance of canary exposure led to reevaluation of the patient's disease. A serum monoclonal IgM and lymphoplasmacytic infiltrate of the bone marrow confirmed the diagnosis of WM. Purified monoclonal IgM from the patient precipitated canary's droppings, implying antibody activity against some antigen found in the droppings.

As discussed previously, 10% to 15% of patients with WM have a cryoglobulin, but less than 5% have symptoms or complications of cryoglobulinemia. Most are type I cryoglobulins (monoclonal IgM), but occasionally mixed Ig complexes in which monoclonal IgM behaves like an antibody against polyclonal IgG (type II) occur in WM (28,53). The mechanism of cryoprecipitation is not well understood, but most clinical manifestations are thought to be related to the physicochemical properties of the cryoprecipitate. However, the cryoprecipitate also may activate complement sequences, resulting in an immune complex vasculitis with ischemia of the skin, nerves, and renal tissues (28).

FUTURE DIRECTIONS

Work continues to understand the pathogenesis of WM, with exciting leads for both genetic and environmental etiologies in at least some, if not at all, cases. Continued study of the similarities and contrasts between WM and the primary autoimmune diseases may lead to a better understanding of the mechanisms of both diseases. Ultimately, this information may lead to improved targeted therapies.

Treatment results remain unsatisfactory, and new approaches are needed. Current therapies have not changed the natural history of the disease. Multiinstitutional trials are essential, given the low incidence of the disease. Radiolabeled monoclonal antibodies, effective in other indolent lymphomas, are under investigation for patients with WM. Work also continues to better define the most effective dose and schedule for the monoclonal antibody rituximab in WM. Ultimately, new strategies for tumor-specific, rational drug development are needed (161).

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34 AMYLOIDOSIS

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Amyloidosis is a disorder of protein folding, characterized by extracellular deposition of abnormal protein fibrils (1). The underlying molecular abnormalities may be either acquired or hereditary, and about 20 different proteins can form clinically or pathologically significant amyloid fibrils *in vivo* (Table 34.1 and Table 34.2). Amyloid deposits also contain glycosaminoglycans, some of which are tightly associated with the fibrils, and a nonfibrillar plasma glycoprotein, amyloid P component (AP). Small, focal, clinically silent amyloid deposits in the aortic wall, brain, heart, seminal vesicles, and joints are a universal accompaniment of aging. However, clinically important amyloid deposits usually accumulate progressively, thereby disrupting the structure and function of affected tissues and leading inexorably to organ failure and death. No treatment yet exists that specifically causes resolution, but intervention that reduces availability of the amyloid fibril precursor proteins may lead to regression of this condition.

TABLE 34.1. Acquired Amyloidosis Syndromes

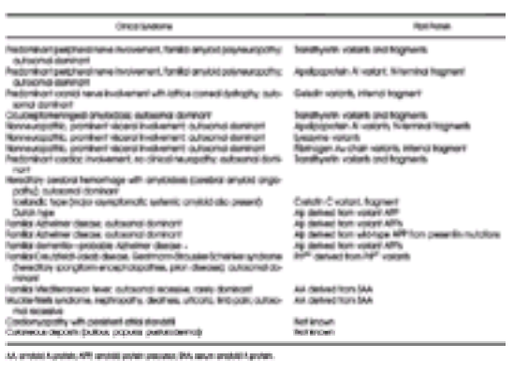

TABLE 34.2. Hereditary Amyloidosis Syndromes

Clinically significant amyloidosis is not rare. Amyloid deposits in the brain and cerebral blood vessels are a central part of the pathology of Alzheimer disease, which is the fourth most common cause of death in the Western world, whereas amyloid is present in the islets of Langerhans of the pancreas in all patients with type 2, maturity-onset, diabetes mellitus. Amyloid deposition in the bones, joints, and periarticular structures eventually affects most patients who receive long-term hemodialysis for end-stage renal failure and is the most frequent cause of serious morbidity among the approximately 800,000 such patients worldwide. Systemic amyloidosis complicating myeloma and other B-cell dyscrasias, or chronic infections and inflammatory diseases, is important because the diagnosis is often difficult and the prognosis is poor, but effective treatments are increasingly available. Hereditary amyloidosis is extremely rare, except in a few geographic foci, but its diversity is remarkable. It is important because of its poor prognosis, the complexity of clinical management, the difficult genetic issues involved, and its considerable value as a

model for understanding the pathogenesis of amyloid deposition.

Although some correlations exist between fibril protein type and clinical manifestations, many forms of acquired and hereditary amyloidosis have little or no concordance between the fibril protein, or the genotype of its precursor, and the clinical phenotype. Evidently, certain genetic and environmental factors, which are distinct from the amyloid fibril protein itself, determine whether, when, and where clinically significant amyloid deposits form. The nature of these important determinants of amyloidogenesis is obscure. Furthermore, the mechanisms by which amyloid deposition causes disease are poorly understood. Whereas a heavy amyloid load is invariably a bad sign, there is often a poor correlation between the local amount of amyloid and the level of organ dysfunction. Active deposition of new amyloid is often associated with enhanced deterioration compared with stable, long-standing deposits. Nascent or newly formed amyloid fibrils, generated *in vitro,* are also cytotoxic to cultured cells, whereas aged or *ex vivo* fibrils are generally inert, although it is not known how this relates to effects *in vivo.*

AMYLOID FIBRILS

Regardless of their diverse protein subunits, amyloid fibrils of different types are remarkably similar: usually straight, rigid, nonbranching, of indeterminate length, and 10 to 15 nm in diameter (2). They are insoluble in physiologic solutions and relatively resistant to proteolysis, and they bind Congo red dye and produce pathognomonic green birefringence when they are viewed in polarized light (3). Electron microscopy reveals that each fibril consists of two or more protofilaments, the precise number varying with the fibril type. The X-ray diffraction patterns of all the different *ex vivo* amyloid fibrils and synthetic fibrils formed *in vitro* demonstrate the presence of a common core structure in the filaments, in which the subunit proteins are arranged in a stack of twisted antiparallel b-pleated sheets lying with their long axes perpendicular to the fibril long axis (4,5). Observations show that many different proteins, including molecules totally unrelated to amyloidosis *in vivo,* can be refolded after denaturation *in vitro* to form typical, stable, congophilic cross-b fibrils (6,7). Although it is not clear why only the 20 or so known amyloidogenic proteins adopt the amyloid fold and persist as fibrils *in vivo,* a major unifying theme that is currently emerging is that in all cases studied the precursors are relatively destabilized (8,9). Even under physiologic or other conditions they may encounter *in vivo,* they populate partly unfolded states involving loss of tertiary or higher-order structure that readily aggregate with retention of b-sheet secondary structure into protofilaments and fibrils. Once the process has started, seeding may also play an important facilitating role, so amyloid deposition may progress exponentially as expansion of the amyloid template “captures” further precursor molecules.

REACTIVE SYSTEMIC (AA) AMYLOIDOSIS

Associated Conditions

Amyloid A protein (AA) amyloidosis occurs in association with chronic inflammatory disorders, chronic local or systemic microbial infections, and occasionally malignant neoplasms. In Western Europe and the United States, the most frequent predisposing conditions are idiopathic rheumatic diseases (Table 34.3). Amyloidosis complicates up to 10% of cases of rheumatoid arthritis and juvenile inflammatory arthritis, although for reasons that are not clear, the incidence is lower in the United States than in Europe. Amyloidosis is exceptionally rare in systemic lupus erythematosus and related connective tissue diseases, as well as in ulcerative colitis, in contrast to Crohn disease. Tuberculosis and leprosy are important causes of AA amyloidosis, particularly where these infections are endemic. Chronic osteomyelitis, bronchiectasis, chronically infected burns, and decubitus ulcers, as well as the chronic pyelonephritis of patients with paraplegia, are other well-recognized associations (Table 34.3). Hodgkin disease and renal carcinoma, which often cause fever, other systemic symptoms, and a major acute-phase response, are the malignant diseases most commonly associated with systemic AA amyloid.

Chronic inflammatory disorders
Rheumatoid arthritis
Juvenile inflammatory arthritis
Ankylosing spondylitis
Psoriasis and psoriatic arthropathy
Reiter syndrome
Adult Still disease
Sohgal syndrome
Crohn disease
Familial Mediterranean fever and other periodic fevers
Chronic microbial infections
Leprosy
Tuberculosis
Bronchiectasis
Chronic pyelonephritis and decubitus ulcers in paraplegia
Osteomyelitis
Whipple disease
Neoplasms
Hodgkin's disease
Renal carcinoma
Castleman disease

TABLE 34.3. Conditions Associated with Reactive Systemic (AA) Amyloidosis

Clinical Features

AA amyloid involves the viscera, but it may be widely distributed without causing clinical symptoms (10). More than 90% of patients present with nonselective proteinuria resulting from glomerular deposition, and nephrotic syndrome may develop before progression to end-stage renal failure. Hematuria, isolated tubular defects, nephrogenic diabetes insipidus, and diffuse renal calcification occur rarely. Kidney size is usually normal but may be enlarged or, in advanced cases, reduced. End-stage chronic renal failure is the cause of death in 40% to 60% of cases, but acute renal failure may be precipitated by hypotension or salt and water depletion after surgical procedures, excessive use of diuretics, or intercurrent infection, and it may be associated with renal vein thrombosis. The second most common presentation is with organ enlargement, such as hepatosplenomegaly or thyroid goiter, with or without overt renal abnormality, but in any case amyloid deposits are almost always widespread at the time of presentation. Involvement of the heart and gastrointestinal tract is frequent but rarely causes functional impairment.

AA amyloidosis may become clinically evident early in the course of associated disease, but the incidence increases with duration of the primary condition. The mean duration of chronic rheumatic diseases such as rheumatoid arthritis, ankylosing spondylitis, or juvenile rheumatoid arthritis before amyloid deposition is diagnosed is 12 to 14 years, although it can present much sooner. For most patients, the prognosis is closely related to the degree of renal involvement and the effectiveness of treatment of the underlying inflammatory condition. In the presence of persistent, uncontrolled inflammation, 50% of patients with AA amyloid die within 5 years of the diagnosis of amyloid deposition; however, if the acute-phase response can be consistently suppressed, the proteinuria can cease, renal function is retained, and the prognosis is much better. The availability of long-term hemodialysis and transplantation prevents early death from uremia *per se,* but amyloid deposition in extrarenal tissues is responsible for a less favorable prognosis than in other causes of end-stage renal failure.

AA protein

The AA protein is a single nonglycosylated polypeptide chain usually of mass 8,000 d and containing 76 residues corresponding to the N-terminal portion of the 104-residue serum AA protein (SAA) (11). Smaller and larger AA fragments, even some whole SAA molecules, have also been reported in AA fibrils. SAA is an apolipoprotein of high-density lipoprotein particles and is the polymorphic product of a set of genes located on the short arm of chromosome 11. SAA is highly conserved in evolution and is a major acute-phase reactant in all species in which it has been studied. Most of the SAA in plasma is produced by hepatocytes in which the synthesis is under transcriptional regulation by cytokines, especially interleukin-1, interleukin-6, and tumor necrosis factor, acting through nuclear factor kB and possibly other transcription factors. After secretion, it rapidly associates with high-density lipoproteins from which it displaces apolipoprotein A-I (apoA-I). The circulating concentration can rise from normal levels of up to 3 mg/L to more than 1,000 mg/L within 24 to 48 hours of an acute stimulus, whereas with ongoing chronic inflammation, the level may remain persistently high. Certain isoforms of SAA, the products of different genes, are predominantly synthesized elsewhere in the body by macrophages, adipocytes, and certain other cells. Although they also associate with high-density lipoproteins, their acute-phase synthesis is stimulated differently, and they presumably have different functions. There is also a closely related family of high-density lipoprotein trace apoproteins that are not acute-phase reactants and that have been designated constitutive SAAs, although they do not form amyloid.

Circulating SAA is the precursor of amyloid fibril AA protein, from which it is derived by proteolytic cleavage (12,13). Such cleavage can be produced by macrophages and by a variety of proteinases, but it is not known whether, in the process of AA fibrillogenesis, cleavage of SAA occurs before or after aggregation of monomers. Persistent overproduction of SAA, causing sustained high circulating levels, is a necessary condition for deposition of AA amyloid, but it is not known why only some individuals in this state develop amyloidosis. In mice, only SAA2, one of the three major isoforms of murine SAA, is the precursor of AA in amyloid fibrils. Human SAA isoforms are more complex, but homozygosity for particular types seems to favor amyloidogenesis, although there may also be ethnic differences (14).

The normal functions of SAA are not known (11), although modulating effects on reverse cholesterol transport and on lipid functions in the microenvironment of inflammatory foci have been proposed. A protein, homologous with SAA, produced by rabbit fibroblasts has been reported to act as an autocrine stimulator of collagenase production *in vitro.* Other reports of potent cell regulatory functions of isolated, denatured, delipidated SAA have yet to be confirmed with physiologic

preparations of SAA-rich high-density lipoproteins. Regardless of its physiologic role, the behavior of SAA as an exquisitely sensitive acute-phase protein with an enormous dynamic range makes it an extremely valuable empiric clinical marker. It can be used to monitor objectively the extent and activity of infective, inflammatory, necrotic, and neoplastic disease. Furthermore, routine monitoring of SAA should be an integral part of the management of all patients with AA amyloidosis or disorders predisposing to it, because control of the primary inflammatory process to reduce SAA production is essential if the amyloidosis is to be halted, enabled to regress, or prevented. Automated immunoassay systems for SAA are available standardized on a World Health Organization International Reference Standard (15).

AMYLOIDOSIS ASSOCIATED WITH IMMUNOCYTE DYSCRASIA (AL AMYLOIDOSIS)

Associated Conditions

Almost any dyscrasia of cells of the B-lymphocyte lineage, including multiple myeloma, malignant lymphomas, and macroglobulinemia, may be complicated by immunoglobulin light-chain (AL) amyloidosis, but most cases are associated with otherwise “benign” monoclonal gammopathy. AL amyloidosis occurs in up to 15% of patients with myeloma, in a lower proportion of those with other malignant B-cell disorders, and probably in fewer than 5% of patients with “benign” monoclonal gammopathy. In some cases, deposition of AL amyloid may be the only evidence of the dyscrasia. A monoclonal paraprotein or free light chains can be detected in serum or urine of only about 90% of patients with AL amyloid, but detection of immunoglobulin gene rearrangement in bone marrow or peripheral blood sometimes confirms a monoclonal gammopathy in the remaining cases. The paraprotein may also appear after presentation and diagnosis of the amyloid, and subnormal levels of some or all serum immunoglobulins or increased numbers of marrow plasma cells may provide less direct clues to the underlying cause. Until recently, it was the practice to diagnose apparently “primary” cases of amyloidosis, with no previous predisposing inflammatory condition or family history of amyloidosis, as AL type by exclusion. However, autosomal dominant hereditary nonneuropathic amyloidosis, particularly that caused by variant fibrinogen a chain, may be poorly penetrant and of late onset, so there may be no family history. The coincident occurrence of a monoclonal gammopathy may then be gravely misleading, and it is essential to exclude by genotyping all known amyloidogenic mutations and to seek positive immunohistochemical or biochemical identification of the amyloid fibril protein in all cases.

Clinical Features

AL amyloidosis occurs equally in men and women, usually after the age of 50 years, but occasionally in young adults. It has a lifetime incidence and is the cause of death of between 0.5 and 1 per 1,000 persons in the United Kingdom. The clinical manifestations are protean, because virtually any tissue other than the brain may be directly involved (16). Uremia, heart failure, or other effects of the amyloid usually cause death within a year of diagnosis, unless the underlying B-cell clone is effectively suppressed.

The heart is affected in 90% of patients with AL amyloidosis, in 30% of whom restrictive cardiomyopathy is the presenting feature and in up to 50% of whom it is fatal. Other cardiac presentations include arrhythmias and angina. Renal AL amyloidosis has the same manifestations as renal AA amyloidosis, but the prognosis is worse. Gut involvement may cause motility disturbances (often secondary to autonomic neuropathy), malabsorption, perforation, hemorrhage, or obstruction. Macroglossia occurs rarely but is almost always pathognomonic. Hyposplenism sometimes occurs in both AA and AL amyloidosis. Painful sensory polyneuropathy with early loss of pain and temperature sensation, followed later by motor deficits, is seen in 10% to 20% of patients, and carpal tunnel syndrome occurs in 20%. Autonomic neuropathy leading to orthostatic hypotension, impotence, and gastrointestinal disturbances may occur alone or together with the peripheral neuropathy, and it has a poor prognosis. Skin involvement takes the form of papules, nodules, and plaques, usually on the face and upper trunk, and involvement of dermal blood vessels results in purpura occurring either spontaneously or after minimal trauma and is common. Articular amyloidosis is rare but may mimic acute polyarticular rheumatoid arthritis, or it may present as asymmetric arthritis affecting the hip or shoulder. Infiltration of the glenohumeral joint and surrounding soft tissues occasionally produces the characteristic “shoulder pad” sign. A rare but serious manifestation of AL amyloidosis is an acquired bleeding diathesis that may be associated with deficiency of factor X and sometimes also factor IX, or with increased fibrinolysis. It does not occur in AA amyloidosis, although in both AL and AA disease, patients may have serious bleeding in the absence of any identifiable factor deficiency.

AL Proteins

AL proteins are derived from the N-terminal region of monoclonal immunoglobulin light chains and consist of the whole or part of the variable (V_L) domain. Intact light chains may rarely be found, and the molecular weight therefore varies between about 8,000 and 30,000 d. The light chain of the monoclonal paraprotein is either identical to, or clearly the precursor of, AL isolated from the amyloid deposits.

AL is more commonly derived from l chains than from k chains, even though k chains predominate among both normal immunoglobulins and the paraprotein products of immunocyte dyscrasias. A new l chain subgroup, l_{VI}, was identified first as an AL protein in two cases of immunocyte dyscrasia–associated amyloidosis before it had been recognized in any other form, and it has subsequently been observed in many more cases of AL amyloidosis. Furthermore, increasing evidence from sequence analyses of Bence Jones proteins of both k and l types from patients with AL amyloidosis, and of AL proteins themselves, indicates that these polypeptides contain unique amino acid replacements or insertions compared with nonamyloid monoclonal light chains. In some cases, these changes involve replacement of hydrophilic framework residues by hydrophobic residues, changes likely to promote aggregation and insolubilization, and in others the monoclonal light chains from patients with amyloidosis have been demonstrated directly to have decreased solubility and a greater propensity for precipitation than control nonamyloid monoclonal light chains (17). The inherent “amyloidogenicity” of particular monoclonal light chains was elegantly confirmed in an *in vivo* model in which isolated Bence Jones proteins were injected into mice (18). Animals receiving light chains from patients with AL amyloidosis developed typical amyloid deposits composed of the human protein, whereas animals receiving light chains from patients with myeloma but without amyloidosis did not.

SENILE AMYLOIDOSIS

Autopsy studies show that some amyloid is present in all persons older than 80 years, but whether this contributes to the aging process or whether it is an epiphenomenon that becomes clinically important only when it is extensive is not known.

Senile Systemic Amyloidosis

Up to 25% of elderly persons have microscopic, clinically silent systemic deposits of transthyretin amyloid involving the heart and blood vessel walls, smooth and striated muscle, fat tissue, renal papillae, and alveolar walls. In contrast to most other forms of systemic amyloidosis, including hereditary transthyretin amyloidosis caused by point mutations in the transthyretin gene, the spleen and renal glomeruli are rarely affected. The brain is not involved. Occasionally, more extensive deposits in the heart, affecting ventricles and atria and situated in the interstitium and vessel walls, cause significant impairment of cardiac function and may be fatal. The transthyretin involved is probably usually of the normal wild type, but cases with transthyretin variants that may be hereditary have been described.

Transthyretin

Transthyretin, formerly known as prealbumin, is a normal nonglycosylated plasma protein, with a relative molecular mass of 54,980 d. It is composed of four identical noncovalently associated subunits, each of 127 amino acids. It is produced by hepatocytes and the choroid plexus and is a significant negative acute-phase protein. Each tetrameric molecule is able to bind a single thyroxine or triiodothyronine molecule, and up to 15% of circulating thyroid hormone is transported in this way. Transthyretin also forms a 1:1 molecular complex with retinol-binding protein, which transports vitamin A.

Transthyretin is encoded by a single copy gene but is appreciably polymorphic, and about 70 different point mutations encoding single-residue substitutions have been identified so far. Normal wild-type transthyretin is an inherently amyloidogenic protein that forms the fibrils in senile systemic amyloidosis, and *in vitro* exposure to reduced pH is sufficient to generate transthyretin amyloid fibrils from the pure protein. Most of the variant forms of transthyretin have been associated with hereditary amyloidosis and show decreased stability *in vitro* compared with the wild type. Transgenic mice expressing human transthyretin Val30Met in the liver develop extensive systemic amyloidosis, but no amyloid deposits have yet been reported in the peripheral nerves, even when the transgene is expressed in the choroid plexus and transthyretin amyloid is deposited in the meninges and choroid plexus. This is another example of the presence of important unknown factors, other than an amyloidogenic protein itself, that determine where and when clinical amyloidosis develops (19).

Individuals heterozygous for transthyretin mutations have a mixture of wild-type and variant transthyretin monomers in their circulating transthyretin, and if they develop amyloidosis, both forms are often present, although the variant may predominate in the amyloid fibrils. Although cleavage fragments of transthyretin are commonly present, intact transthyretin subunits are also found, and fibrillogenesis does not depend on an initial proteolytic step.

Senile Focal Amyloidosis

Microscopic and clinically silent amyloid deposits of different fibril types, localized to particular tissues, are almost always present in elderly persons. The most common form is found in the media of the aortic wall of virtually all persons older than 60 years. The fibrils consist of a 50-residue peptide, named medin, derived by cleavage

from the coagulation factorlike domain of the protein lactadherin (20). Deposits of Ab protein (see later) as amyloid in cerebral blood vessels and intracerebral plaques seen in “normal” elderly brains may or may not represent a harbinger of Alzheimer disease, had the patient survived long enough. Amyloid deposits are present in most osteoarthritic joints at surgery or autopsy, usually in close association with calcium pyrophosphate deposits, affecting the articular cartilage and joint capsule. However, neither the clinical significance of this age-associated articular amyloid nor its biochemical nature is known. The corpora amylacea of the prostate are composed of b_2-microglobulin amyloid fibrils. Amyloid in the seminal vesicles is derived from an as yet unidentified exocrine secretory product of the vesicle cells. Isolated deposits of cardiac atrial amyloid consist of atrial natriuretic peptide. Focal amyloid deposits sometimes present in atheromatous plaques of elderly persons contain fibrils composed of the N-terminal fragment of wild-type apoA-I.

CEREBRAL AMYLOIDOSIS

The brain is a common and important site of amyloid deposition, although possibly because of the blood–brain barrier, there are no deposits in the cerebral parenchyma itself in any form of acquired systemic visceral amyloidosis. However, cerebrovascular transthyretin amyloid may occur in familial amyloid polyneuropathy resulting from the most common transthyretin Val30Met variant, and oculoleptomeningeal amyloidosis is caused by other rare transthyretin variants. The common and major forms of brain amyloid are confined to the brain and cerebral blood vessels, with the single exception of cystatin C amyloid in hereditary cerebral hemorrhage with amyloidosis, Icelandic type, in which major, although clinically silent, systemic deposits are present.

Alzheimer Disease

By far the most frequent and important type of amyloid in the brain is that related to Alzheimer disease, which is the most common cause of dementia. This disease affects more than 3 million persons in the United States and a corresponding proportion of other Western populations. It is generally a disease of the elderly, and its prevalence is therefore increasing. The clinical differential diagnosis of senile dementia and the positive identification of Alzheimer disease are difficult and often of limited precision in life. However, intracerebral and cerebrovascular amyloid deposits are hallmarks of the neuropathologic diagnosis. The amyloid fibrils are composed of Ab protein, a 39- to 43-residue cleavage product of the large amyloid precursor protein (APP) (21). Most cases of Alzheimer disease are sporadic, but some families have an autosomal dominant pattern of inheritance and usually an early onset. About 20 families have causative mutations in the *APP* gene on chromosome 21, and most other kindreds have mutations in the genes for presenilin 1 (chromosome 14) and presenilin 2 (chromosome 1) (22,23). All these mutations are associated with increased production from APP of Ab1-42, the most amyloidogenic form of Ab. Because all persons with Down syndrome (trisomy 21), develop Alzheimer disease if they survive into their 40s, there is evidently a close link among APP, Ab overproduction, Ab amyloidosis, and the pathogenesis of Alzheimer disease, although it remains unclear whether or how Ab *per se,* or the amyloid fibrils that it forms, contributes to the neuronal loss that underlies the dementia. Synthetic Ab fibrils formed *in vitro* are markedly cytotoxic and cause death of cultured cells by apoptosis and necrosis, but to what extent these findings reflect phenomena that may be responsible for neurodegeneration *in vivo* is not clear. Controversy exists about the correlation between the severity of dementia in Alzheimer disease and the extent of amyloid angiopathy and plaques. Nevertheless, the finding that patients with Alzheimer disease caused by APP and presenilin mutations have exactly the same neuropathology as sporadic cases, including tangles, argues strongly that the APP and Ab-protein pathway can be of primary pathogenetic significance.

In addition to the Ab amyloid deposits in the brains of patients with Alzheimer disease and Down syndrome, extensive “amorphous” deposits of Ab are also found throughout the brain. These do not stain with Congo red and are detectable only by immunohistochemical staining. Their significance is unknown. They apparently precede the appearance of histochemically identifiable amyloid but are not necessarily the precursor of it because they are present in areas, such as the cerebellum, in which Ab amyloid is not seen. The nonfibrillar, nonamyloid protein, apolipoprotein E (apoE), is demonstrable in many amyloid deposits, including those of Alzheimer disease. The *ApoE4* gene (chromosome 19), encoding one of the three isoforms of this apolipoprotein, is strongly associated with predisposition to develop Alzheimer disease and with increased amounts of amyloid in the brain, but the underlying mechanisms are unknown.

Another neuropathologic feature of Alzheimer disease and some other neurodegenerative conditions is the neurofibrillary tangle located intracellularly within neuronal cell bodies and processes. These tangles have a characteristic ultrastructural morphology of paired helical filaments, and, although they bind Congo red and then give the pathognomonic green birefringence of amyloid when they are viewed in polarized light, they are completely different structurally from amyloid fibrils. They are composed of an abnormally phosphorylated form of the normal neurofilament protein, t.

Senile Cerebral Amyloidosis and Amyloid Angiopathy

The cerebral blood vessels contain Ab amyloid in up to 60% of aged brains of nondemented persons, and there may also be focal intracerebral Ab amyloid plaques. These deposits are usually clinically silent and may or may not represent harbingers of Alzheimer disease, had the patients survived long enough. Sometimes, the amyloid angiopathy is more extensive, and it is a rare but important cause of cerebral hemorrhage and stroke, to be distinguished from atherosclerotic cerebrovascular disease.

Ab Protein

The fibril protein in the intracerebral and cerebrovascular amyloid of Alzheimer disease, Down syndrome, and hereditary amyloid angiopathy of Dutch type is a 39- to 43-residue sequence derived by proteolysis from the high-molecular-weight precursor protein, the APP, encoded on the long arm of chromosome 21 (22,23). Several isoforms of APP are generated by alternative splicing of transcripts from the 19-exon gene, and yielding three major forms: APP695, APP751, and APP770. These are each single-chain, multidomain glycoproteins with the carboxy-terminal 47 residues within the cytoplasm, a 25-residue membrane-spanning region, and the rest of the molecule lying extracellularly. APP751 and APP770 contain a 56-residue Kunitz-type serine proteinase inhibitor domain encoded by exon 7. After glycosylation and membrane insertion, APPs are cleaved extracellularly by so-called APP secretase activity, close to the transmembrane sequence, releasing, in the case of the isoforms containing the proteinase inhibitor domain, a molecule known as proteinase nexin II, which avidly binds factor XIa, trypsin, and chymotrypsin, as well as epidermal growth factor–binding protein and the g subunit of nerve growth factor. Although mRNA encoding APP695, which lacks the proteinase inhibitor domain, is the predominant species found in the brain, whereas mRNA for APP751 is the most abundant in other tissues, 85% of secreted APP in the brain is proteinase nexin II. APP secreted by a glial cell line is substantially glycosylated with chondroitin sulfate glycosaminoglycan chains. APP also undergoes high-affinity interactions with heparan sulfate. These observations suggest that APP may have important functions in cell adhesion, cell migration, and modulation of growth factor activities. APP proteinase nexin II is present in and released by platelets and probably functions in the clotting cascade.

The amyloidogenic Ab, encoded by parts of exons 16 and 17, corresponds to the part of the APP sequence that extends from within the cell membrane into the extracellular space. Secretase cleavage of APP to release the soluble form therefore cannot generate intact Ab itself or larger fragments containing it. However, there is an alternative processing pathway for APP, in which it is taken up whole by lysosomes and is cleaved to yield fragments that do contain the whole Ab sequence. Furthermore, APP cleaved at the N terminus of Ab and free soluble Ab itself are normally produced by cell lines and by mixed brain cells in culture and are present in the cerebrospinal fluid. However, the source of the Ab in the intracerebral amorphous deposits and of that which aggregates as amyloid fibrils in the brain and cerebral blood vessels is still not known. The 42-residue form of Ab is markedly the most amyloidogenic, and all the mutations in the APP and presenilin genes that are associated with hereditary Alzheimer disease result in increased production of this Ab1-42. Increased availability of the precursor is thus responsible for amyloidogenesis, but the pathogenesis of neuronal damage and dementia is unknown.

Hereditary Cerebral Hemorrhage with Amyloidosis: Hereditary Cerebral Amyloid Angiopathy

DUTCH TYPE

In families originating in a small region on the Dutch coast, the autosomal dominant inheritance of recurrent normotensive cerebral hemorrhages starting in middle age results from deposition of a genetic variant of Ab as cerebrovascular amyloid. These persons also have “amorphous” Ab deposits in the brain and early senile plaques, without congophilic amyloid cores. Multiinfarct dementia occurs in survivors, but some patients become demented in the absence of stroke. Amyloid outside the brain has not been reported.

ICELANDIC TYPE

Cerebrovascular amyloid deposits composed of a fragment of a genetic variant of cystatin C are responsible for recurrent major cerebral hemorrhages starting in early adult life in members of families originating in western Iceland. There is autosomal dominant inheritance, and appreciable but clinically silent amyloid deposits are present in the spleen, lymph nodes, and skin. There is no extravascular amyloid in the brain, and the neurologic deficits, often including dementia, of surviving patients are compatible with their cerebrovascular disease.

CYSTATIN C

Cystatin C (formerly called g-trace) is an inhibitor of cysteine proteinases, including cathepsins B, H, and L. It is encoded by a gene on chromosome 20 and consists of

a single nonglycosylated polypeptide chain of 120 residues. It is present in all major human biologic fluids at concentrations compatible with a significant physiologic role in proteinase inhibition. The normal concentration in cerebrospinal fluid is 6.5 mg/L (range, 2.7 to 13.7, n = 34), but it is much lower (2.7 mg/L; range, 1.0 to 4.7, n = 9) in patients with the Icelandic type of hereditary cerebral amyloid angiopathy, in whom fragments of the Gln 68 genetic variant of cystatin C form the amyloid fibrils. This reduced concentration is useful diagnostically and is evident even in presymptomatic carriers of the cystatin C gene mutation. The point mutation that causes the disease encodes a Gln for Leu substitution in the mature protein, and the amyloid fibril protein consists of the C-terminal 110 residues of the variant. This amino-terminally truncated form is not detectable in the cerebrospinal fluid of affected patients, a finding suggesting that cleavage takes place either in close proximity to fibril deposition or is a postfibrillogenic event. The variant cystatin C is less stable than the wild type and readily forms fibrils *in vitro.* It is not known whether cerebral hemorrhage in cystatin C amyloidosis is caused simply by the damaging effects of vascular amyloid deposition or whether deficiency in inhibitory capacity for cysteinase proteinases also plays a part.

Cerebral Amyloid Associated with Prion Disease

The neuropathology of a group of progressive, invariably fatal spongiform encephalopathies that are transmissible and in some cases are hereditary sometimes includes intracerebral amyloid plaques and amyloid cerebral angiopathy. These diseases, sporadic and familial Creutzfeldt-Jakob disease, the familial Gerstmann-Sträussler-Scheinker syndrome, and kuru, are caused by prions (PrP^{Sc}), conformational isoforms of the normal physiologic cellular prion protein (PrP^{C}) (24). The human diseases are closely related to several animal diseases: scrapie of sheep and goats; transmissible encephalopathy of mink, elk, and male deer; and bovine spongiform encephalopathy. New-variant Creutzfeldt-Jakob disease is apparently the result of transmission of bovine spongiform encephalopathy to humans (25). The significance of amyloid *per se* in these disorders is not clear, because it is not always detectable histologically and is not seen, for example, in fatal familial insomnia or in bovine spongiform encephalopathy, which is apparently a result of transmission of ovine scrapie to cattle. When scrapie or its human counterpart is transmitted to experimental animals by inoculation of affected brain tissue, the development of intracerebral amyloid depends on the strain of infectious agent and the genetic background of the recipient. Even when amyloid is present in the brain, it is not seen elsewhere, for example, in the spleen, although the spleen is a rich source of the infective agent. However, when the infective agent is exhaustively and highly purified from brain or spleen, it forms typical congophilic amyloid fibrils, composed of the proteinase resistant subunit that is the prion, PrP^{Sc}; and when amyloid deposits are present in affected brains, they immunostain with antiprion antibodies. The amyloid fibril protein is thus directly related to the cause of the encephalopathy, but gross amyloid deposition is evidently not necessary for expression of disease. Neuronal damage may perhaps be caused by cytotoxic prefibrillar PrP^{Sc} aggregates or by other mechanisms entirely (26). This situation is different from that of the extracerebral amyloidoses and of cystatin C and nonhereditary cerebral amyloid angiopathies, in which amyloid deposition is invariably present when there is clinical disease.

HEREDITARY SYSTEMIC AMYLOIDOSIS

Familial Amyloid Polyneuropathy

Familial amyloid polyneuropathy is an autosomal dominant syndrome with onset at any time from the second decade onward, characterized by widespread progressive amyloid deposition leading to peripheral and autonomic neuropathy and varying degrees of visceral involvement affecting the heart, kidneys, thyroid, adrenals, and frequently the vitreous of the eye. There is usually amyloid deposition in blood vessel walls and the connective tissue matrix throughout the body. In addition to the major foci in Portugal, Japan, and Sweden, familial amyloid polyneuropathy has been reported in most ethnic groups throughout the world. Considerable variation exists in the age of onset, rate of progression, and involvement of different systems, although within families the pattern is usually consistent. The disease is characterized by remorseless progression, and the disorder is invariably fatal. Death results from the effects and complications of peripheral or autonomic neuropathy or from cardiac or renal failure.

Familial amyloid polyneuropathy is caused by mutations in the gene for the plasma protein transthyretin, formerly known as prealbumin, the most frequent of which causes Met for Val substitution at position 30 in the mature protein, but more than 60 amyloidogenic mutations have been described (19). There is often little correlation between the underlying mutation and the clinical phenotype, which is evidently determined by other genetic and possibly also environmental factors, although in a few cases certain mutations are uniquely associated with particularly aggressive or relatively organ-limited disease. The amyloidogenic transthyretin mutations are not always penetrant, and even asymptomatic Met30 homozygotes older than 60 years have been reported. Rare kindreds with the apoA-I Arg26 variant, which usually causes nonneuropathic amyloidosis, may present with prominent peripheral neuropathy resembling transthyretin familial amyloid polyneuropathy.

Familial Amyloid Polyneuropathy with Predominant Cranial Neuropathy

Originally described in Finland but now reported in other ethnic groups, this autosomal dominant hereditary amyloidosis presents in adult life with cranial neuropathy, lattice corneal dystrophy, and distal peripheral neuropathy (27). Patients may have skin, renal, and cardiac manifestations, and microscopic amyloid deposits are widely distributed in connective tissue and blood vessel walls, although life expectancy approaches normal. The amyloid fibrils are derived from variants of the actin-modulating protein, gelsolin, encoded by point mutations. Individuals homozygous for these mutations have severe renal amyloidosis in addition to the usual neuropathy.

Gelsolin

Gelsolin (mass 90,000 d) is a widely distributed cytoplasmic protein that binds actin monomers, nucleates actin filament growth, and severs actin filaments. Alternative transcriptional initiation and message processing from a single gene on chromosome 9 are responsible for synthesis of a secreted form of gelsolin (mass 93,000 d), which circulates in the plasma at a concentration of about 200 mg/L. Its function in the blood is not known but it may be related to clearance of actin filaments released by dying cells.

In the Finnish type of hereditary amyloidosis, the amyloid fibril protein is a 71-residue fragment of variant gelsolin with Asn substituted for Asp at position 15, corresponding to residue 187 of the mature molecule, and the same mutation has been discovered in affected kindreds from different ethnic backgrounds. One Danish family with the same phenotype has a different mutation at the same nucleotide, predicting a Tyr for Asp substitution at residue 187. Synthetic and recombinant peptides, including the Asn for Asp187 substitution, are less soluble than the wild-type sequence and readily form amyloid fibrils *in vitro* (28).

Nonneuropathic Systemic Amyloidosis

The patterns of organ involvement and overall clinical phenotype vary among families in this rare autosomal dominant syndrome of major systemic amyloidosis without clinical evidence of neuropathy. The kidneys are often most severely affected, leading to hypertension and renal failure, but the heart, spleen, liver, bowel, connective tissue, and exocrine glands may all be involved. After clinical presentation, usually from the second decade onward, one sees inexorable progression to organ failure, which requires transplantation, or to death. The amyloid proteins identified so far are genetic variants of apoA-I, lysozyme, and fibrinogen Aa chain. Although symptoms usually start in early adulthood, presentation may be as late as the sixth decade in a few kindreds, and growing evidence suggests incomplete penetrance in many families with fibrinogen Aa-chain variants.

Apolipoprotein A-I

ApoA-I is the most abundant apolipoprotein of high-density lipoprotein particles and participates in their central function of reverse cholesterol transport from the periphery to the liver. ApoA-I variants are extremely rare and may be phenotypically silent or may affect lipid metabolism. However, nine different variants of apoA-I, including single- and multiple-residue substitutions and deletions, have been associated with amyloidosis (29,30,31 and 32). Although it is inherited as an autosomal dominant, and usually highly penetrant, trait, there are marked variations in age and manner of presentation even in the same family and in different kindreds with the same mutation. The amyloid fibril protein consists, in all cases studied, of the first 90 or so N-terminal residues even when the causative variant residues are more distal. Wild-type apoA-I is also amyloidogenic and forms the deposits associated with atheromatous plaques in the elderly (33), and the various amyloidogenic mutations presumably encode sequence changes that render apoA-I less stable or more liable to cleavage to yield the fibrillogenic N-terminal fragment.

Lysozyme

Lysozyme (EC 3.2.1.17) is the classic bacteriolytic enzyme of external secretions, discovered by Fleming in 1922. It is also present at high concentration in articular cartilage and in the granules of polymorphonuclear leukocytes, and it is the major secreted product of macrophages. Lysozymes are present in most organisms in which they have been sought, although their physiologic role is not always clear. The complete structures of hen egg white and human lysozymes are known to atomic resolution and their catalytic mechanism, epitopes, folding, and other aspects of structure-function relationship have been analyzed exhaustively. This understanding contrasts with the absence of detailed three-dimensional structural information on all other amyloid fibril proteins and their precursors, except transthyretin and b_2-microglobulin. There is only one copy of the lysozyme gene in the human genome, and no mutations in the coding region other than those causing amyloidosis are known, nor is any disease associated with lysozyme other than amyloidosis. The amyloidogenic mutations encode Ile56Thr in one family and Asp67His in others

(34,35). These dramatic changes in residues that are extremely conserved throughout the lysozyme and related a-lactalbumin protein families destabilize the native fold so the variants readily populate partly unfolded states even under physiologic conditions and spontaneously aggregate *in vitro,* and evidently also *in vivo,* into amyloid fibrils (9). A polymorphic variant of lysozyme has been discovered, but its relationship with amyloidosis has yet to be elucidated.

Fibrinogen Aa Chain

Four different mutations in the fibrinogen Aa chain have been reported to cause systemic amyloidosis. All encode sequence changes located in the protease-sensitive C-terminal portion of the chain: Glu526Val and Arg554Leu are single-residue substitutions, whereas 4904delG and 4897delT produce frame shifts and premature termination of the protein (36,37,38 and 39). The amyloid fibrils consist of peptides from the C-terminal portion of the Aa chain encompassing the variant region; and in the case of the 4897delT mutation, the protein is a hybrid of normal wild-type sequence and a novel sequence encoded as a consequence of the mutation-induced frame shift. Haplotype analysis suggests that all the genes encoding the Glu526Val variant may be derived from a common founder. A rapidly increasing number of kindreds is being identified in which penetrance is so limited, or presentation is so late in life, that no family history is obtained (40,41). This form of systemic amyloidosis is thus likely to be much more common than previously recognized.

Cardiac Amyloidosis

Cardiac amyloidosis, without overt involvement of other viscera or neuropathy, progressing inexorably to death, is associated with certain transthyretin gene mutations and is inherited as an autosomal dominant with variable penetrance (Table 34.2). By far the most common variant is Ile122 transthyretin, which occurs in 4% of African-Americans and frequently causes cardiac amyloidosis from the sixth decade onward (42).

Familial Mediterranean Fever

Familial Mediterranean fever is an autosomal recessive disorder caused by mutations in the gene on chromosome 16 that encodes a neutrophil-specific protein of unknown function, called pyrin or marenostrin (43). The disease is characterized by recurrent episodes of fever, abdominal pain, pleurisy, or arthritis, and it predominantly occurs in non-Ashkenazi Jews, Armenians, Anatolian Turks, and Levantine Arabs. In Sephardic Jews of North African origin and in the other ethnic groups except Armenians and to a lesser extent Ashkenazi Jews, untreated familial Mediterranean fever is eventually complicated in a high proportion of cases by typical systemic AA amyloidosis. Furthermore, some patients with familial Mediterranean fever present with AA amyloidosis before they have experienced any symptoms, and this circumstance is consistent with the finding that a substantial acute-phase plasma protein response is frequently present even in asymptomatic persons. The different incidence of amyloid in patients with familial Mediterranean fever from different ethnic groups is not wholly explained by their specific pyrin gene mutations and is another illustration of the unknown genetic determinants of clinical amyloidosis.

HEMODIALYSIS-ASSOCIATED AMYLOIDOSIS

Almost all patients with end-stage renal failure who are maintained on hemodialysis for more than 5 years develop amyloid deposits composed of b_2-microglobulin (44). These deposits are predominantly osteoarticular and are associated with carpal tunnel syndrome, large joint pain and stiffness, soft tissue masses, bone cysts, and pathologic fractures. Renal tubular amyloid concretions may also form. The serious clinical problems associated with b_2-microglobulin amyloidosis constitute the major morbidity in patients undergoing long-term dialysis. Furthermore, in some such patients, more extensive deposition occurs, most commonly in the spleen but also in other organs, and a few cases of death associated with systemic b_2-microglobulin amyloid have been reported. The b_2-microglobulin is derived from the high plasma concentrations that develop in renal insufficiency and are not cleared by dialysis. This type of amyloid also occurs in patients undergoing continuous ambulatory peritoneal dialysis and has even been reported in a few patients with chronic renal failure who had not received dialysis.

b_2-Microglobulin

b_2-Microglobulin is a nonglycosylated, nonpolymorphic single-chain protein of 99 residues with a single intrachain disulfide bridge (relative molecular mass 11,815 d) encoded by a single gene on chromosome 15. It becomes noncovalently associated with the heavy chain of major histocompatibility class I antigens and is required for transport and expression of the complex at the cell surface. Amino acid sequence homology places b_2-microglobulin in the superfamily that includes immunoglobulins, T-cell receptor a and b chains, Thy 1, major histocompatibility class I and II molecules, and secretory component. Its three-dimensional structure is a typical b barrel with two antiparallel pleated sheets comprising three and four strands respectively, and it closely resembles an immunoglobulin domain.

b_2-Microglobulin is produced by lymphoid and various other cells in which it stabilizes the structure and function of class I antigens at the cell surface. When these complexes are shed by cleavage of the heavy chain at the cell surface, free b_2-microglobulin is released. The circulating concentration of b_2-microglobulin is 1 to 2 mg/L, and the protein is rapidly cleared by glomerular filtration and then is catabolized in the proximal renal tubule. Impairment of renal function is associated with retention of b_2-microglobulin and increased circulating levels because no other site exists for its catabolism. Daily production of b_2-microglobulin is about 200 mg, and in patients with end-stage renal failure who are undergoing hemodialysis, plasma b_2-microglobulin levels rise to and remain at levels of about 40 to 70 mg/L. Isolated unaltered b_2-microglobulin can form amyloidlike fibrils itself *in vitro,* and most studies of *ex vivo* b_2-microglobulin fibrils show the whole intact molecule to be the major subunit, although fragments and altered forms of b_2-microglobulin have also been reported.

ENDOCRINE AMYLOIDOSIS

Many tumors of APUD (amine precursor uptake and decarboxylation) cells that produce peptide hormones have amyloid deposits in their stroma. These are probably composed of the hormone peptides, and in the case of medullary carcinoma of the thyroid, the fibril subunits are derived from procalcitonin. In insulinomas, the amyloid fibril protein is a novel peptide, called islet amyloid polypeptide (IAPP), and also amylin, which was first identified in that site and was subsequently shown to be the fibril protein in the amyloid of the islets of Langerhans in type 2, maturity-onset diabetes (45). IAPP is an almost universal feature of the pancreatic islets in type 2 diabetes and becomes more extensive with increasing duration and severity of the disease. Although the amyloid itself is probably not initially responsible for the metabolic defect in this form of diabetes, progressive amyloid deposition leading to islet destruction probably does contribute to the pathogenesis (46).

Islet Amyloid Polypeptide

IAPP amylin is a 37-residue molecule encoded by a gene on chromosome 12 and with 46% sequence homology to the neuropeptide, calcitonin gene–related peptide (CGRP). IAPP is produced in the b cells of the pancreatic islets of Langerhans and is stored in and released from their secretory granules together with insulin. It has been reported to modulate insulin release and to induce peripheral insulin resistance, vasodilatation, and lowering of plasma calcium, but neither its physiologic role nor its contribution to diabetes is known.

Amyloidogenicity of IAPP depends on the amino acid sequence between residues 20 and 29, as shown by *in vitro* fibrillogenesis with synthetic peptides (47). The synthetic decapeptide IAPP20-29 and even the hexapeptide IAPP25-29, Gly-Ala-Ile-Leu-Ser-Ser, form amyloidlike fibrils *in vitro,* whereas other IAPP fragments do not. A correlation also exists between conservation of this sequence and deposition of IAPP amyloid in the islets of diabetic animals of different species. However, the role of the amyloid in diabetogenesis remains to be established. In the degu, a South American rodent, spontaneous diabetes is associated with islet amyloid composed of insulin, and xenogeneic insulin can also form amyloid in humans at sites of repeated therapeutic insulin injections.

RARE LOCALIZED AMYLOIDOSIS SYNDROMES

Amyloid deposits localized to the skin occur in both acquired and hereditary forms. Primary localized cutaneous amyloidosis presents in adult life as macular or papular lesions, the fibrils of which may be derived from keratin. Hereditary cutaneous amyloid lesions are rare, of unknown fibril type, and sometimes are associated with other, nonamyloid, multisystem disorders. Amyloid deposits in the eye cause local problems in the cornea (corneal lattice dystrophy) or conjunctiva, whereas orbital amyloid presents as mass lesions that can disrupt eye movement and the structure of the orbit. In one such case, the fibril protein has been identified as a fragment of IgG heavy chain.

Localized foci of AL amyloid can occur anywhere in the body, in the absence of systemic AL amyloidosis, the most common sites being the skin, upper airways and respiratory tract, and urogenital tract. These foci may be associated with a local plasmacytoma or B-cell lymphoma producing monoclonal immunoglobulin, but often the cells, which must be present to produce the amyloidogenic protein, are scattered inconspicuously in the affected tissue. The clinical problems caused by these space-occupying amyloidomas are usually cured by surgical resection, but surgery is not always possible.

GLYCOSAMINOGLYCANS

Amyloidotic organs contain more glycosaminoglycans than normal tissues, and at least some of this material is a tightly bound, integral part of the amyloid fibrils. These fibril-associated glycosaminoglycans are heparan sulfate and dermatan sulfate in all forms of amyloid that have been investigated. Fibrils isolated by water extraction and separated from other tissue components contain 1% to 2% by weight of glycosaminoglycans, none of which is covalently associated with the fibril protein. In systemic AA and AL amyloidosis, the only forms in which this has been studied so far, one sees marked restriction of the heterogeneity of the glycosaminoglycan chains, a finding suggesting that particular subclasses of heparan and dermatan sulfates are involved (48). Immunohistochemical studies demonstrate the presence of proteoglycan core proteins in all amyloid deposits, and these are closely related to fibrils at the ultrastructural level. However, in isolated fibril preparations, much of the glycosaminoglycan material is free carbohydrate chains, and it is not yet clear whether this represents aberrant glycosaminoglycan metabolism related to amyloidosis or is just an artifact of postmortem core protein degradation.

The significance of glycosaminoglycans in amyloid remains unclear, but their universal presence, intimate relationship with the fibrils, and restricted heterogeneity all suggest that they may be important. Glycosaminoglycans participate in the organization of some normal structural proteins into fibrils, and they may have comparable fibrillogenic effects on certain amyloid fibril precursor proteins. Furthermore, the glycosaminoglycans on amyloid fibrils may be ligands to which serum AP (SAP), another universal constituent of amyloid deposits, binds.

AMYLOID P COMPONENT AND SERUM AMYLOID P COMPONENT

Amyloid deposits in all different forms of the disease, both in humans and in animals, contain the nonfibrillar glycoprotein AP (49). AP is identical to and is derived from the normal circulating plasma protein, SAP, a member of the pentraxin protein family that includes C-reactive protein. Human SAP is secreted only by hepatocytes, is a trace constituent of plasma (women: mean, 24 mg/L; standard deviation, 8, range, 8 to 55, n = 274; men: mean, 32 mg/L; standard deviation, 7; range, 12 to 50, n = 226), and is not an acute-phase reactant. Nevertheless, apart from the fibrils themselves, AP is always by far the most abundant protein in all amyloid deposits.

SAP consists of five identical noncovalently associated subunits, each with a molecular mass of 25,462 d, which are noncovalently associated in a pentameric disclike ring. The tertiary fold of the subunit is dominated by antiparallel b sheets, forming a flattened b barrel with jellyroll topology and a core of hydrophobic side chains (50). This is the so-called “lectin fold,” shared with a variety of other animal, plant, and bacterial carbohydrate-binding proteins (lectins). SAP is a calcium-dependent ligand-binding protein, the best-defined specificity of which is for the 4,6-cyclic pyruvate acetal of b-D-galactose, but it also binds avidly and specifically to DNA, to chromatin, to glycosaminoglycans, particularly heparan and dermatan sulfates, and to all known types of amyloid fibrils. This last is the interaction responsible for the unique, specific, accumulation of SAP in amyloid deposits. Aggregated, but not native, SAP also binds specifically to C4-binding protein and fibronectin from plasma, although SAP is not complexed with any other protein in the circulation. In addition to being a plasma protein, SAP is also a normal constituent of certain extracellular matrix structures. It is covalently associated with collagen and other matrix components in the lamina rara interna of the human glomerular basement membrane and is present on the microfibrillar mantle of elastin fibers throughout the body (51,52).

No deficiency of SAP has been described, and it has been stably conserved in evolution. It has a single copy of its gene on chromosome 1 and no polymorphism of the amino acid sequence, and the single biantennary oligosaccharide chain attached to Asn32 is the most invariant glycan of any known glycoprotein. These indications that SAP is likely to have important physiologic functions have been confirmed by the finding that mice with targeted deletion of the SAP gene spontaneously develop marked antinuclear autoimmunity and immune complex glomerulonephritis (53). Studies of these SAP knock-out mice also show that SAP is involved in host resistance to a wide range of infections.

The SAP molecule is highly resistant to proteolysis, and, although SAP is not itself a proteinase inhibitor, its binding to amyloid fibrils *in vitro* protects them against proteolysis (54). Once bound to amyloid fibrils *in vivo,* SAP persists for prolonged periods and is not catabolized at all, in contrast to its rapid clearance from the plasma (half-life 24 hours) and prompt catabolism in the liver. These observations suggest that SAP may contribute to persistence of amyloid deposits *in vivo;* and, indeed, SAP knock-out mice show retarded and reduced induction of experimental AA amyloidosis, a finding confirming that SAP is significantly involved in the pathogenesis of amyloidosis (55).

Other Proteins in Amyloid Deposits

Certain plasma proteins other than the fibril proteins themselves and SAP have been detected immunohistochemically in some amyloid deposits. These proteins include a_1-antichymotrypsin, some complement components, apoE, and various extracellular matrix or basement membrane proteins. None of these match the universality or the quantitative or selective importance of SAP, and their role, if any, in the pathogenesis of amyloid deposition or its effects is not known, although experiments in transgenic and knock-out mice indicate that apoE may play a crucial role in Ab amyloid deposition.

DIAGNOSIS AND MONITORING OF AMYLOIDOSIS

Until recently, amyloidosis was an exclusively histologic diagnosis, and red-green birefringence of deposits stained with Congo red and viewed in polarized light remains the current standard. Furthermore, immunohistochemical staining of amyloid-containing tissue is the simplest method of identifying the amyloid fibril type. However, biopsies provide extremely small samples and therefore cannot provide information on the extent, localization, progression, or regression of amyloid deposits. A major advance in the clinical evaluation of amyloidosis has been the development of radiolabeled SAP as a specific tracer for amyloid (56,57). Combined scintigraphic imaging and metabolic analyses with labeled SAP have provided a wealth of new information on the natural history of many different forms of amyloid and their response to treatment.

Histochemical Diagnosis of Amyloid

BIOPSY

Amyloid may be an incidental finding on biopsy of the kidneys, liver, heart, bowel, peripheral nerve, lymph node, skin, thyroid, or bone marrow. When systemic amyloidosis is suspected clinically, biopsy of rectum or subcutaneous fat is the least invasive. When biopsy is performed by experienced physicians, amyloid can usually be detected in these sites in more than 90% of cases of systemic AA or AL disease. However, routine histopathologic examination has a substantially lower detection rate (i.e., many false-negative results) as well as a significant incidence of false-positive results. This situation reflects both laboratory technique and observer expertise, as well as variations in biopsy size. Direct biopsy of a clinically affected tissue is more likely to be positive but is more invasive.

CONGO RED AND OTHER HISTOCHEMICAL STAINS

Many cotton dyes, fluorochromes, and metachromatic stains have been used, but Congo red staining, and its resultant red-green birefringence when viewed with high-intensity polarized light, is the pathognomonic histochemical test for amyloidosis (3). The stain is unstable and must be freshly prepared at least every 2 months. Section thickness of 5 to 10 µm and inclusion in every staining run of a positive control tissue containing modest amounts of amyloid are critical.

IMMUNOHISTOCHEMISTRY

Although many amyloid fibril proteins can be identified immunohistochemically, the demonstration of amyloidogenic proteins in tissues does not, on its own, establish the presence of amyloid. Congo red staining and red-green birefringence are always required, and immunostaining may then enable the amyloid to be classified. Antibodies to SAA protein are commercially available and always stain AA deposits; the situation is similar with anti–b_2-microglobulin antisera and hemodialysis-associated amyloid. In AL amyloid, the deposits are stainable with standard antisera to k or l in only about half of all cases, probably because the light-chain fragment in the fibrils is usually the N-terminal variable domain, which is unique for each monoclonal protein. Immunohistochemical staining of transthyretin, Ab, and prion protein amyloid may require pretreatment of sections with formic acid or alkaline guanidine or deglycosylation.

ELECTRON MICROSCOPY

Amyloid fibrils cannot always be convincingly identified ultrastructurally, and electron microscopy alone is not sufficient to confirm the diagnosis of amyloidosis.

PROBLEMS OF HISTOLOGIC DIAGNOSIS

The tissue sample must be adequate (e.g., the inclusion of submucosal vessels in a rectal biopsy specimen), and failure to find amyloid does not exclude the diagnosis.

The unavoidable sampling problem means that biopsy cannot reveal the extent or distribution of amyloid. Experience with Congo red staining is required if clinically important false-negative and false-positive results are to be avoided. Immunohistochemical staining requires positive and negative controls, including demonstration of specificity of staining by absorption of positive antisera with isolated pure antigens.

Nonhistologic Investigations

A two-dimensional echocardiography image showing small, concentrically hypertrophied ventricles, generally impaired contraction, dilated atria, homogeneously echogenic valves, and “sparkling” echodensity of ventricular walls, is virtually diagnostic of cardiac amyloidosis. However, clinically significant restrictive diastolic impairment may be difficult to detect even by comprehensive Doppler and other functional studies. Imaging after injection of isotope-labeled calcium-seeking tracers has poor sensitivity and specificity and is of no clinical use.

In patients with known or suspected hereditary amyloidosis, the gene defect must be characterized. If amyloidotic tissue is available, the fibril protein may be known, and the corresponding gene can then be studied; however, if no tissue containing amyloid is available, screening of the genes for known amyloidogenic proteins must be undertaken.

Biochemical and immunochemical screening tests for the presence in the plasma of amyloidogenic variant protein products of mutant genes also exist (e.g., for transthyretin and apoA-I variants), but molecular genetic analysis of DNA is easier to perform and is the most direct approach. However, regardless of the DNA results, it is desirable, if possible, to identify the respective protein in the amyloid directly.

Serum Amyloid P Component as a Specific Tracer in Amyloidosis

The universal presence in amyloid deposits of AP, derived from circulating SAP, is the basis for the use of radioisotope-labeled SAP as a diagnostic tracer in amyloidosis (56,57). No localization or retention of labeled SAP occurs in healthy persons or in patients with diseases other than amyloidosis. Radioiodinated SAP has a short half-life (24 hours) in the plasma and is rapidly catabolized with complete excretion of the iodinated breakdown products in the urine. However, in patients with systemic or localized extracerebral amyloidosis, the tracer rapidly and specifically localizes to the deposits, in proportion to the quantity of amyloid present, and it persists there without breakdown or modification. For clinical purposes, highly purified SAP is isolated from the plasma of single accredited donors and is oxidatively iodinated under conditions that preserve its function intact. The medium-energy, short half-life, pure g emitter iodine-123 is used for scintigraphic imaging, and the long half-life isotope iodine-125 is used for metabolic studies. The dose of radioactivity administered (less than 4 mSv) is well within accepted safety limits, and more than 2,000 studies have been completed without any adverse effects. In addition to high-resolution scintigraphs, the uptake of tracer into various organs can be precisely quantified, and, together with highly reproducible metabolic data on the plasma clearance and whole-body retention of activity, the progression or regression of amyloid can be monitored serially and quantitatively (58).

Important observations regarding amyloid (which have been made for the first time *in vivo*) include the following: the different distribution of amyloid in different forms of the disease; amyloid in anatomic sites not available for biopsy, such as the adrenals and spleen; major systemic deposits in forms of amyloid previously thought to be organ limited; a poor correlation between the quantity of amyloid present in a given organ and the level of organ dysfunction; a nonhomogeneous distribution of amyloid within individual organs; and evidence for rapid progression and sometimes regression of amyloid deposits with different rates in different organs. Examples of major regression of amyloidosis, when it has been possible to reduce or eliminate the supply of fibril precursor, are encouraging. Labeled SAP studies thus make a valuable contribution to the diagnosis and management of patients with systemic amyloidosis, and these are available routinely for all patients with known or suspected cases of amyloidosis in the British National Health Service National Amyloidosis Centre at the Royal Free Hospital, London.

MANAGEMENT OF AMYLOIDOSIS

Although no treatments yet exist that specifically promote the mobilization of amyloid, substantial advances have been made in the management of systemic amyloidosis, particularly the institution of active measures to support failing organ function while attempting reduce the supply of the amyloid fibril precursor protein. Serial SAP scintigraphy in more than 1,000 patients with various forms of amyloid has confirmed that control of the primary disease process, or removal of the source of the amyloidogenic precursor, usually results in regression of existing deposits and recovery or preservation of organ function (58,59). This finding strongly supports aggressive intervention, and relatively toxic drug regimens or other radical approaches can be justified by the poor prognosis. Such an approach, leading to reduced morbidity rates and improved survival periods, is the basis for the establishment of the British National Health Service National Amyloidosis Centre. However, clinical improvement in amyloidosis is often delayed long after the underlying disorder has remitted, a finding reflecting the gradual regression of the deposits that is now recognized to occur in most patients. Continuing production of the amyloid precursor protein should be monitored as closely as possible long term, to determine the requirement for and intensity of treatment for the underlying primary condition. In AA amyloidosis, such monitoring involves frequent estimation of the SAA level, and in AL amyloidosis, it requires monitoring of monoclonal plasma cell proliferation and immunoglobulin light-chain production.

The treatment of AA amyloidosis ranges from potent antiinflammatory and immunosuppressive drugs in patients with rheumatoid arthritis to lifelong prophylactic colchicine administration in patients with familial Mediterranean fever to surgical intervention in conditions such as refractory osteomyelitis and tumors in Castleman disease. The alkylating agent chlorambucil can induce rapid and complete remission of inflammatory activity in many patients with rheumatoid and juvenile chronic arthritis (10), but its use must be considered carefully because it is not licensed for this indication, it is potentially carcinogenic, and it causes infertility.

Treatment of AL amyloidosis is based on the treatment of myeloma, although the plasma cell dyscrasias in AL are often subtle. Prolonged low-intensity cytotoxic regimes such as oral melphalan and prednisolone are beneficial in about 20% of patients. Dose-intensive infusional chemotherapy regimens such as vincristine, doxorubicin (Adriamycin) and dexamethasone and autologous peripheral blood stem cell transplantation are currently being evaluated with far more promising early results (60). However, extremely rigorous patient selection for organ transplantation is essential because the procedure mortality rate is high in persons with multiple amyloidotic organ involvement, especially patients with autonomic neuropathy, severe cardiac amyloidosis, or a history of gastrointestinal bleeding, and those older than 55 years.

Patients with the disabling arthralgia of b_2-microglobulin amyloidosis may respond partially to nonsteroidal antiinflammatory drugs or glucocorticoids, but even the most severe symptoms usually vanish rapidly after renal transplantation. The basis for this remarkable clinical response is unclear because, although renal transplantation rapidly restores normal b_2-microglobulin metabolism, regression of b_2-microglobulin amyloid may not be evident for many years (61).

Hepatic transplantation is effective in patients with familial amyloid polyneuropathy associated with transthyretin gene mutations because the variant amyloidogenic protein is produced mainly in the liver (62). Successful liver transplantation has now been reported in hundreds of patients with this condition, and although the peripheral neuropathy usually only stabilizes, autonomic function can improve substantially, and the associated visceral amyloid deposits have been shown to regress in most cases (63). Important questions remain about the timing of the procedure, but, so far, early intervention seems advisable.

Supportive therapy remains critical in systemic amyloidosis, with the potential for delaying target organ failure, maintaining quality of life, and prolonging survival while the underlying process can be treated. Rigorous control of hypertension is vital in renal amyloidosis. Surgical resection of amyloidotic tissue is occasionally beneficial, but, in general, a conservative approach to surgery, anesthesia, and other invasive procedures is advisable. Should any such procedure be undertaken, meticulous attention to blood pressure and fluid balance is essential. Amyloidotic tissues may heal poorly and are liable to bleed. Diuretics and vasoactive drugs should be used cautiously in patients with cardiac amyloidosis because these drugs can reduce cardiac output substantially. Dysrhythmias may respond to conventional pharmacologic therapy or to pacing. Replacement of vital organ function, notably dialysis, may be necessary; and cardiac, renal, and liver transplant procedures have a role in selected cases.

Finally, certain different therapies aimed specifically at inhibiting amyloid fibril formation or promoting fibril regression are currently under development and will be evaluated clinically within the next few years. These approaches, directed at generation of precursor proteins, the protein folding process, formed fibrils, glycosaminglycans, and SAP, offer hope that amyloidosis may become a treatable condition in the future.

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35 EOSINOPHILIA

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Various infectious, allergic, neoplastic, and other, often idiopathic, diseases can be associated with increased eosinophil numbers in the peripheral blood or tissues (Table 35.1). Eosinophilia develops when specific stimuli enhance eosinophilopoiesis (Chapter 22). Among the three eosinophil growth factor cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5, IL-5 is principally responsible for increases in eosinophilopoiesis (Chapter 22). With helminth parasites, the parasite-elicited eosinophilia is attributable to IL-5 produced by the Th2 subset of $CD4^+$T lymphocytes that can also elaborate a restricted number of other cytokines, including IL-4, which may promote heightened immunoglobulin E (IgE) production. Increases in IgE are often, but not uniformly, found in association with eosinophilia, notably in both allergic diseases and helminth infections. In several idiopathic eosinophilic disorders, concomitant increases in IgE are not present, and the immune mechanisms responsible for the eosinophilia remain to be ascertained.

TABLE 35.1. Eosinophil-Associated Diseases and Disorders

Based on published studies establishing the normal range of blood eosinophils, eosinophil numbers in excess of their usual level of less than 450/µL of blood define blood eosinophilia. Quantitation of blood eosinophils does not require the use of absolute eosinophil counts and can be calculated from differential cell counts of Wright-stained blood smears (1). Blood eosinophil numbers exhibit a mild diurnal variation, being higher in the early morning and falling as endogenous glucocorticoid levels increase (2). In addition, blood eosinophilia may fluctuate. Blood eosinophil numbers can be decreased by stress (3), as well as by intercurrent bacterial and viral infections (see later). With leukopenia in another white cell line, the percentages of eosinophils in the differential leukocyte count may be elevated without constituting a true increase in the absolute magnitude of circulating eosinophils.

Blood eosinophil numbers do not necessarily indicate the extent of eosinophil involvement in affected tissues. Eosinophils normally are principally tissue-dwelling leukocytes predominantly distributed in specific tissues, including the respiratory, gastrointestinal, and lower genitourinary tracts (Chapter 22). In addition, eosinophils are recruited into tissue sites in various diseases processes, and the tissue eosinophilia need not be reflected by the concomitant level of blood eosinophilia. Moreover, routine histopathologic staining with eosin lacks sensitivity to detect eosinophils in tissues, and techniques that use the cationic protein content in eosinophil-specific granules are more sensitive for fully enumerating eosinophils in tissues. Fluorescent staining techniques, based on granule fluorescence after staining with Fisher Giemsa stain (4,5), or fluorescein isothiocyanate (6), help to detect tissue eosinophilia. In tissues, loss of morphologically intact eosinophils, resulting from degranulation or cytolysis (7), leads to underestimation of eosinophils, whose prior presence can be evidenced by immunofluorescent detection of residual eosinophil granule cationic proteins, including major basic protein and eosinophil cationic protein (8).

In patients with eosinophilias of various causes, circulating blood eosinophils can exhibit morphologic and functional alterations consequent to being "activated" *in vivo* (9) (Chapter 22). Morphologic alterations include cytoplasmic vacuolization, alterations in granule numbers and size, and losses within specific granules of eosinophil major basic protein–containing cores or matrix. In the idiopathic hypereosinophilic syndrome (HES), the extent of cytoplasmic vacuolization and of hypogranularity has been correlated with the development of cardiac disease (2). A propensity for endomyocardial damage is a recognized complication of various diseases marked by sustained eosinophilia. This cardiac involvement can include early necrosis and subsequent formation of intraventricular thrombi and endomyocardial fibrosis with secondary mitral or tricuspid regurgitation. The damage to the heart is the same whether the eosinophilia is from HES (10,11 and 12) or other causes. Eosinophilic diseases associated at times with endomyocardial damage include the following: eosinophilic leukemia (13); eosinophilia with carcinomas or Hodgkin or non-Hodgkin lymphomas (14,15); eosinophilia from GM-CSF or IL-2 administration (16) or drug-reactions (17); and eosinophilia from helminth infections such as trichinosis, visceral larva migrans, and filariases (18,19,20,21 and 22). More recently, endomyocardial involvement has been recognized in association with eosinophilic Churg-Strauss vasculitis (23). Although diverse eosinophilic diseases can cause the same forms of cardiac disease, most patients with eosinophilia develop no evidence of endomyocardial damage. Thus, the pathogenesis of eosinophil-mediated cardiac damage involves both heightened numbers of eosinophils and some activating events, as yet ill-defined, that promote eosinophil-mediated endomyocardial damage. Patients with sustained eosinophilia should be monitored by echocardiography for evidence of cardiac disease.

The approach to a patient with eosinophilia is directed by a synthesis of information from the patient's history, the nature and types of associated clinical findings and organ involvement, and pertinent laboratory and imaging studies. Because eosinophilia (usually mild) is frequently a concomitant of common allergic diseases, these diseases should be considered, as should adverse reactions to medications. Second, because eosinophilia is common with helminthic parasite infestation, these infections should be evaluated, based on pertinent travel or other exposure histories and on associated clinical manifestations. Potentially occult strongyloidiasis merits special attention, because this infection may present with eosinophilia (24,25), it can persist for decades after acquisition, and it can cause potentially fatal dissemination if glucocorticoids are administered (26). A serologic test for *Strongyloides* helps to ascertain that occult infection with this parasite neither is the cause of eosinophilia nor is likely to disseminate with subsequent glucocorticoid administration (24). Finally, other eosinophil-associated syndromes are considered based on specific organs involved (e.g., eosinophilic pneumonia, eosinophilic gastroenteritis) or other clinical and pathologic features associated with eosinophilia.

ALLERGIC DISEASES ASSOCIATED WITH EOSINOPHILIA

Eosinophilic diseases in this category include atopic and related diseases and drug-induced eosinophilias, two of the more common causes of eosinophilia.

Atopic and Related Diseases

Peripheral blood eosinophilia may be associated with allergic rhinitis but is less common than nasal eosinophilia as an indicator of nasal allergy (27). Nasal eosinophilia, which correlates with the signs and symptoms of allergic rhinitis (28), helps to distinguish allergic rhinitis from viral infections and vasomotor rhinitis and is an indicator of responsiveness to topical glucocorticoids (27,29). Nasal eosinophilia may be present in patients with other conditions, including patients with asthma who have no symptoms of nasal allergy. Patients with nasal polyposis also have lesional and nasal secretion eosinophilia (30), and some manifest the triad of asthma, aspirin sensitivity, and nasal polyposis. Eosinophilia of involved tissues is frequent in chronic sinusitis (31).

Asthma has long been recognized to be associated with eosinophilia (2). Usually, low-grade blood eosinophilia is common, especially in allergic asthma, and in both allergic and nonallergic asthma, eosinophil numbers are increased in airway tissues (32).

Medication-Related Eosinophilias

The administration of diverse therapeutic agents, including herbal or “natural” therapies (33,34 and 35), can elicit eosinophilia. Although these reactions are often considered to represent hypersensitivity reactions, in most instances of drug-associated eosinophilia, the mechanisms leading to eosinophilia are not yet defined. Eosinophilia may develop without other manifestations of adverse drug reactions, such as rashes or drug fevers. In addition, drug-induced eosinophilia may be associated with distinct clinicopathologic patterns in which tissue eosinophilia accompanies drug-induced diseases that are characteristically limited to specific organs with or without associated blood eosinophilia. In the absence of organ involvement, blood eosinophilia by itself need not mandate cessation of drug therapy, if such therapy is medically indicated. Drug-induced blood eosinophilia, however, should prompt an evaluation to determine whether organs, including the lungs, kidneys, and heart, are involved in the eosinophil-associated drug reaction. If organ involvement develops, cessation of drug administration is necessary.

Some cytokines used as therapeutic agents are potential causes of eosinophilia. GM-CSF, but not granulocyte colony-stimulating factor, stimulates eosinophilopoiesis as well as the development of neutrophils. GM-CSF can cause prominent blood and tissue eosinophilia and, less commonly, eosinophil-associated diseases, including eosinophilic pneumonia (36) and eosinophilic endomyocardial fibrosis (16). IL-3 administration also stimulates eosinophil production (37). Administration of IL-2– or IL-2–stimulated lymphocytes frequently is followed by the development of eosinophilia (38,39), most likely from IL-2–stimulated production of IL-5 (40,41). Eosinophilic myocarditis and endocardial thrombosis has developed as a complication of high-dose IL-2 therapy (42). IL-4 can induce mild eosinophilia (43).

Diverse agents are capable of eliciting the development of pulmonary eosinophilia, including many antimicrobial agents and nonsteroidal antiinflammatory agents (NSAIDs) (44,45). The clinical patterns of presentation vary, and some patients have presented with acute or chronic eosinophilic pneumonia. Blood eosinophilia is usually, but not always, present, and if blood eosinophilia is absent, sputum or bronchoalveolar lavage (BAL) fluid eosinophilia is necessary to help make the diagnosis (45). A distinct eosinophilic pleuropulmonary reaction can be caused by dantrolene (46), and it is associated with pleural and blood eosinophilia.

In drug-induced acute interstitial nephritis, eosinophilia is common in the involved kidneys, urine, and, at times, blood. In addition to eosinophilia, fever, rash, and arthralgia support the diagnosis, but they are commonly absent in cases of drug-induced acute interstitial nephritis (47). Agents that elicit acute interstitial nephritis include methicillin and other penicillin congeners, NSAIDs, cimetidine, sulfonamides, captopril, allopurinol, phenytoin, rifampin, ciprofloxacin, aztreonam, triazolam, and warfarin (Coumadin). Eosinophiluria is not uniformly present in all patients with drug-induced interstitial nephritis. The sensitivity of eosinophiluria for acute interstitial nephritis is 40% to 60% (47,48), and when more than 10% urine eosinophils are present, the positive predictive value is 48% to 57% (48,49).

Acute necrotizing eosinophilic myocarditis is a serious but uncommon type of hypersensitivity myocarditis (50). Reactions to medications, such as ranitidine (51) or penicillin, are responsible in some cases. A syndrome of hepatitis with eosinophilia can be a manifestation of reactions to drugs, including minocycline, choline magnesium trisalicylate, halothane, methoxyflurane, salicylazosulfapyridine, ranitidine, carbamazepine, phenytoin, sulfa antibiotics, and trovafloxacin (52). Other medication-related eosinophilic responses include drug-induced hypersensitivity vasculitis (53), as well as forms of gastroenterocolitis elicited by medications, including clozapine (54) and NSAIDs (55). Patients with the syndrome of asthma, nasal polyps, and aspirin sensitivity often have eosinophilia (56). Adverse reactions to contaminated L-tryptophan in “natural” medications was previously a cause of an eosinophilia-myalgia syndrome (33,34). Not all medication-elicited eosinophilias are serious, however. Asymptomatic drug-induced eosinophilia occurs and need not cause cessation of medication usage (57,58).

INFECTIOUS DISEASES ASSOCIATED WITH EOSINOPHILIA

Eosinophilia accompanies only specific infectious diseases. Acute bacterial, viral, or protozoan parasitic infections characteristically produce eosinopenia. Even in patients with eosinophilia from helminth parasitic or allergic diseases, the development of bacterial or viral infections suppresses blood eosinophilia during these intercurrent infections. This suppression results from heightened endogenous glucocorticoid production as well as from inflammatory mediators released during these infections (59). Eosinopenia, with either serious bacterial infections or marked inflammation, accounts for the absence of otherwise expected eosinophilia in some patients with helminthic infections (26). Elevated or even normal blood eosinophil numbers in a febrile patient strongly suggest a noninfectious cause, perhaps an eosinophilic syndrome–related illness or adrenal insufficiency.

Parasitic Infections

Infections with a diversity of multicellular helminth parasites characteristically elicit eosinophilia (26). The magnitude of the eosinophilic response to helminths is determined both by the host's immune response and by the parasite, including its distribution, migration, and development in the infected host. The level of eosinophilia parallels the extent of tissue invasion by helminthic larvae or adults. For several helminthic infections, migrations of infecting larvae through the tissues are greatest early in infections; hence, the magnitude of the elicited eosinophilia is greatest during these early phases. For established infections, tissue eosinophilia around helminths may not be accompanied by blood eosinophilia when the organism is antigenically sequestered within tissues (e.g., intact echinococcal cysts) or is limited solely to the intestinal lumen (e.g., adult *Ascaris,* tapeworms). Intermittent leakage of fluids from echinococcal cysts can transiently stimulate increases in blood eosinophilia and elicit allergic (urticaria, bronchospasm) or anaphylactic reactions (60,61).

Consideration of helminth causes of eosinophilia is guided by the clinical findings and by a geographic history of potential exposure to infection (26). Several helminths can lead to long-lasting eosinophilia. Although stool examinations help to identify enteric helminths, many helminths capable of eliciting eosinophilia dwell in tissues or blood. Some tissue- or blood-dwelling helminths that are not diagnosable by stool examinations but can cause marked eosinophilia require diagnostic examinations of blood or biopsied tissues or specific serologic tests (26). Infections with these characteristics include filarial infections, trichinosis, and visceral larva migrans. Among the helminths, the principal parasite that needs to be evaluated is *Strongyloides* because, as noted earlier, it may cause fatal disseminated infections in patients unwittingly given glucocorticoid therapy. Enzyme-linked immunosorbent assay serologic testing is useful in detecting strongyloidiasis even when fecal examinations are unrevealing (62).

In contrast to infections with helminth parasites, infections with single-celled protozoan parasites do not characteristically elicit blood eosinophilia, except for two enteric protozoan organisms: *Dientamoeba fragilis* and *Isospora belli* (26).

Specific Fungal Infections

Two fungal diseases are associated with eosinophilia: aspergillosis, in the form of allergic bronchopulmonary aspergillosis (63) (Chapter 70), and coccidioidomycosis (26). Blood eosinophilia, peaking during the second or third week of illness, occurs with primary coccidioidal infection (64). Coccidioidal infection should be in the differential diagnosis of eosinophilic pneumonia (65). Eosinophilia, at times prominent, also may develop in patients with disseminated coccidioidomycosis (66,67). Coccidioidal infections also are a common cause of eosinophilic meningitis (68).

Human Immunodeficiency Virus and Other Retroviral Infections

Eosinophilia may accompany human immunodeficiency virus (HIV) infections for several reasons. First, leukopenia may lead to an increased eosinophil percentage without true eosinophilia. Second, adverse reactions to medications may elicit eosinophilia, and therapy with GM-CSF can stimulate eosinophilia. Third, patients with acquired immunodeficiency syndrome are at risk of developing adrenal insufficiency from cytomegalovirus and other infections, and eosinophilia may reflect the adrenal insufficiency (69). In addition, often modest eosinophilia is observed in some HIV-infected patients (70,71). Eosinophilia also accompanies eosinophilic folliculitis in HIV infection. Finally, marked hypereosinophilia has developed in patients with HIV infection, including some with the hyperimmunoglobulin E syndrome and some with exfoliative dermatitis (72,73,74 and 75). Eosinophilia frequently develops in patients with human T-cell lymphocytotrophic virus type I (HTLV-I) infections (76).

MYELOPROLIFERATIVE AND NEOPLASTIC DISEASE

Eosinophilia may occur with certain neoplastic diseases, as well as with a heterogeneous disorder, idiopathic HES.

Idiopathic Hypereosinophilic Syndrome

Idiopathic HES is not a single entity but rather a constellation of leukoproliferative disorders characterized by sustained overproduction of eosinophils (77,78). The three diagnostic criteria for this syndrome are the following: (a) eosinophilia in excess of 1,500 eosinophils/μL of blood persisting for longer than 6 months; (b) lack of an identifiable parasitic, allergic, or other cause of eosinophilia and an absence of other, even idiopathic, eosinophilic syndromes clinically distinct from HES; and (c) signs and symptoms of organ involvement (77). Not all patients with prolonged eosinophilia develop organ involvement, and many have benign courses. Some patients with HES exhibit features common to myeloproliferative disorders, including elevated vitamin B_{12} levels, abnormal leukocyte alkaline phosphatase scores, splenomegaly, cytogenetic abnormalities, myelofibrosis, anemia, erythrocyte abnormalities including teardrop forms, myeloid dysplasia, and basophilia. Patients with these features are less likely to respond to prednisone and are more likely to require alternative therapies. However, patients with HES rarely have expansions of cell lines other than eosinophils to the extent seen in myeloproliferative disorders and do not usually develop myelofibrosis severe enough to be associated with pancytopenia or acute leukemia. The clinical manifestations, severity, and therapeutic responsiveness vary for different patients with HES. Because the foregoing diagnostic criteria are sufficiently broad to include a diversity of eosinophilic disorders, HES represents a collection of varied disorders. In some patients with HES, the disorder has been correlated either with clonal expansions of $CD4^+CD3^-CD8^-$ Th2-like lymphocytes (79,80 and 81) or with other aberrant T cells elaborating IL-5 (82). A patient with eosinophilia associated with polyclonal expansion of activated $CD3^+$ T cells expressing natural killer (NK) cell markers (CD16 and CD56), associated with IL-2 and IL-15 overproduction, has been reported (83). In other patients, however, it appears that overproduction of IL-5 is not solely responsible for the eosinophilia (77). Clonal abnormalities in the eosinophil lineage have been reported in few patients (84,85 and 86). For many patients with HES, the causes of the eosinophilia are not currently understood.

In addition to eosinophilia and other hematologic abnormalities, the organ system most commonly involved is the heart (77). Cardiac damage progresses through three stages: the first involves acute necrosis in the early weeks, the second involves the development of endocardial thrombi over many months, and the third is the fibrotic stage after 2 or more years of disease. The first stage is frequently clinically occult. There is damage to the endocardium and infiltration of the myocardium with eosinophils and lymphocytes with histopathologic evidence of myocardial necrosis, eosinophil degranulation, and eosinophil microabscesses. A similar acute eosinophilic myocarditis can develop with hypersensitivity reactions and may be more fulminant (50). In patients with acute-stage myocardial necrosis, splinter hemorrhages may be prominent; but clinical cardiac findings are often absent, although rare deaths can result from acute progressive cardiac disease (12). Echocardiography and angiography detect no abnormalities in this stage, and endomyocardial biopsy is needed to make the diagnosis (12). Glucocorticoid therapy during the acute stage may help to control and prevent the evolution of myocardial fibrosis (12).

The second stage of heart disease, the formation of thrombi along the damaged endocardium, affects either or both ventricles and rarely the atria (10,77). Outflow tracts near the aortic and pulmonic valves are usually spared. Finally, in the fibrotic stage, progressive scarring may lead to entrapment of the chordae tendineae with the development of mitral or tricuspid valve regurgitation and endomyocardial fibrosis producing restrictive cardiomyopathy (77). Patients with HES often present at the later thrombotic and fibrotic stages. Common manifestations include dyspnea, chest pain, signs of left or right ventricular congestive heart failure, murmurs of mitral regurgitation, cardiomegaly, and T-wave inversions (10). Echocardiography is valuable in detecting intracardiac thrombi and the manifestations of fibrosis, which include thickening of the posterior mitral valve leaflet and its attachment to a thickened posterior wall, as well as increases in intensities of endomyocardial echoes in areas of endomyocardial fibrosis (77). Cardiac catheterization demonstrates increased right and left ventricular end-diastolic pressures, and angiography can demonstrate valvular incompetence as well as delineate apical obliteration or irregularities (12). Cardiac biopsies confirm the diagnosis, although in patients with intense fibrosis in late disease, biopsy instruments may fail to obtain samples.

The risks of developing cardiac disease in two series of patients with HES were not related to the extent of eosinophilia or to the duration of HES (87,88). Patients who developed evident cardiac disease were more likely to be male, to be HLA-Bw44 positive, and to have splenomegaly, thrombocytopenia, elevated levels of vitamin B_{12}, hypogranular or vacuolated eosinophils, and abnormal early myeloid precursors in their blood (88). Those patients with HES who were free of cardiac disease tended to be female and to have angioedema, hypergammaglobulinemia, elevated serum levels of IgE, or circulating immune complexes (88).

In HES, neurologic involvement can take three forms: embolic disease originating from the heart; diffuse encephalopathy; and peripheral neuropathy, especially mononeuritis multiplex (89). Other organs systems that can be involved in HES include the skin, liver, spleen, gastrointestinal tract, and lungs (77).

No specific diagnostic tests exists for HES. Early reports emphasized the mortality related to this disorder, but many of these deaths resulted from congestive heart failure and complications of endomyocardial damage (90). For many patients, if the sequelae of organ damage, especially to the heart, can be managed, HES can have a prolonged course over decades. For those patients with eosinophilia without organ damage, no therapy need be administered. For those requiring therapy, prednisone is the initial agent, administered at 60 mg per day in adults. Patients more likely to respond to prednisone alone are those with angioedema, urticaria, or elevated serum IgE levels and those who experience prolonged eosinopenic responses to single doses of prednisone (91,92). Patients less likely to respond to prednisone include those with splenomegaly and with cardiac or neurologic dysfunction at the time of presentation (91). For those not responsive to prednisone, daily hydroxyurea was previously shown to be effective (93), but the currently preferred therapy for HES is interferon-a (94). Medical management of cardiac complications, including arrhythmias and congestive heart failure, and surgical replacement of damaged valves are important and effective measures in the longer-term management of HES (77).

Eosinophilia with Tumors or Leukemias

Eosinophilic leukemia is distinctly uncommon. Acute eosinophilic leukemia can be distinguished from HES when one notes a marked increase in the number of immature eosinophils in the blood or bone marrow (with more than 10% blast forms in the marrow), infiltration of tissues with immature cells of predominantly eosinophilic type, and a clinical course similar to that of other acute leukemias, including pronounced anemia, thrombocytopenia, and susceptibility to infections (2,95). In addition, patients with eosinophilic leukemias often exhibit a clonal cytogenetic abnormality. Eosinophilia is a characteristic of the M4Eo subtype of acute myeloid leukemia, having the common M4 characteristic of chromosomal 16 abnormalities. In addition, several rare forms of eosinophilic leukemia, each with specific cytogenetic and molecular genetic abnormalities, have been recognized (95). Eosinophilia may accompany chronic myelogenous leukemia but is uncommon with acute lymphoblastic leukemia. Eosinophilia may accompany some lymphomas, including Hodgkin disease, especially the nodular sclerosing form (96), T-cell lymphoblastic lymphoma, and adult T-cell leukemia/lymphoma (95). A few patients with carcinomas, especially of mucin-producing epithelial cell origins, have associated blood and tissue eosinophilia. Eosinophilia may accompany angioimmunoblastic lymphadenopathy, mycosis fungoides, Sézary syndrome, and lymphomatoid papulosis (82,97). Eosinophilia occurs in about 20% of patients with systemic mastocytosis and may be the presenting finding in the absence of cutaneous manifestations (98).

ORGAN SYSTEM INVOLVEMENT AND EOSINOPHILIA

Eosinophilic syndromes limited to specific organs, such as eosinophilic pneumonia (99) or eosinophilic gastroenteritis (100), characteristically do not extend beyond their own target organ. Therefore, these syndromes lack the multiplicity of organ involvement often found with HES and do not have the predilection to develop secondary, eosinophil-mediated cardiac damage, for reasons that are not known. These distinct eosinophilic syndromes therefore can usually be separated from HES, although individual patients may on occasion present with overlapping features that complicate classification.

Skin and Subcutaneous Diseases

Various cutaneous diseases can be associated with increased numbers of blood eosinophils, including atopic dermatitis, blistering disorders such as bullous pemphigoid, drug reactions, and two diseases associated with pregnancy, herpes gestationis and the syndrome of pruritic urticarial papules and plaques of pregnancy. Eosinophilic pustular folliculitis is seen mostly in patients with HIV infections and in those treated for hematologic malignant disease or after bone marrow transplantation (101,102). For patients with cutaneous involvement and eosinophilia, angiolymphoid hyperplasia with eosinophilia and Kimura disease (103), eosinophilic cellulitis (Wells syndrome) (104), eosinophilic fasciitis (105), and eosinophilic pustular folliculitis can be differentiated on the basis of the histopathologic features of biopsied lesions. A distinct syndrome of unknown origin, episodic angioedema with eosinophilia, is characterized by recurring episodes of angioedema, urticaria, fever, and marked blood eosinophilia (106). The clinical course of this disease, with its prominent periodic occurrences of angioedema and eosinophilia, and its lack of association with cardiac damage distinguish it from HES.

Gastrointestinal Diseases

Eosinophilic gastroenteritis represents a heterogeneous collection of disorders in which one may note eosinophilic infiltration of the mucosa, the muscularis, or the serosa, the last of which can lead to eosinophilic ascites (100). Although some patients have demonstrable intolerance to milk proteins, the disease usually remains idiopathic but usually responds to glucocorticoid treatment. Common symptoms include abdominal pain, diarrhea, vomiting, and nausea. Although ultrasound or computed tomography can detect areas of intestinal wall thickening, gastric or intestinal biopsy is needed to make the diagnosis.

Eosinophils are present in the lesions of collagenous colitis and ulcerative colitis, but blood eosinophilia is usually absent. Gastrointestinal eosinophilia elicited by intestinal helminths and eosinophilic enterocolitis resulting from hypersensitivity reactions to medications must be excluded in patients with these diseases who have tissue eosinophilia.

Pulmonary Eosinophilias

Blood eosinophilia can infrequently accompany pleural fluid eosinophilia, which is a nonspecific response seen with various disorders including trauma and even repeated thoracenteses (107). Several pulmonary parenchymal disorders may be associated with eosinophilia. Helminth parasites are responsible for four forms of eosinophilic lung disease (26). The first is Loeffler syndrome, which is marked by blood eosinophilia, eosinophilic patchy pulmonary infiltrates that appear and resolve over weeks, and, at times, bronchospasm. This syndrome is typically caused by those helminth parasites (*Ascaris lumbricoides* and, less commonly, hookworm and *Strongyloides*) that migrate through the lungs early in their developmental life cycles (26). Stool examinations are not helpful because the pulmonary response is elicited by infecting larval forms months before productive egg laying from later adult stages would begin in the intestines. The diagnosis is made on epidemiologic grounds or, less commonly, by detecting larvae in sputum or BAL fluids (26,108). The second form of helminth-induced lung disease is the syndrome of tropical pulmonary eosinophilia, which develops in a few patients infected with lymphatic-dwelling filarial species (26,109). Clinically, this syndrome is characterized by marked blood eosinophilia, paroxysmal nonproductive cough, wheezing, and occasional weight loss, lymphadenopathy, and low-grade fevers. On chest radiographs, increased bronchovesicular markings, diffuse interstitial lesions 1 to 3 mm in diameter, or mottled opacities, usually more prominent in lower lung fields, are common. Patients have markedly increased numbers of blood and alveolar eosinophils and elevations in both total serum IgE and antifilarial antibodies. A third form of helminth-induced lung disease is caused by helminths that invade the pulmonary parenchyma, notably lung flukes that cause paragonimiasis (26). The fourth form of lung disease is caused by larger than usual numbers of helminth organisms that are carried hematogenously into the lungs. Examples include schistosomiasis, trichinosis, and larva migrans (26). Bronchopulmonary aspergillosis constitutes another type of eosinophil-associated pulmonary disease.

Two forms of idiopathic eosinophilic pneumonia are recognized. In chronic eosinophilic pneumonia, patients typically exhibit peripheral pulmonary infiltrates that may extend across lobar fissures (110). Blood eosinophilia is present in most, but not all, patients. This disease of unknown origin is responsive to glucocorticoids but is prone to relapse (111). An acute form of eosinophilic pneumonia, manifest by fever, pulmonary infiltrates, and respiratory insufficiency, is diagnosable by demonstrating eosinophils in BAL fluids or by lung biopsy (112). Acute eosinophilic pneumonia responds to glucocorticoid treatment and does not relapse.

The major vasculitis associated with eosinophilia is Churg-Strauss syndrome (113). A history of asthma, nonfixed pulmonary infiltrates, blood eosinophilia greater than 10%, paranasal sinus abnormalities, mononeuropathy or polyneuropathy, and a blood vessel biopsy specimen demonstrating extravascular eosinophils characterize this syndrome (114). For patients with vasculitis, the presence of four or more of these six criteria yielded a sensitivity of 85% and a specificity of 99.7% for Churg-Strauss vasculitis (114). Late-onset asthma is common and may be antecedent for several years. Asthma, eosinophilia, and, at times, transient pulmonary infiltrates antedate the development of systemic vasculitis in about half the cases. Pulmonary involvement is seen in almost all patients, and pulmonary infiltrates occur in three fourths of patients. Nasal and sinus involvement is common. Glucocorticoid treatment of asthma may mask the evolution of Churg-Strauss syndrome (115). Use of anti–cysteinyl leukotriene agents for asthma has been associated with the onset of the Churg-Strauss syndrome. In many, but not all, patients, these agents have permitted diminished glucocorticoid dosing for asthma, but the precise relationship of these drugs with the development of this syndrome remains to be delineated (116). In addition to frequent pulmonary disease, neurologic, cutaneous, cardiac, and gastrointestinal organ involvement is common (113).

Medications and other drugs are capable of eliciting pulmonary eosinophilia (45). Commonly implicated medications include NSAIDs and antimicrobial medications. Toxic agents, including those from occupational exposures, also can cause pulmonary eosinophilia (45). Each of these reactions has a defined etiologic stimulus and hence differs from idiopathic and other eosinophilic diseases, but the clinical presentation of drug- and toxin-elicited pulmonary eosinophilias can resemble that of other forms of pulmonary eosinophilia, including acute or chronic eosinophilic pneumonia.

Rheumatologic Disorders

Of the various forms of vasculitis, only two are commonly associated with eosinophilia. The principal eosinophil-related vasculitis is Churg-Strauss syndrome, as discussed in the previous section. Cutaneous necrotizing eosinophilic vasculitis with hypocomplementemia and eosinophilia is a distinct vasculitis of small dermal vessels that are extensively infiltrated with eosinophils. This form of vasculitis may occur in patients with connective tissue diseases (117). In addition, eosinophilia may uncommonly accompany rheumatoid arthritis itself but is more commonly the result of adverse reactions to treatment medications (including NSAIDs, gold, and tetracyclines) or concomitant vasculitis. A syndrome, characterized by the association of nodules, eosinophilia, rheumatism, dermatitis, and swelling (NERDS), includes prominent paraarticular nodules, recurrent urticaria with angioedema, and tissue and blood eosinophilia [Zenarola, 1995 #5320].

Immunologic Disorders

Some primary immunodeficiency syndromes are associated with eosinophilia. The hyper-IgE syndrome is characterized by the following: recurrent staphylococcal abscesses of the skin, lungs, and other sites; pruritic dermatitis; hyperimmunoglobulinemia E; and eosinophilia of the blood, sputum, and tissues (118). Eosinophilia is characteristic of Omenn syndrome, combined immunodeficiency with hypereosinophilia (119).

Infiltration of eosinophils accompanies rejection of lung (120), kidney (121), and liver (122,123 and 124) allografts. Tissue and blood eosinophilia occurs early in the rejection process, and eosinophil counts and granule protein levels (in urine or BAL fluids and in involved allograft tissues) have correlated with prognosis, severity, and response to rejection therapy.

Endocrine Diseases

Because glucocorticoids normally exert eosinopenic effects, the loss of endogenous adrenoglucocorticoid production in Addison disease, adrenal hemorrhage, or hypopituitarism can cause blood eosinophilia (125,126).

Other Causes of Eosinophilia

The syndrome of atheromatous cholesterol embolization can be associated with eosinophilia and eosinophiluria (127,128). Uncommonly, kindreds with hereditary eosinophilia have been recognized (129). Irritation of serosal surfaces can be associated with eosinophilia, and related diseases can include Dressler syndrome, eosinophilic pleural effusions (107), peritoneal and, at times, blood eosinophilia developing during long-term peritoneal dialysis (130), and perhaps the eosinophilia that may follow abdominal irradiation (131).

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36 MASTOCYTOSIS

Dean D. Metcalfe, M.D.

Mastocytosis is a relatively rare disease characterized pathologically by mast cell hyperplasia in the skin, bone marrow, lymph nodes, liver, gastrointestinal tract, and spleen. Clinical features include pruritus, urtication, nausea, vomiting, abdominal pain, diarrhea, headache, flushing, and vascular instability. Urticaria pigmentosa, the most common skin manifestation of mastocytosis, was described by Nettleship in 1869 (1). Excessive numbers of mast cells were recognized in the lesions of urticaria pigmentosa by Unna in 1887 (2). In 1949, Ellis reported the case of a child with urticaria pigmentosa, with the first postmortem evidence suggesting that mast cell hyperplasia in skin could be associated with systemic disease (3). Since then, the clinicopathologic diversity of mastocytosis has been increasingly recognized. These developments have been accompanied by a rapid expansion in knowledge of the growth, differentiation, and biologic expression of mast cells, including the recognition of the origin of mast cells from $CD34^+$ human pluripotent cells (4), the description of mast cell growth factors including interleukin-3 (IL-3) (5) and kit ligand or stem cell factor (SCF) (6), the recognition that SCF is the one essential growth factor for human mast cells (7), and the description of various pleomorphic and redundant mast cell–derived mediators (8).

The prevalence of mastocytosis is unknown. It has been reported to be the diagnosis in 1 patient per 1,000 to 8,000 new cases seen in a dermatology clinic (9). Mastocytosis can occur at any age, and its distribution between men and women is approximately equal. Approximately 50 cases of familial mastocytosis have been reported (10). Most cases of mastocytosis are sporadic.

CLINICAL SIGNS AND SYMPTOMS

Urticaria pigmentosa is the most common skin manifestation of mastocytosis in both children and adults. The individual lesions of urticaria pigmentosa manifest as small, yellow-tan to reddish-brown macules or slightly raised papules. Lesions are less likely to appear on the palms, soles, face, and scalp. Mild trauma, such as scratching or rubbing of the lesions, may provoke urtication and erythema at the site of the macules; this is known as the Darier sign. Pruritus occurs and may be exacerbated by changes in temperature, skin friction, ingestion of hot beverages or spicy foods, ethanol, and certain drugs.

In diffuse cutaneous mastocytosis, the entire cutaneous integument is involved. The skin is normal to yellow brown and is diffusely thickened. Diffuse cutaneous mastocytosis usually occurs before the age of 3 years. Children with urticaria pigmentosa or diffuse cutaneous mastocytosis may experience bullous eruptions with hemorrhage (11). Blisters erupt spontaneously or occur in association with infection or immunization. Blisters may appear at birth and thus are in the differential diagnosis of neonatal disorders with blisters.

Telangiectasia macularis eruptiva perstans is a rare form of mastocytosis. In this pattern of disease, the skin is covered with tan to brown macules and patchy erythema. Telangiectasias are observed (12). Solitary lesions called mastocytomas are rare. Their onset is generally before the age of 6 months, and in most cases these lesions spontaneously involute.

Gastrointestinal symptoms develop in up to 80% of adult patients with systemic disease (13). The most common gastrointestinal symptom is abdominal pain, followed by diarrhea, nausea, and vomiting. Peptic ulcer disease occurs in up to approximately 40% of adult patients with systemic disease, with some evidence of malabsorption in approximately 30% (13). Malabsorption is usually not severe and is manifested primarily as mild steatorrhea with impaired absorption of D-xylose or vitamin B_{12}.

Approximately 60% of patients with mastocytosis have evidence of liver disease. Severe liver disease, however, is uncommon, except in patients with aggressive forms of mastocytosis (14). The most common chemical abnormality is an elevated alkaline phosphatase level, which directly correlates with hepatomegaly, splenomegaly, liver mast cell infiltrations, and fibrosis. Patients with systemic disease frequently exhibit splenic involvement manifested by splenic enlargement (15), and spleens weighing more than 700 g are seen in patients with severe disease. Portal hypertension and ascites associated with liver fibrosis, nodular regenerative hyperplasia, portal venopathy, and venoocclusive disease are unusual. Peripheral lymphadenopathy occurs in up to 26% of patients with systemic disease, and central lymphadenopathy is seen in 19% of patients at the time of diagnosis (15).

Hematologic abnormalities are frequent in patients with mastocytosis sent to referral centers such as the Mayo Clinic in Rochester, Minnesota, and the National Institutes of Health in Bethesda, Maryland. In one study, 3 of 13 patients with isolated cutaneous disease had anemia, thrombocytosis, eosinophilia, or lymphopenia. Twenty-two of 32 patients with systemic disease had at least one of the following: anemia (50%), thrombocytopenia (19%), thrombocytosis (9%), leukopenia (9%), leukocytosis (31%), eosinophilia (21%), monocytosis (18%), lymphopenia (32%), or lymphocytosis (14%) (16). Hematologic abnormalities were infrequently reported in other studies (17,18), a finding suggesting that, in general, the prevalence of hematologic abnormalities in patients with mastocytosis is less than the data from large referral centers suggest.

Skeletal changes are believed to contribute to musculoskeletal pain in up to 28% of patients. Radiographically detectable lesions are present in up to 70% of patients with systemic disease. The proximal long bones are most often affected, followed by the pelvis, ribs, and skull. Skeletal scintigraphy (bone scan) is more sensitive than a radiographic survey in detecting and locating active lesions (19). In addition, both the degree of abnormalities on the initial bone scan and the progression of scintigraphic abnormalities with serial scanning correlate with more aggressive forms of mastocytosis. In patients with severe or advanced disease, pathologic fractures may occur.

Patients with mastocytosis may report cutaneous flushing. In severe cases, vascular collapse may occur. Flushing is variably provoked by alcohol, aspirin, exercise, or infection.

Headache, decreased attention span, memory impairment, irritability, difficulty in concentration, inability to work, problems in interpersonal relations, poor motivation, confusion, anger, and depression have been reported as mental manifestations of mastocytosis in adults (20). In contrast, in one study that assessed behavioral problems in a group of pediatric patients with mastocytosis, no clear behavioral disorder was found to exist in children with mastocytosis compared with other medically ill children (21).

PATHOBIOLOGY

Human mast cells arise from $CD34^+$ progenitor cells (4), which are kit^+ and $CD13^+$ (22) under the influence of SCF. Mast cell survival in tissues is believed to depend on the local production of SCF by stromal cells (6). SCF then acts in concert with other locally produced factors to determine the final mast cell phenotype.

The identification of mast cells as hematopoietically derived cells whose growth and differentiation depends at least in part on SCF suggests that one cause of mastocytosis may relate to an overproduction of mast cell growth factors. Production of SCF by keratinocytes in the skin of patients with urticaria pigmentosa (23) could be one explanation of the mast cell hyperplasia in such lesions (24). Our group has been unable to demonstrate that the levels of SCF are elevated in sera or skin blister fluids from patients with mastocytosis.

An alternate hypothesis about the origin of mastocytosis is that activating mutations in kit permit the evolution of mastocytosis by driving excess proliferation of mast cell precursors and by facilitating mast cell survival. This idea was suggested by early data on mast cell growth and development that showed that more mast cells could be found in culture in the presence of SCF when mast cell progenitors were obtained from patients with mastocytosis compared with hematologically normal donors (25). Subsequent analysis of c-*kit* obtained from peripheral blood mononuclear cells of patients with mastocytosis revealed that a subset of these patients with a hematologic disorder had a point mutation at amino acid 816 resulting in substitution of Val for Asp (26). This mutation was identical to 1 of 2 mutations reported in the HMC-1 cell line and had been shown to cause ligand-independent phosphorylation of kit (27). The subsequent identification of this mutation in the skin, but not in the bone marrow or blood, of a child with mastocytosis (28) contributed to the idea that lesional skin of patients with mastocytosis, in which cells bearing the mutation would be concentrated, should be the best tissue in which to identify mutated kit. Subsequently, it was found that of 11 adults examined, all had an activating mutation in

codon 816 in lesional skin. Among the children examined, 4 with severe disease had codon 816 activating mutations; 3 had a dominant inactivation mutation substituting lysine for glutamic acid in position 839. Three children with sporadic disease had no mutations. No c-*kit* mutations were found in 3 patients with familial mastocytosis (29). Follow-up studies have also shown that the c-*kit* mutation can be identified in lymphocytes and myelomonocytic cells as well as in mast cells (30). Thus, it appears that in most adults with mastocytosis, an activating mutation at codon 816 is present and may permit mastocytosis to develop. Additional genetic polymorphisms, mutations, and chromosomal changes may then determine the eventual disease course. In children, the situation is more varied, and other causes of disease are possible. The identification of mutations in kit in mastocytosis may eventually aid in assigning prognosis and in developing a more objective classification for mastocytosis (31).

The mast cell hyperplasia in mastocytosis is associated with evidence of abnormal levels of circulating mast cell mediators, including histamine (32) and mast cell tryptase (33). Mediators, including histamine, tryptase, heparin, tumor necrosis factor-a, prostaglandin D_2 (PGD_2), leukotriene C_4, cytokines, and platelet-activating factor, undoubtedly contribute to the pathogenesis of mastocytosis.

The prevalence of atopy in persons with urticaria pigmentosa is similar to that in the general population (34). However, total serum IgE levels are significantly lower. This finding may relate to an increased adsorption of circulating IgE to an increased number of tissue mast cells.

The skin of patients with urticaria pigmentosa (Fig. 36.1) contains increased numbers of mast cells, with highest concentrations under the pigmented lesions (Fig. 36.2). Mast cells first appear to concentrate near vessels and with time appear at greater distances from vessels (24). Small vessel hyperplasia is attributed to mast cell heparin and mast cell cytokines that facilitate vessel proliferation. Blisters in children associated with cutaneous disease contain not only histamine, but also PGD_2 and platelet-activating factor (35). Diffuse cutaneous mastocytosis is associated with a remarkable diffuse mast cell infiltrate throughout the dermis. Patients with significant dermal infiltration with mast cells may have prolonged bleeding times resulting from mast cell–associated heparin (36).

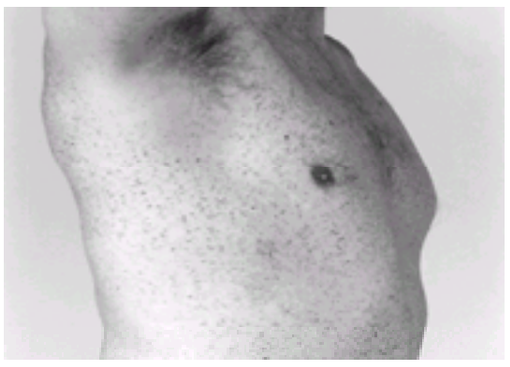

Figure 36.1. Urticaria pigmentosa in an adult patient with indolent disease. (See Color Figure 36.1.)

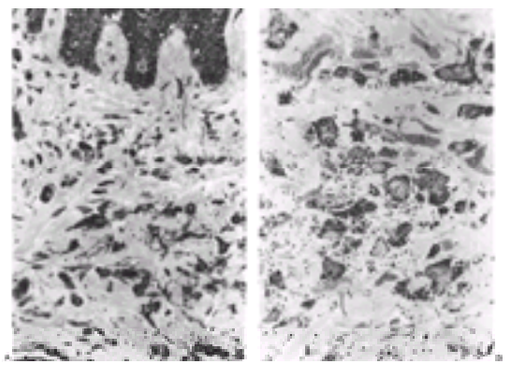

Figure 36.2. A and **B:** Light micrographs of a skin lesion from a patient with urticaria pigmentosa showing mast cell proliferation in the dermis. A specimen of lesional skin was obtained by biopsy, embedded in epoxy resin, sliced 1-µm thick, and stained with Wright-Giemsa stain. (**A** ×520; **B** ×1,310). (Courtesy of Dr. John P. Caulfield.)

Most patients with dyspeptic-type abdominal pain have evidence of gastric hypersecretion (13). The plasma concentration of histamine correlates with the basal acid output but not with the maximal acid output. These findings are consistent with the hypothesis that elevated systemic levels of mast cell–derived histamine lead to increased gastric acid secretion, which, in turn, promotes gastritis and peptic ulcer disease.

Nonpeptic abdominal pain, located in the lower abdomen, is not relieved by antacids and is not associated with gastric hypersecretion. This pain is often cramping and may result from the local production of lipid-derived mediators. Evidence of malabsorption may be found in association with these complaints. Diffuse small intestinal mucosal dysfunction has been proposed as the basis of malabsorption (37). A structural basis for this intestinal mucosal dysfunction has been suggested by the finding of blunted villi and an increase in mucosal mast cells in the mucosa (13,37). No differences in gastric emptying studies or mouth-to-cecum transit time were found between patients with systemic mastocytosis with or without diarrhea or nondyspeptic abdominal pain and control subjects (13). An increase in intestinal transit thus does not appear to contribute to symptoms.

Gastrointestinal peptides alter gastrointestinal transit and motility; they also can degranulate mast cells and can lead to the production of mast cell cytokines (38). Thus, mast cells may release mediators in response to neuropeptides or may release neuropeptides themselves that can affect motility. An elevated level of vasoactive intestinal peptide was observed in one patient with mastocytosis (39). A second study measured gastrointestinal peptide concentrations in six patients with mastocytosis with diarrhea or nondyspeptic abdominal pain and control subjects; no significant differences were found in mean plasma concentrations of neurotensin, substance P, or motilin. Patients had lower mean plasma concentrations of gastrin and substance P than did control subjects (13). These results do not exclude the possibility that increased or altered local release of these peptides is relevant.

In lymph nodes, mast cell infiltrates are most common in the paracortex, followed by the follicles, the medullary cords, and the sinuses. Early infiltrates are exemplified by clusters of mast cells. Eosinophils accompany mast cell infiltrates in lymph node tissues in approximately one half of the patient's lymph nodes (15). Blood vessel proliferation sometimes occurs in lymph nodes in the paracortical areas infiltrated by mast cells. Some patients exhibit extramedullary hematopoiesis.

Tissue fibrosis is most frequently found in the liver (14) and bone marrow (16). Liver fibrosis may be minimal, moderate, or severe. Fibrotic patterns include periductal patterns and portal-to-portal fibrosis. Various degrees of trabecular fibrosis are common in the spleen (15). This fibrosis may relate to the ability of mast cells to produce certain connective tissue components (40), and mast cells synthesize transforming growth factor-b and other agents that may promote fibrosis (8).

The bone marrow is the most useful biopsy site for establishing the diagnosis of systemic mastocytosis (41,42 and 43). Examination of the bone marrow reveals mast cell infiltrates and allows examination of the hematopoietic marrow, which provides important prognostic information. The morphologic appearance of mastocytosis-related bone marrow infiltrates in trephine core biopsy sections is unique. Infiltrates in the bone marrow are usually focal, although they may be diffuse. Focal mastocytosis lesions are most commonly situated paratrabecularly (Fig. 36.3). Perivascular and parafollicular distributions are the next most common. Lesions consist of nodular aggregates of spindle-shaped mast cells, frequently accompanied by lymphocytes and eosinophils. Mast cells have oval to elongated nuclei and eosinophilic cytoplasm. They may or may not appear granular by light microscopy with hematoxylin and eosin stains. The bone marrow lesions are cellular in early stages of the disease. As the disease progresses, the number of mast cells may decrease, and the lesions may become fibrotic. Mastocytosis infiltrates in the bone marrow may be associated with osteosclerotic or osteolytic changes in the bone trabeculae.

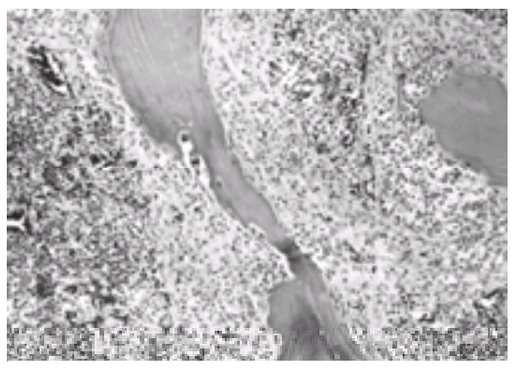

Figure 36.3. Light micrograph of a bone marrow specimen from a patient with systemic indolent mastocytosis. The specimen is stained with hematoxylin and eosin (×400).

Special stains are often used to confirm the presence of mast cells. The metachromatic staining of mast cell granules decreases during routine processing of specimens. Optimal staining is achieved with immunohistochemistry to detect mast cell tryptase.

Prognostic features of bone marrow in mastocytosis have been reported. Hypercellular bone marrow with a decreased percentage of fat cells is a significant predictor of poor prognosis (43). The hypercellular marrow, in most cases, is caused by an increase in hematopoietic elements or extensive mast cell infiltration. Most patients with mastocytosis with a hypercellular hematopoietic marrow have an associated hematologic disorder. Approximately one third of all adult patients referred to the Mayo Clinic or the National Institutes of Health have associated hematologic disorders, defined by traditional criteria (16,43), including dysmyelopoietic syndromes, myeloproliferative disorders, *de novo* acute leukemia, malignant lymphoma, and chronic neutropenia. Chromosomal abnormalities in mastocytosis have been reported but are not consistent (44). Patients with mastocytosis with hematologic disorders have significantly reduced 5-year survival rates (43). Although systemic mast cell lesions in patients with hematologic disorders may be discovered incidentally during examination of bone marrow biopsy specimens, most patients present with manifestations of mastocytosis including urticaria pigmentosa, organomegaly, increased excretion of histamine metabolites, and elevated levels of serum mast cell tryptase; the hematologic disorder is detected after mastocytosis is diagnosed.

The relative number of mast cells in the bone marrow aspirate is not always a useful measure of pathologic mast cell infiltrates. In some cases, no mast cells are found in the bone marrow aspirate despite evidence of mastocytosis in trephine core biopsy sections. Mast cell hyperplasia in bone marrow occurs in conditions other than systemic mast cell disease, including uremia (45), osteoporosis (46), and hematologic conditions such as lymphomas, preleukemias, and leukemias (47,48 and 49). Because the number of mast cells varies in persons with and those without mastocytosis, no criteria exist to distinguish mast cell hyperplasia from mastocytosis solely on the basis of the bone marrow aspirate.

Bone marrow infiltration with mast cells is associated with radiographically detectable lesions. Osteoporosis is common (50). Bone changes may result from the action of mast cell–derived heparin, proteases, cytokines, and lipid-derived mediators on bone deposition and on osteoclast and osteoblast function.

DIAGNOSIS

Mastocytosis is diagnosed on the basis of presentation, physical examination, laboratory studies, and histologic findings. This information is also used to classify disease and to assign prognosis (51) (Table 36.1). Patients with cutaneous or indolent mastocytosis have a good prognosis, whereas patients in the other groups tend to develop more complications and to exhibit a more rapid disease course. Indolent mastocytosis is defined by the presence of syncope, elevated levels of mast cell–derived mediators in blood or urine, cutaneous disease, ulcer disease, malabsorption, bone marrow involvement, skeletal disease, hepatosplenomegaly, and lymphadenopathy. Most patients diagnosed as having mastocytosis are placed in the indolent category. Most patients with indolent disease experience symptom progression but can be managed successfully for decades with medications that provide symptomatic relief. The next most common form of mastocytosis is that associated with a hematologic disorder. In this group, examination of the bone marrow and peripheral blood reveals the hematologic abnormality. The prognosis of these patients is determined by the course of the associated hematologic disorder. Patients in the following category have an aggressive form of mastocytosis but do not have a distinctive hematologic disorder or mast cell leukemia. These patients have poor prognostic features, they have a rapid increase in mast cell number, and they are difficult to manage medically. One subset of patients with aggressive mastocytosis has a distinct syndrome termed *lymphadenopathic mastocytosis with eosinophilia* because of the pronounced eosinophilia, hepatosplenomegaly, and lymphadenopathy. Mast cell leukemia is a rare form and has the most fulminant disease course (52). The peripheral blood smear reveals numerous immature mast cells. Mast cell leukemia is distinguished from the other categories by its unique pathologic and clinical picture.

Cutaneous mastocytosis
1. Urticaria pigmentosa
2. Diffuse cutaneous mastocytosis
3. Mastocytoma of the skin
Systemic mastocytosis without an associated hematologic disorder
1. Indolent
2. Smoldering
Systemic mastocytosis with an associated hematologic disorder
1. Myeloproliferative
2. Myelodysplastic
Systemic aggressive mastocytosis
Mast cell leukemia
Mast cell sarcoma
Extracutaneous mastocytoma

Adapted from WHO classification.

TABLE 36.1. Classification of Mastocytosis

Most patients with indolent mastocytosis have either urticaria pigmentosa or diffuse cutaneous mastocytosis. Urticaria pigmentosa is less common in patients with mastocytosis and an associated hematologic disorder and in those with aggressive mastocytosis, and it is rarely observed in patients with mast cell leukemia. The clinical diagnosis of urticaria pigmentosa should be confirmed with a skin biopsy (12,24). Although there is often a 15- to 20-fold increase in mast cells beneath the urticaria pigmentosa lesion, occasionally only a 2- to 4-fold increase in mast cells occurs. Similar 2- to 4-fold increases in mast cells have been found in patients with recurrent anaphylaxis (24), scleroderma (53), chronic urticaria (54), and prolonged antigenic contact (55). Thus, the diagnosis of urticaria pigmentosa cannot be made solely on the basis of small increases in dermal mast cells. Cutaneous responses to intradermal histamine are unchanged in patients with urticaria pigmentosa (56).

Mastocytosis should be suspected in patients without skin lesions in the presence of one or more of the following features: unexplained ulcer disease or malabsorption, radiographic or technetium-99 bone scan abnormalities, hepatomegaly, splenomegaly, lymphadenopathy, peripheral blood abnormalities, and unexplained flushing or vascular collapse. Elevations in the levels of plasma (32,57) or urinary histamine or histamine metabolites (58), PGD_2 metabolites in the urine (59), and plasma mast cell tryptase (33) are not diagnostic, but they suggest the possibility of mastocytosis. Plasma mast cell tryptase is actually a mixture of a- and b-tryptases. Most commercial assays measure total tryptase. However, clinical data demonstrate that a-tryptase is the form elevated in mastocytosis, whereas b-tryptase becomes elevated during a systemic allergic reaction. As these tests become available, it may be possible to distinguish more clearly between mastocytosis and anaphylaxis resulting from an allergic reaction (33).

In the absence of skin lesions, patients suspected of having mastocytosis should undergo a bone marrow biopsy and aspiration to confirm the diagnosis and to determine the disease category. Patients with urticaria pigmentosa or diffuse cutaneous mastocytosis should also undergo this procedure if they have peripheral blood abnormalities, hepatomegaly, splenomegaly, or lymphadenopathy, to determine whether they have an associated hematologic disorder. Mast cell leukemia is a distinct clinical entity. The bone marrow is hypercellular, with diffuse infiltration with atypical mast cells (52). In patients with mast cell leukemia, more than 10% of the circulating nucleated cells are atypical mast cells, and this percentage may increase to more than 90% of all circulating nucleated cells during the course of the disease. Mast cell leukemia differs from the rare case of aggressive mastocytosis with a terminal leukemic phase, in which circulating mast cells appear late in the course of the disease and the percentage of circulating cells that are mast cells is relatively low. Histologic study of other tissue specimens such as lymph nodes, spleen, liver, and gastrointestinal mucosa may help to define the extent of mast cell involvement. Surrogate plasma markers of bone marrow disease, including soluble

kit and soluble CD25, have been described, and the circumstances in which progressive elevations of these molecules correlate with more severe bone marrow disease (60). These tests are not yet widely available.

A 24-hour urine study of 5-hydroxyindoleacetic acid and urinary metanephrines may be necessary to eliminate the possibility of a carcinoid tumor or pheochromocytoma. Patients with mastocytosis do not excrete increased amounts of 5-hydroxyindoleacetic acid. Idiopathic anaphylaxis and flushing must also be considered. Angioedema, which may occur in patients with anaphylaxis, is rarely present in patients with mastocytosis. Patients with such anaphylaxis, flushing, and angioedema do not have histologic evidence of significant mast cell hyperplasia and should have normal plasma histamine or tryptase levels between episodes (33).

TREATMENT

The control of mast cell mediator–induced signs and symptoms is a major objective in the treatment of patients in all categories of mastocytosis. H_1 receptor antagonists reduce pruritus, flushing, and tachycardia. Pruritus is also reduced by efforts to retain skin moisture. H_2 receptor antagonists and proton pump inhibitors are used to treat gastric hyperse cretion and to prevent gastritis and peptic ulcer disease. Hypotensive episodes are treated with epinephrine (61). If hypotensive episodes are frequent, there may be value in prophylaxis with H_1 and H_2 antihistamines. Patients should be trained to medicate themselves with epinephrine and to seek medical assistance promptly if they experience a hypotensive episode. Hypotension has been observed after insect stings (62), as well as after administration of contrast media. If subcutaneous epinephrine is inadequate in the treatment of the hypotensive episode, intensive therapy as for anaphylaxis must be instituted.

Aspirin and other nonsteroidal antiinflammatory drugs have therapeutic value in the treatment of recurrent hypotension and possibly severe flushing in that they block the synthesis of PGD_2 (59). However, aspirin and the other nonsteroidal antiinflammatory drugs may exacerbate gastritis in patients with mastocytosis. Some patients also have a severe hypotensive response to aspirin ingestion. Because of these difficulties, aspirin should be added to the antihistamine regimen only when symptoms are severe and are otherwise uncontrolled. Aspirin should be administered initially under controlled circumstances, with personnel and equipment available for resuscitation.

Methoxsalen with long-wave ultraviolet radiation relieves pruritus and whealing after 1 to 2 months of treatment (63). Improvement is associated with a transient decrease in dermal mast cells. Pruritus recurs 3 to 6 months after therapy is discontinued. Some patients report a diminution in the number or intensity of cutaneous lesions after repeated exposure to natural sunlight. Topical glucocorticoids may be applied under occlusion for 8 hours a day over 8 to 12 weeks for the treatment of urticaria pigmentosa or diffuse cutaneous mastocytosis. Lesions eventually recur after therapy is discontinued (64), although the treatment may lead to improvement in cutaneous lesions for up to 1 year.

The treatment of intestinal disease is determined by the degree of cramping, diarrhea, and malabsorption. Anticholinergic agents may give partial relief. Oral cromolyn sodium is of value, particularly in the management of abdominal symptoms (17,18,65). In patients with severe malabsorption, systemic glucocorticoids are effective. Ascites, which is more often seen in mastocytosis with an associated hematologic disorder and in aggressive mastocytosis, is also difficult to control. Portal hypertension in one patient was successfully managed with a portacaval shunt (66). Another patient with exudative ascites was treated successfully with systemic glucocorticoid therapy (67).

Patients with mastocytosis and an associated hematologic disorder are managed as dictated by the specific hematologic abnormality (68). Chemotherapy has not been shown to produce remission or to prolong survival in patients with mast cell leukemia, and it has no place in the treatment of indolent mastocytosis. The results of one study suggested that splenectomy may improve the length of survival in patients with forms of mastocytosis associated with poor prognosis (69).

Patients with aggressive mast cell disease have been variably reported to respond to interferon-a (70,71). Anecdotal reports have described a partial remission with interferon-g (72,73).

Other experimental approaches include the use of antihistamines with mast cell–stabilizing properties such as ketotifen and azelastine. Ketotifen relieves the pruritus and whealing associated with mastocytosis. However, in a double-blind, placebo-controlled trial of ketotifen versus hydroxyzine in the treatment of pediatric mastocytosis, ketotifen offered no advantage over hydroxyzine (74). Similarly, in a double-blind trial of azelastine versus chlorpheniramine in the treatment of adults with mastocytosis, azelastine, although effective in relieving skin symptoms, offered no advantage over chlorpheniramine (75).

The management of patients with mastocytosis who require surgical procedures must take into account the potential for mast cell activation and subsequent release and generation of potent mediators such as histamine, PGD_2, leukotriene C_4, and platelet-activating factor, which, in concert, may cause severe hypotension and cardiac arrhythmias. Therefore, anesthetic management should include avoidance of histamine-releasing drugs, immediate accessibility to pharmacologic agents including epinephrine to reverse an acute severe attack, and consideration of premedication with H_1 and H_2 antihistamines. With these precautions, patients with mastocytosis may be carried safely through operative procedures (76,77).

PROGNOSIS

Prognosis depends on the category of mastocytosis to which a patient is eventually assigned. Certain variables are strongly associated with poor survival (16,43): constitutional symptoms, anemia, thrombocytopenia, abnormal liver function test results, lobated mast cell nuclei, a low percentage of fat cells in the bone marrow biopsy specimen, and an associated hematologic disorder. Other variables that may be associated with poor prognosis are the presence of hepatomegaly or splenomegaly and the absence of urticaria pigmentosa and skin and bone symptoms.

Patients with indolent mastocytosis who have only skin involvement have the best prognosis. Isolated urticaria pigmentosa in children resolves in at least 50% of patients by adulthood (78). Urticaria pigmentosa in adults with indolent mastocytosis usually evolves into systemic disease. Occasional patients convert to mastocytosis with an associated hematologic disorder; these patients have a course that depends largely on the prognosis of the specific hematologic disorder. Survival time for patients with aggressive mastocytosis is years with intense symptom management. The mean survival time of patients with mast cell leukemia is usually less than 6 months.

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37 RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease that develops in genetically predisposed persons. It primarily affects the synovial membranes of multiple joints in a characteristic accumulative and symmetric fashion. If untreated, RA can lead to joint destruction and systemic injury, resulting in disability, joint deformity, decreased life expectancy, and high medical care–related costs.

The prevalence of RA for most of the population groups in the world is about 0.5% to 1% (1). This value is found regardless of urban or rural status and the presence or absence of distinct environmental conditions. A few population groups have an elevated prevalence, notably Native American tribes, such as the Pima (5.5%), Chippewa (6.8%), and Yakima (3.4%) (2). In contrast, lower rates are found in some Chinese (0.3%), Southeast Asians (0.2%), and certain African tribes (0.3%) (3). Genetic and not environmental factors appear to account for the differences in prevalence. The genetic differences include the frequency of human leukocyte antigen (HLA)-DRB1 alleles that encode the shared epitope and likely other genes within and outside the major histocompatibility complex (MHC) gene region.

STAGES IN THE DEVELOPMENT OF RHEUMATOID ARTHRITIS

The development of RA progresses through a series of stages from genetic predisposition to frank joint destruction. A general scheme for the development of the disease through six hypothetical stages is illustrated in Fig. 37.1. In each of these stages, from susceptibility genes to interaction with the developmental mechanisms of the joint, it appears that normal biologic processes and genes controlling them are subverted or exploited to result in the inappropriate process that results in RA. To date, no evidence has emerged of a distinctly pathogenic gene allele that is involved in the disease process. It is conceivable that the susceptibility genes are in fact normal allelic polymorphisms that, in the presence of the other susceptibility alleles, will culminate in the development of the disease.

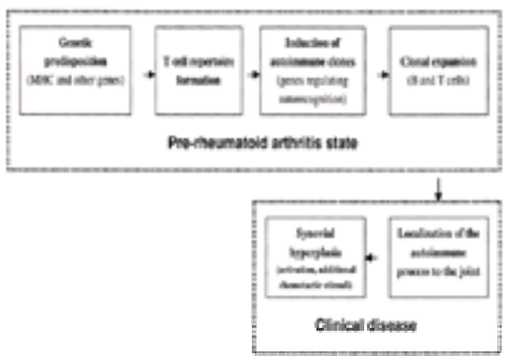

Figure 37.1. Six hypothetical stages in the development of rheumatoid arthritis.

Genetic Predisposition

There is a clear genetic basis for disease susceptibility. There appears to be a complex mode of inheritance under polygenic regulation. A major part of the genetic contribution to disease susceptibility is provided by certain MHC genes, most notably, the class II alleles encoding a common amino-acid sequence motive in the third diversity region of the HLA-DR molecule, initially designated the *shared epitope.* It is probable that these genes act to determine susceptibility by serving as the selective elements for the T-cell receptor (TCR) repertoire. We suggest that RA should be considered a disease in which alleles of a number of germline genes,

including those within and outside the MHC, establish an initial susceptibility state. For the MHC genes and likely some other genes that determine susceptibility, this will be through their interaction with the totality of self-peptides, resulting in the formation of a somatic T-cell repertoire that contains a proportion of autoreactive T-cell clones. The state of the genetically dependent, but not determined somatic immune genome, is the outcome of exposure to the range of self-peptides and processing pathways, which are presented by MHC molecules that are all under genetic control. The intricate series of stochastic events involved in the selection, expansion, and regulation of these repertoires makes the action of the susceptibility genes highly epistatic and extremely difficult to define (4).

Although MHC genes are central to the development of RA, several non-MHC genes (*loci*) also appear necessary. These latter genes have not yet been identified, however (5,6 and 7). Rodent models of autoimmune arthritis also support the concept of a complex mode of inheritance with both a dominant role for MHC alleles and clear involvement of multiple non-MHC genes (8,9,10,11,12,13,14 and 15).

Development of the Autoimmune Response

The autoimmune response underlying RA is envisioned as centered on the cognitive interaction of autoreactive T cells and B cells in response to the presentation of self-peptides by MHC class II molecules that contain the shared epitope. The nature of the peptide involved in this immune recognition event is unknown, however, and it will be argued in a later section that there may not be a single inciting peptide. Moreover, the initial triggering event that results in the abrogation of the state of tolerance in the autoreactive clones is unknown, if it indeed exists. The role of environmental factors in disease susceptibility, and in the specific evolution of the T-cell and B-cell repertoires toward those capable of sustaining development of the response that ultimately leads to RA, are also largely undefined.

One of the features of the autoimmune response that may antedate the development of overt RA is the production of certain autoantibodies. of these, rheumatoid factor (RF), an autoantibody directed to the Fc region of immunoglobulin G (IgG), is the best-studied marker of this autoimmune response. Newer work showing that the autoantibodies of RA may recognize other nonimmunoglobulin molecules will also be discussed. Although RFs can occur in normal immune responses, in RA it is commonly present in large quantities and in multiple isotypes, and it shows extensive somatic mutation as evidence of antigen-driven selection. All these features reflect the fact that the kind of RF found in RA is an intrinsic result of the cognitive T- and B-cell interactions that uniquely reflect the autoimmune response of RA. Increasing evidence is being accumulated to show that in some persons the development of RF precedes the appearance of clinically detectable RA by months to years. In these persons, the development of RF is the first marker for the development of the autoimmune response (16,17). There is also strong positive evidence that implicates RF as an intrinsic component of the immune response in RA derived from prospective epidemiologic studies. Aho and associates followed up on 7,217 persons and identified 21 instances of new RA. In 15 of these individuals, elevated titers of RF were identified in the stored preillness serum sample obtained up to 3 years before evidence of arthritis. In the six RF-negative persons, the preillness sample had been obtained more than 4 years before development of RA (16). Additionally, RFs were demonstrable among 39% of unaffected first-degree relatives in multicase RA families, and their occurrence was strongly associated with HLA-DR4 (18). Whereas the pathogenic role for RFs remains uncertain, the weight of evidence strongly favors the presence of RFs or related autoantibodies as a characteristic of the immune process leading to RA.

Localization of the Autoimmune Response to the Joint

Rheumatoid arthritis can have profound systemic features, such as lung disease, pericarditis, splenomegaly, vasculitis, and granulomatous nodule formation. Its dominant feature is the localization of the autoimmune response to the synovial membrane and initiation of joint disease. This localization could reflect an immune response driven by a synovial antigenic peptide. Alternatively, and perhaps more likely, the systemic nature of the disease and the lack of an identified suitable candidate joint-specific antigenic peptide suggest that the peptide, or peptides, driving the autoimmune response may be more generally distributed. In this view, localization of the autoimmune response to the joint would be largely attributable to the particular biology of the synovium. The joint is organized to accomplish enhanced immune surveillance of the joint cavity and removal of cartilaginous debris resulting from wear and tear. Recent studies on the gene expression phenotype of the fibroblast lineage synoviocytes identified several molecules that are likely involved in the histologic patterning of the joint and normal joint function (19,20). Information about the genes expressed in RA synoviocyte lines provided several insights into the biology of the joint and how features of this biology are subverted by the process of RA. The identification of these gene products provides evidence of how the synoviocyte can chemoattract T cells and B cells to the synovial membrane and interact with them. These interactions could underlie the development and intensification of the autoimmune response and foster its localization to the joint, as will be discussed later.

Initiation of Arthritis and Synovial Hyperplasia

In addition to the potential role of fibroblast lineage synoviocytes in the afferent limb of the autoimmune response underlying RA, these synoviocytes respond to the presence of this immune response in the joint by undergoing marked hyperplasia. This is likely a paracrine-mediated response to products such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), which are elaborated largely by the monocyte/macrophage component of the autoimmune response. This component is highly dependent on transcription factors, such as NF-kB. Information is being obtained about the nature of the synoviocyte cell membrane receptors that provide the molecular basis of cell–cell and cell–matrix interactions involved in both the patterning of the normal joint and the development of hyperplasia.

Development of Sustained Arthritis

Conceivably, the autoimmune process required to initiate the arthritic process also requires local synovial changes that include macrophage activation, synovial fibroblast hyperplasia, and increased expression of genes that will contribute to the persistence of the inflammatory process in the joint. Extensive neovascularization also is required for arthritis persistence. The dominant role of lymphocytes in early stages of the illness appears somewhat diminished after initiation of the disease. There is evidence to suggest that, at some point after onset of the disease, monocytes and macrophages and synovial fibroblasts take a more central role in the persistence of the disease. Perhaps sustained disease develops in patients with abnormalities in downregulatory pathways that otherwise would have controlled the autoimmune inflammatory process.

Joint Destruction

The cartilage and contiguous bone of the joint become altered and ultimately are destroyed by the hyperplastic synovial lining cells. They elaborate a variety of cytokines capable of reprogramming the patterns of gene expression of bone and cartilage through paracrine mechanisms and elaborate degradative enzymes capable of altering the matrix of the connective tissue. Moreover, cell—cell interactions may be involved. Among the insights into the biology of the joint provided by the gene expression studies is the fact that intimal synoviocytes are closely related to mesenchymal stem cells. This finding raises the question of whether the hyperplastic synoviocytes are embryologic progenitors of the cartilage and bone cells that they seemingly seek to destroy in later stages of the disease. The biologic significance of the destructive interaction between synoviocytes, bone, and cartilage assumes new importance when considered in this light. It also raises the possibility of the development of new therapeutic approaches aimed at inducing the hyperplastic synoviocytes to differentiate into the cells they seek to destroy.

RESTRUCTURING THERAPEUTIC STRATEGIES

In recent years, growing knowledge of the immunopathology of RA as well as its genetic basis has contributed to the development of new therapeutic agents, some of which have been demonstrated to be quite effective in the treatment of disease. An accumulating body of evidence supports the concept that early institution of therapy with disease-modifying antirheumatic drugs (DMARDs), like methotrexate or agents that block effector molecules like TNF-a, is beneficial. These agents slow disease progression, decrease joint damage, improve quality of life (21,22 and 23), and enhance survival (24). We appear to be at the beginning of a most promising phase of drug development for this disease.

This chapter is an interpretative presentation of certain aspects of the current understanding of the pathophysiology and genetic basis of RA as well as its clinical characteristics and treatment. Although the general features of the etiology and pathogenesis of RA are understood along the lines of the overall paradigm of stages in the development of the disease, a number of vexing questions about the nature of the disease remain unanswered and are emphasized here. A major theme of the chapter is subversion of normal physiologic elements and mechanisms into disease processes.

GENETIC PREDISPOSITION

Formal Genetics

From the perspective of the rheumatologist caring for a patient with RA, the illness usually appears to be a sporadic event that often makes its appearance only well into adulthood. Yet, as with many diseases in which autoimmune reactions are found, there is a clear genetic basis associated with particular MHC alleles, non-MHC alleles, sex status, as well as the participation of still undefined stochastic processes. Family and twin studies on the occurrence of RA permit an estimation of the contributions of heredity and environment and characterization of some general features of the genetic predisposition. There is definite familial aggregation, with the

risk of finding an affected relative of a patient with RA estimated at 2.3 (25).

The I_{MZ} (prevalence of disease among monozygotic twins divided by the prevalence of the disease in the general population) can be viewed as an estimate of the maximum genetic risk for the disease. In RA, the I_{MZ} has been estimated to be between 12 and 62. Comparison of the elevated concordance rates for RA in monozygotic versus to dizygotic twins further supports the genetic basis of the disorder; however, the concordance rate in monozygotic twins is not absolute, being estimated between 15% and 30% in varying populations, at different times, and using different ascertainment methods (26). The frequency with which RA is found in the second twin when it is identified in the first is 15.4% for monozygotic and 3.6% for dizygotic twins according to findings in the study of Silman et al. (26). The concordance rate was slightly higher, that is, 16.9%, when twin pairs who were RF positive were studied. When these data are reported in terms of cases per 1,000 years of observation, the prevalence rates are 12 for monozygotic twins, 2.2 for dizygotic twins, and 0.5 for the general population. One factor influencing concordance is that twins develop RA at different times. Silman et al. showed that the concordance rate increases with time from the day when the first twin develops RA, when it is essentially zero, to a rate of approximately 40% when 30 years of follow-up are available (27). This finding suggests the importance of stochastic factors in the causation of RA, a point that is discussed subsequently. This does not necessarily imply that environmental factors play a predominant role because, as is discussed in more detail subsequently, MHC genes act in a developmentally complex manner to select the TCR repertoire with which they will interact. This stochastic somatically generated T-cell repertoire is the key to the immune system's ability to function in health as well as in autoimmune disease.

MHC Class II Genes

IDENTIFICATION of SUSCEPTIBILITY WITH PARTICULAR MHC ALLELES THAT ENCODE THE SHARED EPITOPE

The recognition of class II MHC molecules over two decades ago and the appreciation of their involvement in the regulation of the immune response were the basis for a large number of genetic epidemiologic studies on the relationship of the susceptibility of RA to particular MHC specificities. The history of the development of this knowledge provides an important insight into the way HLA operates to determine susceptibility. Initially, these relationships were defined by using serologic techniques (28). RA was strongly associated with the HLA-DR4 specificity in initial studies in Northern American and Northern European Caucasoid populations and certain other ethnic groups; however, this association was not replicated in Southern European and Eastern Mediterranean Caucasoids (29,30,31,32,33 and 34). The highest relative risks for DR4 and RA were found in Mexicans and the Chippewa Nation of American Indians, being 14.6 and 13.4, respectively (2,35), although some of their potential genetic antecedents had much lower relative risks, such as Southern Spaniards 1.8, Japanese 2.9, and Siberians 4.0 (36,37 and 38). As a group, the information emerging from these generally excellent studies was dismayingly different from population to population. The application of a monoclonal antibody (mAb) 109d6 to patients with RA identified an epitope shared by a proportion of RA patients (39,40). Although this antibody did not recognize HLA-DR4, its use came at an important moment during the period of divergent conclusions regarding DR4 because it provided renewed motivation to pursue the notion that the MHC alleles involved in RA susceptibility shared a structural feature. Ultimately, it provided additional insight into the nature of the amino acid motif responsible for the shared epitope.

With the anticipation that the structural differences responsible for the different subtypes of DR4 delineated by MLC typing (41,42) would illuminate the problem of RA susceptibility, an extensive effort was made to sequence the relevant DQ and DR subregion genes in the DR4 haplotype (43). RA susceptibility was associated only with certain HLA-DR4 alleles, including HLA-DRB1*0401, *0404, and *0405, but not *0402 and *0403 (44). The predominant alleles encoding the HLA-DR4 specificity vary in different populations. Thus, if a particular ethnic group has a high frequency of HLA-DRB1*0401, as in Central and Northern Europeans, *0404, as in Scandinavians, or *0405, as in Asians, only in those populations will RA be associated with HLA-DR4. Conversely, if the population has a high frequency of HLA-DRB1*0402 or *0403, as in Southern European or Eastern Mediterranean peoples, then RA will not be associated with the HLA-DR4 specificity. This was followed by an analysis of the structure of DR1 alleles in individuals with RA by Merryman et al. (45).

Detailed analysis of DR4 haplotypes was begun at the level of molecularly defined epitopes recognizable by monoclonal antibodies (39,46,47). In parallel, these haplotypes were dissected in terms of their gene structure (43,45,47). This effort provided the information leading to the recognition that all the alleles associated with RA susceptibility encoded a sequence of leu-gln- arg/lys- ala at positions 67, 70, 71, and 74 of the DRB1 chain. This relationship initially was reported by Gregersen et al. (44) as the shared epitope hypothesis. It was one of the first instances of an attempt to relate the molecular basis of disease susceptibility to a particular defined conformation or structure in an MHC molecule. The designation of this sequence motif as an “epitope” reflected the earlier data on serologic associations and attempted to communicate that the motif was a hypothetical structure that determined disease susceptibility through its function in the MHC molecule. Notably, any allele that encoded a negatively charged amino acid in this motif, including HLA-DRB1*0402 and *0403, was not associated with susceptibility to RA. Supporting the identification of this region with susceptibility, other alleles, such as HLA DRB1*0101, that were associated with RA susceptibility also exhibited this same shared motif of amino acids (28).

The subsequent delineation of the crystallographic structure of the MHC class II molecule revealed that the shared epitope motif of amino acids occupies a prominent position at the apex of the upwardly arching rim of the HLA-DR molecule formed by the a-helix (Fig. 37.2). Thus, the shared epitope forms a major portion of the pocket that binds the fourth amino acid side chain (peptide-4, or P4) of the peptide bound by the MHC molecule. Moreover, the prominent position of the shared epitope motif on the face of the molecule facing the abTCR also implied that this structure would interact with the abTCR. The implication of this structural feature is that the shared epitope operates during the embryologic development of the T-cell repertoire as an important selective structure. Here it both regulates binding of the particular self-peptides presented by the MHC molecule and directly interacts with abTCR contact residues. It appears that a abTCR clonal repertoire selected by MHC molecules with neutral or positive charges in the shared epitope region is capable of developing the autoreactive state that results in RA. Conversely, the T-cell repertoire selected by MHC molecules with negative charges in this region is not capable of mediating RA.

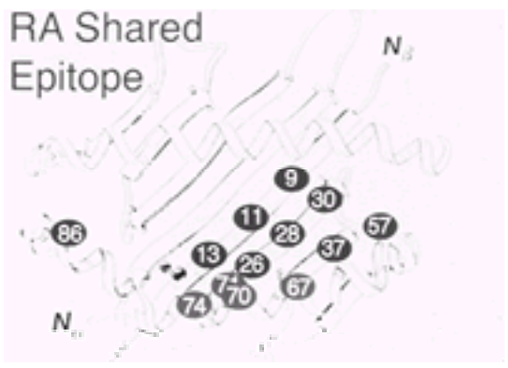

Figure 37.2. Human leukocyte antigen DR locus (HLA-DR) and the “shared epitope” location in the a helix, amino-acid positions 67 through 74.

Subsequently, particularly strong support for the identification of RA susceptibility with the shared epitope was provided by epidemiologic studies in various Native Americans delineating an association with DRB1*1402, which encodes the HLA-DR6 (Dw16) serologic specificity but also encodes the shared epitope sequence motif (48). The genetic susceptibility of RA in Native Americans is of particular interest because the predominant HLA-DR4 allele is HLA-DRB1*0403. This allele is decreased in frequency in RA, whereas the DRB1*1402 is present in 91% of Native Americans with RA (49). Further work proved that the actual epitope recognized by mAb 109d6 was a slightly different structure from the shared epitope described already, which led to both the identification of DR10 as an allele related to RA susceptibility and expansion of the concept of the shared epitope (47,50,51). Figure 37.3 shows that the critical part of the epitope recognized by mAb 109d6 is determined by the presence of arginine at two polymorphic residues at positions 70 (arg) and 71 (arg), probably in conjunction with the side chains of the surrounding nonpolymorphic residues. This consensus structure differs from the center of the postulated sequence of the shared DR4-DR1 epitope leu-gln-arg/lys-ala by the substitution of an arg (R) for gln (Q) at position 70. In the case of DR10, which is clearly associated with RA, the analogous sequence is leu-arg- arg- ala. It seems likely that both the DR4-DR1 leu-gln-arg/lys-ala sequence and the leu-arg-args-ala sequence are involved in determining susceptibility. The consensus sequence between the DR4-DR1 sequences motif and the DR10 sequence motif is leu-arg/gln-arg/lys- ala, (Fig. 37.3), emphasize the importance of positively charged amino acids in this region of the MHC molecule for disease susceptibility. The crystallographic image of HLA-DRB1*0401 presenting two different type II collagen peptides is depicted in Fig. 37.4.

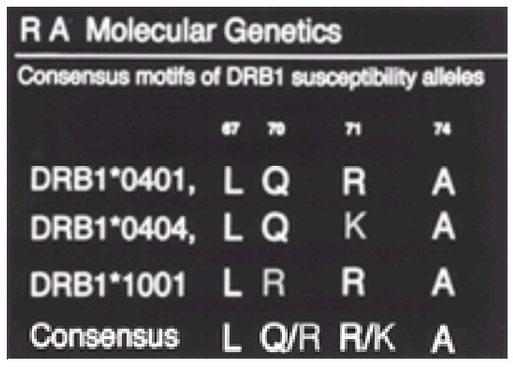

Figure 37.3. Human leukocyte antigen (HLA)-DRB1) “shared epitope” consensus motifs.

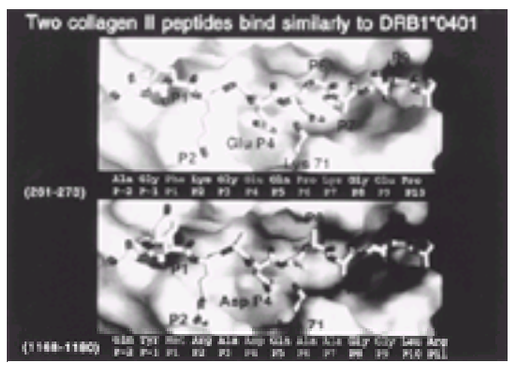

Figure 37.4. Crystallographic image of human leukocyte antigen (HLA)-DRB1*0401 presenting two different type II collagen peptides. (From Dessen et al., *Immunity* 1997; 7(4):473–481, with permission.)

More recently, it was demonstrated that the overexpression of either one of two shared-epitope alleles (HLA-DRB1*0101 and *0401) in transgenic mice makes the animals highly susceptible to collagen-induced arthritis (CIA) (52,53 and 54). These findings provided additional evidence to support the importance of these alleles in disease regulation.

CONTRIBUTION OF MHC GENES TO SUSCEPTIBILITY

The I_s approach can be used to estimate the relative contribution of HLA alleles to susceptibility. Using a published series of HLA-typed RA-affected sibling pairs, a figure of 1.8 is derived (55). This value can be expressed as a proportion of the total I_s for the condition. An approximate figure of 37% is derived (56). This is, of course, the lowest estimate of the contribution of HLA alleles. This estimate does not take into account unshared pairs of alleles that are, however, equivalent in a functional immunologic sense, that is, identical by function, such as the presence of the shared epitope. The calculation nevertheless suggests that although HLA alleles, as we currently understand them, are a large single genetic factor for RA susceptibility, a considerable portion of the genetic component in RA may be non-HLA derived (56). Alternatively, a portion of the susceptibility could depend on currently unrecognized functional similarities of different HLA molecules.

The presence of the shared epitope on the class II alleles associated with susceptibility to RA is found in most individuals with RA. The frequency of shared epitope positivity in RA varies from population to population because the frequency of those alleles varies in different ethnic populations, from about 25% to 90% (28,49,57,58). Because genetic and disease prevalence data are not uniformly available in enough ethnically defined populations, RA develops in roughly from 1 in 20 to 1 in 100 of those inheriting alleles that encode the shared epitope. Reciprocally, RA develops in from 1 in 500 to 1 in 1,000 persons who lack a shared epitope allele.

There is evidence in certain populations that the severity of RA may be influenced by the allele involved in determining susceptibility. In a prospective 6-year study of the course of RA in HLA-typed individuals, the frequency of DR4 was 59.2% in those who had progressive disease and 34.8% in those with mild disease (59). DR4 positivity was associated with nearly all measures of severity, including joint scores, functional assessment, radiologic classification, and use of second-line drugs. Similar results were found in Japanese patients (60). In another study, HLA-DR1 was increased only in those with mild RA (61,62). Further support for an association between the presence of the shared-epitope alleles and increased disease severity in both RF-positive and -negative patients comes from studies done predominantly in tertiary care centers in both established (59,63,64) and recent-onset disease (65,66,67 and 68). Other reports are not consistent with these findings (69), particularly in community-based studies (70,71), emphasizing that this question requires additional study.

POSSIBLE MECHANISMS FOR THE ACTION OF THE SHARED EPITOPE

Multiple Equivalent Peptides

The identification of the shared epitope motif emphasizes the general functional equivalence of several otherwise structurally disparate alleles, at least from the perspective of encoding the molecular basis of susceptibility to RA. So what is the implication of this observation in terms of the trimolecular complex of MHC, peptide, and TCR involved in a presumptive immune recognition event? The implications for this association are not easy to translate into a conventional peptide-binding model. The fact that the DRB1 alleles encoding the shared epitope motif differ in all peptide-binding pockets other than that binding the P4 side chain argues against the recognition of a single peptide in RA. It is more likely that multiple equivalent peptides that share the P4 side chain, but perhaps differ elsewhere, are recognized in RA patients with different DRB1 alleles and are the putative driving peptides.

Stochastic Somatic Genetic Mechanism

The location of the shared epitope on the face of the molecule interacting with the TCR also directs attention to the potential role of the shared epitope in selecting the CD4 abTCR repertoire, as mentioned. This suggests that a subset of the CD4 T-cell ab TCR repertoire is critically dependent on the presence of the shared epitope structure. This subset of the abTCR repertoire likely mediates the critical immune recognition events in RA. Because the somatic generation of the repertoire is stochastic, this introduces a stochastic somatic genetic mechanism between the germline genes and the expression of the RA susceptibility phenotype.

This stochastic somatic genetic mechanism likely accounts for the much less than complete concordance exhibited by monozygotic twins who are identical at the germ line but differ in terms of the somatic genetics of TCR repertoire formation and its peripheral maintenance.

In the calculation of the proportional effect of the environment on susceptibility, the degree of nonconcordance between monozygotic twins is used to imply a significant role for environmental factors. In the case of RA and other autoimmune diseases, however, it is likely that much of the non–germ line “environmental” contribution actually comes from the stochastic somatic genetics of TCR repertoire formation. Exposure to a variety of environmental bacterial or viral antigens could cause further expansion of the TCR clone through cross-reactive mechanisms. It should be emphasized that the MHC susceptibility genes are not pathologic variants, nor does the process of TCR repertoire selection appear to be intrinsically pathologic, as shown by the healthy non–disease-concordant twins.

ISSUE OF SHARED EPITOPE “HOMOZYGOSITY” AND RA SEVERITY

Given the relatively high frequency of alleles in the general population encoding the shared epitope, a considerable proportion of persons with RA would be expected to be “homozygous” for two shared epitope alleles based on chance occurrence. There is evidence that persons who have inherited two alleles that encode shared epitopes have a higher risk of developing RA, approximately twice that of those with a single shared epitope allele (72,73). These data suggest that the contribution of HLA may fit better with a recessive model or, less likely, an additive dominant model (74). Bearing on the importance of sharing two HLA alleles is a study of concordance among members of a sibship for RA, which is 15.5% if the two siblings share two HLA haplotypes, 7.1% for one, and 5.2% for no sharing (75). The HLA status of a twinship has a major role in RA concordance. In DR4-negative monozygotic twins, the concordance rate was 6%. For twins who are DRB1*0401/0404

(Dw4/Dw14), the concordance rate was elevated to nearly 50%. A clear dose effect was evident when twins were stratified according to whether they had zero, one, or two haplotypes encoding the shared epitope, the concordance being, respectively, 5%, 13%, and 28% (26). The heightened susceptibility is entirely consistent with the preceding discussion of the role of susceptibility alleles on repertoire and self-peptide antigen presentation.

Whether a double dose of susceptibility alleles or a particular susceptibility allele contributes a higher risk for more severe disease remains unanswered. The biochemical studies of Nepom et al. (30) on early onset RA provided the first suggestion that a double dose of susceptibility alleles or a particular susceptibility allele might contribute a higher risk for severe disease. They described persons who had DR4 encoded by both MHC haplotypes usually involving Dw14 (*0404 or *0408) or Dw4 (*0401); this notion was expanded by several groups (76,77). Weyand et al. (64) studied 102 patients with seropositive, erosive RA by genotyping for both HLA-DRB1 alleles. They were categorized according to the expression of one or two disease-linked HLA-DRB1 alleles; 46% had a double dose of the shared epitope. Twenty-eight patients expressed HLA-DRB1*04 variants on both alleles, and 19 combined an HLA DRB*04 variant with HLA-DRB1*0101 or DRB1*1402. Nodular disease was present in 100% of patients typed as HLA-DRB1*04/04 and in 59% of patients typed as HLA-DRB1*04 and who had inherited only a single dose of the disease-linked sequence. Other extraarticular manifestations were present in 61% and 11% of these two patient groups, respectively. Joint surgery was required in 61% and 25% of the two groups, respectively. Although in this population the Dw4/Dw14 heterozygosity was not as prominent as in the Nepom study, the same fundamental conclusion was reached, that is, that two doses of the susceptibility alleles were associated with increased disease severity. Subsequently, Weyand et al. also demonstrated that the presence of shared epitope alleles correlated with increased disease severity in RF-negative patients as well (78).

In other studies, however, the effect of two doses of susceptibility alleles is often relatively modest or not detectable. For example, in a prospective, population-based study of 532 British patients with inflammatory polyarthritis, the most obvious effect of the presence of alleles encoding the shared epitope was on the development of erosions for those who carried at least one DRB1 shared epitope allele. Only a slightly greater effect was seen in those “homozygous” for two shared epitope-bearing alleles (79). Furthermore, studies based in nontertiary care centers (70,71) and studies of African-American (80) and Hispanic-American (81) patients with RA found no association between the presence of shared epitope alleles and increased disease severity. There is also the issue of recent-onset disease versus long-duration disease where there is also conflicting data, with several studies describing increased disease severity in shared epitope-positive patients (67,68), whereas others find no such increased risk (69). In view of the importance of the question and the disparate results of studies in different ethnic groups, this question of gene dose effect and disease severity remains an area of interest.

NON-HLA GENES WITHIN THE MHC

In addition to the HLA-DRB1 contribution, other non-HLA MHC genes may be involved in the disease susceptibility. Mulcahy et al. reported that the TNFc1 alleles encoded in the MHC in the class III region contribute to RA susceptibility independently of the shared epitope (82). Although the mechanism of the contribution of this polymorphism is not delineated, in view of the importance of TNF in the pathogenesis of RA, it is a potentially important observation. One additional study of multiplex RA families provides support for the involvement of this allele in susceptibility (7).

Because of its involvement in peptide processing in antigen-presenting cells, HLA-DM, an interesting RA candidate gene, has been studied. There have been three studies from France. Two found an increased frequency of HLA-DMA*0103 in RA (83,84), and the third one identified an increased frequency of HLA-DMB*0101-0101 homozygote genotype among RA patients: 70.8% compared with 50% of the controls (85). Two other studies, one from Canada (86) and one from Thailand (87), however, found no associations between RA and HLA-DMA or DMB alleles. This association remains controversial.

The fact that HLA-DQ8 (DQA1*0301 and DQB1*0302) transgenic mice develop severe CIA (88) led Zanelli et al. to propose that this gene would be critical in RA (89). This group found that peptides derived from RA-associated HLA-DRB1 alleles fail to induce a DQ8-restricted T-cell response and hypothesized that HLA-DQ8 presentation of shared-epitope-derived peptides has a critical role in shaping the pathogenic T-cell repertoire. Although this hypothesis is interesting, it has not been supported by recent studies (68,90,91).

Quadri et al. found an association between the HSP70-1B promoter allele and RA (92). These authors did not make adjustments for the presence of the shared epitope alleles, however, and their findings could have been accounted for by linkage disequilibrium between the HSP70-1B and the shared epitope alleles.

Non-MHC Genes

STRATEGIES FOR IDENTIFYING NON-MHC SUSCEPTIBILITY GENES THAT REGULATE DIFFERENT STAGES OF DISEASE DEVELOPMENT

The identification of HLA-DRB alleles associated with susceptibility followed from the knowledge that these genes regulate aspects of immune responsiveness. Additional genes regulating cellular functions in each one of the hypothetical stages of RA development also could be candidate susceptibility/severity genes and potential targets for therapy. Identification of these additional genes among the estimated 30,000 genes constituting the human genome is challenging, however, several approaches facilitate this endeavor.

Linkage analysis is an approach to gene identification that can be used to map regulatory genes without prior knowledge of their location in the genome. This powerful technique identifies the cosegregation between a phenotype and genotype. It is critical to have a well-defined phenotype before the mapping effort. RA is a heterogeneous disease with manifestations that may vary from one patient to the other, creating potential confusing factors. Because large numbers of multicase families or affected sibling pairs are required for linkage studies, the fact that most RA patients do not have affected first-degree relatives necessitates that the studies be performed at a multiinstitutional center. Furthermore, because the usual age of onset of RA is between age 40 and 50, most RA patients do not have both parents alive for transmission disequilibrium testing, another type of linkage/association testing. Despite the difficulties involved in this kind of analysis, the studies published so far have been encouraging (see following discussion).

A second approach that may facilitate the genetic dissection of RA is an extension of the classic clinical investigation approach of delineating the pathophysiology of the disease. This involves searching for the genetic control of particular immunologic or biologic manifestations of RA that are termed *subphenotypes*. For instance, whether certain genes are responsible for particular elements in the distinctive fibroblast lineage intimal synoviocyte phenotype or in RF production. For example, a certain gene may be important to the capacity of a fibroblast lineage intimal synoviocyte to degrade cartilage and its subphenotype and that property contributes to RA susceptibility. Because other different genes also may contribute to RA susceptibility, the contribution of this particular gene to the complete phenotype of RA is more difficult to identify. By deconstructing the complex phenotype of RA into a series of distinct subphenotypes, the likelihood of identifying the genetic basis of RA should be enhanced.

A third approach is to use animal models of autoimmune arthritis in inbred rodent strains to identify a candidate gene or locus that regulates susceptibility or severity. The massive efforts in analyzing the genomes of a number of species provided detailed information regarding homologous (syntenic) chromosomal regions among each of the various species. This facilitates moving from studying a region in a rodent genome to the homologous region in the human genome. The utility of the animal models derives from the large numbers of offspring that can be generated in a short time from controlled crosses among inbred strains that differ in the susceptibility to disease (8,9,10,11,12,13,14 and 15). This minimizes genetic heterogeneity and controls environmental factors that may interfere with the genetic analyses of RA in humans. Additionally, the use of animal models has been considered critical to prove a phenotypic effect caused by a particular genomic interval in polygenic diseases. The possibility of genetic manipulations through the breeding of congenic animals carrying a single or multiple susceptibility loci in different genetic backgrounds, and the adjustments for the presence of different MHC genes create a unique experiment, decreasing the number of confounding factors, which could not be achieved in human studies. This strategy has been used in the study of several forms of autoimmune diseases, including autoimmune arthritis, and among other advantages permits a clearer study of gene-to-gene interactions (*epistasis*). Interestingly, most of the susceptibility or disease severity regulatory loci mapped by different groups in autoimmune arthritis in mice or rats are located to regions involved in the regulation of other forms of autoimmune diseases. This suggests that at least some of these loci may contain genes common to more than one autoimmune disease. Rodent models of autoimmune arthritis also provide a better opportunity to study different time points during disease development, from very early prearthritic stages to chronic and erosive disease. For example, two inbred rat strains that share the same MHC haplotype, DA and ACI, have a similar autoimmune response to autologous rat collagen immunization, as measured by the IgG anti-rat collagen antibodies, but only one (DA rat) develops CIA. Analysis of the non-MHC genes responsible for the localization of the autoimmune response to the joints identified one regulatory locus that reached logarithm of odds (LOD) scores significant for linkage and three others that reached suggestive levels (8). An analysis of the genes located within those genomic intervals may provide candidate genes for testing in RA. Similar to the approach in humans, the identification of subphenotypes in experimentally induced disease also should facilitate this approach to gene identification in animal models.

A fourth approach for additional gene discovery efforts has been the use of differential gene subtraction or expression strategies to identify candidate genes (20,93,94,95 and 96). The rationale motivating this approach is that the identification of a gene highly differentially expressed in a cell type involved in RA implies the possibility that the overexpression has either a germline or somatic genetic basis and may identify certain candidate susceptibility genes. This strategy would be of particular importance in dissecting the regulation of different stages of disease course, particularly the later stages, where T cells may be less conspicuously involved, with a more central role for macrophage and fibroblast lineage intimal synoviocytes (97,98 and 99). A similar strategy could be used to determine which genes, downregulation or upregulation, are critical for clinical improvement and response to drug therapy.

Each of these four approaches results in the identification of specific candidate genes. The relevance of these genes to RA susceptibility may be tested simply by the

statistical methodology of association tests, similar to studies that demonstrated the role of HLA-DRB1 shared epitope alleles in RA susceptibility.

NON-HLA SUSCEPTIBILITY AND SEVERITY GENES

In the case of non-MHC genomic regions regulating disease susceptibility, linkage studies involving families with multiple affected first-degree relatives identified a number of candidate regions. This is a particularly promising moment in RA genetics because of the immense effort being put into the human genome project and identification of susceptibility genes in large cohorts of polygenic diseases, including RA. Most of the loci identified by one group could not be replicated by the other, however, which emphasizes the need for extensive cross validation of the results in independent study populations. Indeed, to date, only limited cross validation of the results from study to study has been done.

The first study was done with a relatively small number of families and identified linkage to chromosome 7 region 7q35, where the TCRb chain gene is located (100). Linkage to this region has not been confirmed in subsequent studies. Whereas other genes, such as IL-2, IL-4, IL-10, corticotropin releasing hormone (CRH), and natural resistance-associated macrophage protein-1 (NRAMP-1) have been studied in association or linkage analyses, the results were either negative or achieved borderline statistical significance (101,102 and 103). A recent study of cytokine genes found no association of the IL-1-receptor antagonist (IL-1RA) and IL-10 genes but did find an increased frequency of one IL-4 gene (5q31-33) polymorphism (RP1) in RA patients compared with controls, but the result did not reach significant *p* values (104). The same study found that the IL-1b exon 5 allele E2 correlated with increased risk for erosive disease in the shared-epitope–positive patients [odds ratio (OR) of 8.2] (104).

The European Consortium on RA Families studied 114 affected sibling pairs from 97 families and reported a genome-wide scan that identified significant linkage with only the HLA region and nominal linkage for 19 markers in 14 other regions (6). Four of the loci implicated in insulin-dependent diabetes mellitus (IDDM) overlap with these regions. Two of these loci (18q22-23 and 3q13) were further analyzed in a second set of 194 RA sib pairs from 164 nuclear families. The *p* values for the 3q13 locus improved and reached levels suggestive of linkage, although not definitive, and appeared to interact with the HLA locus. Candidate genes for the chromosome 3 locus include CD80 and CD86, molecules involved in the costimulation of T cells. Also, this study estimated the HLA and the chromosome 3 locus contribution to RA to be 33% and 16%, respectively.

In a study of 41 Japanese families with at least one affected sib pair, Shiozawa et al. identified significant evidence for linkage with loci located in chromosomes 1p36, 8q21, and Xq24-q27 (5). Chromosomes 1 and X loci overlap with two of the loci identified by the European Consortium.

A third genetic analysis of multiplex RA families by Bali et al. (7) focused on certain candidate gene regions and the MHC. These investigators found evidence of independent effects of the shared epitope and TNFc in disease susceptibility, supporting the previous findings of Mulcahy et al. (82). These researchers also found *p* values suggestive of linkage in different regions, with the most significant ones being 2p13-p11.1 and 15q15. Using a relative pair analysis, but not the sib pair analysis, Bali et al. also found *p* values suggestive of linkage at 1p12-q21 and 10q23-q24. of these loci, only 2p13 overlaps a locus described by the European Consortium. Currently, the North American Consortium for RA is collecting 1,000 affected sib pairs, which is expected to identify loci that contribute with a small relative risk and to validate some of the loci previously described. This consortium will also address a significant portion of the issue of sample power to detect linkage.

SEX AND SEX CHROMOSOMES

Several intriguing relations have been found between gender, gonadal hormones, and RA. These are discussed subsequently in the section entitled “Other Features of RA, Pregnancy-associated Remission.” RA, like many other autoimmune diseases, is more common in women than in men; the generally accepted ratio is 3:1. It is extremely rare before puberty, and its incidence peaks in women around the perimenopause years. Some women with RA experience a striking remission during pregnancy, and epidemiologic studies showed that pregnancy has a protective effect on susceptibility. This finding suggests that sex hormones, or factors associated with the inheritance of two X chromosomes, could favor the expression of RA in female subjects. Although both the European consortium (6) and the Japanese study (5) identified a susceptibility locus in chromosome X, Xq24-q27, there has been limited insight into the genes responsible for this effect in humans. Studies of experimental arthritis in animals support the importance of gender and hormonal influences. Recent work in an F2 intercross between DA (collagen-induced arthritis [CIA] susceptible) and ACI (CIA-resistant) rats demonstrated that the presence of the Y chromosome was significantly protective against disease development and reduced disease severity (8). Joe et al. studied CIA in congenic rats (105), and their results suggested that certain non-MHC regulatory loci regulate disease severity in a sex-dependent manner. It remains unknown whether the susceptibility gene's effect is dependent on gonadal hormones or on epistatic interactions with genes located either in the X or the Y chromosomes.

NON-MHC LOCI REGULATING RODENT MODELS OF AUTOIMMUNE ARTHRITIS

Several models of autoimmune arthritis have been used to gain insight into the pathogenesis of RA. These consist of CIA, complete Freund's adjuvant-induced arthritis (AIA), pristane-induced arthritis (PIA), incomplete Freund's adjuvant oil-induced arthritis (OIA), and proteoglycan-induced arthritis (PROIA). Using either intercrossing or backcrossing strategies between susceptible and resistant inbred animals, different investigators identified a number of regulatory genes, a significant percentage of which map to regions homologous to those regulating RA or other autoimmune diseases in humans or rodents (106). These findings further exemplify the relevance of the rodent studies, which are likely to facilitate the gene discovery effort in RA.

INITIATION OF THE AUTOIMMUNE RESPONSE

The fact that RA usually begins decades after birth raises the question of what precipitates the development of the disease. This question is common to studies on all autoimmune diseases and concerns the lack of information about how “tolerance to self” is broken to result in autoimmunity and subsequently in an autoimmune disease. That is, we do not understand the ongoing processes involved in the regulation of autorecognition and how these operate in the sculpting of lymphocyte repertoires. The regulation of the self-tolerance and the self-immune recognition is one of the likely key steps toward explaining why identical twins, who share the same germ-line genome, are not fully concordant for the expression of the disease [see Ollier and Winchester for a more extensive discussion of the initiation of autoimmunity (4)].

In RA, the coefficient of familial clustering, l_s, estimated from the ratio of the disease prevalence among siblings and to its prevalence in the general population (107), ranges from 5 to 10 (56). By comparison, conditions such as IDDM and primary biliary cirrhosis (108) have l_s values of 15 and 100, respectively. The lower l_s in RA likely reflects an enhanced dependency on events involved in the generation of the somatic immune repertoire capable of self-reactivity or perhaps lower disease gene penetrance as another somatic regulatory factor.

Given the presence of a MHC molecule with the shared epitope, in order for disease to occur, a potentially autoreactive clone first must arise in the positive selection phase of repertoire formation. This T-cell clone would likely be selected on the peptide that ultimately could drive the rheumatoid process. Then the second requirement is that the clone must evade negative selection. These processes may be largely stochastic or dependent in part on the action of additional undiscovered genes. The potentially autoreactive clone is now in the quiescent state of clonal ignorance, which is one form of “tolerance,” and it remains unexpanded and inactivated. Activation and expansion of this clone could be the consequence of any of a large number of events. These might include exposure to a superantigen, stimulation by a mimetic peptide from an infectious agent, and pure bystander cytokine-mediated activation of the autoreactive clone by physiologic immune responses.

Regulatory events may inhibit this clonal expansion, presenting another possible opportunity for the effect of novel genes on the disease process and a further contribution to the low level of penetrance. Antigenic cross-reactivity between the shared epitope and prokaryotic protein sequences (109) have been proposed to act as a “drive” at these latter levels of repertoire expansion. The leu-gln-arg/lys-ala shared epitope sequence, along with the intervening amino acids, is present in both gp110, an Epstein–Barr virus EBV glycoprotein (110), and dnaJ, an *Escherichia coli* heat shock protein (111). This could lead to the activation and expansion of low affinity and potentially self-reactive T cells, capable of recognizing heat shock proteins expressed in the inflamed joint (109) that might drive and precipitate a sustained arthritis. Although these molecular mimicry hypotheses are interesting, their role in disease pathogenesis remains to be determined.

Specific Infectious Agents

The appearance of RA in a previously healthy person led many clinical investigators to search for an infectious agent, with the hypothesis that the synovial immune response was evidence of chronic infection of the joints. Attempts to isolate organisms such as mycoplasmas and numerous other bacteria, including diphtheroids, streptococci, and L-phase bacteria from joint fluids, however, have been unsuccessful or attributed to laboratory contamination. Similar negative conclusions have been reached about the role of parvoviruses, oncornaviruses, coronavirus, and myxoviruses in RA (112,113,114,115,116 and 117).

Other viruses also received special attention, particularly EBV. Sera of patients with RA often have antibodies to a nuclear antigen expressed in EBV-infected B lymphocytes demonstrable by a precipitation reaction (118). The nuclear antigen was designated RANA (RA-associated nuclear antigen), and its main epitope recognized by the RANA antibodies is the glycine/alanine repeat sequence on the EBV p62 nuclear antigen-1 (EBNA-1). The possibility has been considered that this molecular mimicry between an epitope encoded by EBV and an autoantigen could contribute to the breakdown of tolerance and autoimmunity in RA (119). The

subsequent finding that there was enhanced spontaneous outgrowth of EBV-infected B-lymphoblastoid cell lines from RA peripheral blood lymphocytes due to a T-cell deficiency in suppressing their emergence (120,121) reinforced interest in EBV. However, this latter observation was explained by a secondary nonspecific deficiency in T-cell function common to most chronic autoimmune diseases (122), making specific infection by this agent unlikely to be the etiology of the disease.

Nevertheless, the fact that EBV infection has occurred could alter the T-cell repertoire in a selective manner in that person because of the example of molecular mimicry between EBV gp110 and the “shared epitope” on the MHC molecules. Both contain the amino acid motif leu-gln-arg-ala-ala (123). It is possible that persons who inherit this MHC structure have developed a T-cell repertoire enriched for clonal receptors that recognize “self” during the phase of positive selection of the repertoire. Exposure of persons who have the T-cell repertoire biased by the presence of the shared MHC epitope to this EBV sequence might be anticipated to have a different consequence in terms of enhancing specific clonal responses than would occur in persons who lack the shared epitope.

Both EBV RNA and DNA have been identified in the synovium of RA patients (124,125), in both leukocytes and nonleukocyte lining cells. Koide et al. established a spontaneous EBV-infected synovial fibroblast cell line from an RA patient (126). T cells reactive to EBV lytic antigens also were identified in the RA synovium (127). It remains unknown whether these findings are in fact important in the regulation of synovitis and whether similar findings are present in other tissues, representing a nonspecific homing of EBV-infected leukocytes to the synovial membrane.

Retroviral infection as an etiologic factor is another controversial topic. No evidence of the presence of retroviruses has been obtained (128,129). In southern Japan, however, where human T-cell leukemia virus-1 (HTLV-1) is endemic, infection by this agent may induce an erosive polyarthritis, rheumatoid factor negative, that resembles RA. The *tax* gene is the likely candidate to initiate alterations in gene expression in the infected cells through a transactivation mechanism (130,131). This is suggested by the spontaneous erosive arthritis seen in *tax*-transgenic mice (132). Moreover, Trabandt et al. suggested the presence of increased levels of retrovirally related sequences in RA synovial tissues (133). Although further work is certainly warranted, at this time there is no conclusive evidence that implicates a microorganism as a causative agent in RA.

Sex Hormones as Modulators of the Immune Response

Male patients with RA are reported to have lower levels of testosterone than controls (134,135), although cause and effect have not been analyzed. The peak of disease onset around the perimenopause period in women suggests a correlation between decreasing levels of gonadal hormones and disease onset and that both estrogen and testosterone could have a suppressive role on the immune responses that cause RA. Both estrogens and androgens are, in fact, capable of modifying the immune response, including the production of T-helper cell 2 (Th2) cytokines by autoreactive T cells (136). Synovial macrophages and synovium-infiltrating CD29+ memory CD8+ T cells express estrogen receptors (137), suggesting that estrogens could directly modulate some functions of these cells. Based on this evidence, gonadal hormone therapy has been tested in RA; however, the results were less than optimum.

FEATURES OF THE AUTOIMMUNE RESPONSE

There are three central areas of knowledge about the autoimmune response in RA: First, the basis of disease susceptibility is defined by class II MHC alleles encoding the shared epitope. Second, the features of RF production, along with other autoantibodies by the B-cell component, reflect extensive evidence of cognate T-cell help, including somatic mutation of RF to higher affinity, high titer, and polyclonality, along with the finding of extensive isotype switching. Third, activated T cells elaborate cytokines such as IL-1 capable of altering the pattern of gene expression in synovial fibroblasts and macrophages. Taken together, these events imply the paradigm of an immune recognition event that involves the presentation of peptide by MHC class II molecules to CD4 T-cells. These T-cells then participate in the provision of antigen-specific cognate help to B cells as well as activating monocytes and other cells by paracrine mechanisms of delayed-type hypersensitivity.

Consistent with this paradigm, the tissues beneath the synovial lining cell layer become the site of a remarkable and often well-organized infiltration of both T and B cells that in every respect appear to be engaged in a purposeful immune response. Germinal centers develop, and RAG genes are expressed by the infiltrating lymphocytes (138), suggesting that B cells undergo local affinity maturation and that T-cells may be selected for optimum cognate interaction. The pattern and degree of lymphocytic infiltration are variable among individuals, however, as well as during the course of disease. Indeed, sometimes the lymphocytic infiltration regresses late in the course of the illness while the intimal synovial lining hyperplasia persists. Lymphocytes are often also a conspicuous cell in the synovial fluid, where they are accompanied by neutrophils. Unfortunately, although the description of the immune processes found in the synovial tissues is becoming increasingly comprehensive, consistent, and elegant, there is still no clear notion of what peptides are driving the T-cell or indeed the B-cell component of the response.

As discussed in the introductory section of this chapter, there is considerable evidence, based on the appearance of RF in healthy persons before the development of joint disease, to suggest that the autoimmune response of RA may be initiated without first affecting the joints. It will be argued that localization of the autoimmune process to the joints and its subsequent intensification appear likely to be fostered by the unique biology of the synovial lining. Thus, in addition to being the target of the efferent effects of the autoimmune response, the synovial lining appears also to participate in the afferent circuits, leading to its localization and intensification. These aspects of the interaction between the immune response and the synovial lining are discussed in the following section on the synovium, which describes the features of the T-cell and B-cell response.

T-lymphocytes

Phenotype and Activation State

The T-lymphocytes in the synovial tissue and fluid consist of relatively equal proportions of CD4 and CD8 cells. The T cells of both the blood, and particularly the joint fluid, express a variety of activation markers; however, there is a curious dichotomy in the expression of the T-cell activation markers HLA-DR and IL-2 receptor (CD25). The major proportion of the T-cells expressing HLA-DR molecules as activation markers are found in the CD8 T-cell lineage, whereas CD4 T-cells predominantly express IL-2 receptors (CD25) as their activation marker (139,140). As anticipated, both the activated CD4 and CD8 T cells in the joint exhibit a predominant memory cell phenotype of $CD45RO^{bright}$ and CD29 (141). Moreover, both CD4 and CD8 T-cell repertoire in the synovium contain oligoclonal expansions, reflecting an antigen-driven process (142,143,144 and 145). Conversely, in the blood, the highest level of IL-2 receptor (CD25) expression is on the CD45RO-negative naive compartment of CD4 T cells (146). Cells of this phenotype are considered not to have had previous exposure to antigenic stimulation. It may be that expression of IL-2 receptor is the initial step in the induction of the CD4 T cell into the CD45RO-positive, CD29-positive memory cell phenotype.

Most T cells infiltrating rheumatoid synovium express the chemokine receptors CXCR3 and CCR5 (147). These receptors are preferentially expressed in Th1 cells (148), a fact consistent with the functions of the T cells described in the autoimmune paradigm. IP-10, a chemokine ligand for CXCR3, is produced by RA synovial fibroblast cultures (149) whereas Mig, another CXCR3 binding chemokine, has not yet been studied in the rheumatoid synovium. The CCR5 chemokine ligands regulated on activation normal T-cell expressed and secreted (RANTES) (150,151), macrophage inflammatory protein-1a (MIP-1a) (151,152), and MIP-1b (151) are expressed in large amounts by the RA synovium. As discussed subsequently, synoviocytes produce stomal cell–derived factor-1 (SDF-1), a chemokine that binds to the CXCR4 chemokine receptor expressed by naive T cells as well as members of the monocyte lineage. Antibodies to RANTES ameliorated adjuvant-induced arthritis in rats (153), suggesting that the production of RANTES may be generally important in the biology of synovitis. The concept that chemokines have an important role in the localization of antigen-specific T cells and in the inflammatory process to the synovial membrane is suggested by several facts. These include production of chemokines by synovial fibroblasts, that the infiltrating lymphocytes express the corresponding receptors for the cytokines, and the improvement of arthritis in experimental animal models with the use of antichemokine therapy.

ROLE OF T CELLS

Given the degree of circumstantial evidence supporting a pathogenic role for T cells in RA, the expectation of investigators was that the T-cell compartment in the synovium would resemble an *in vitro* stimulated T-cell culture in terms of signaling and cytokine production. The characteristics of the T cells infiltrating synovium include hyporesponsiveness, reflected by a reduced response to mitogenic stimulation and decreased Ca^{2+} influxes (154,155); impaired TCRz-chain–mediated signaling (156); a lower T-cell proliferation rate; and reduced expression of T-cell–derived cytokines, such as IL-2 and interferon-g (IFN-g) (157). These findings initially raised the question of how critical T cells really are in RA and led to the postulate that T cells might have a more critical role in early stages of RA, but during the chronic stages, monocytes and synovial fibroblasts would be the cells coordinating the inflammatory process. Smeets et al., however, identified no significant difference between synovium-infiltrating T cells from early and late stages of RA, and both groups had low percentages of T cells expressing activation markers or IFN-g (158). Moreover, studies in CIA showed increased numbers of T cells and oligoclonal expansions during the early stages of the disease, followed by a polyclonal T-cell infiltrate (159) with increasing numbers of monocytes and monocyte/macrophage-derived cytokine production (160).

A more likely explanation for this seeming degree of hyporesponsiveness in RA synovia is that this is the physiological state of lymphocytes in a chronic ongoing immune response *in vivo*. CTLA-4 is expressed in the T-cell-activation cascade and serves to downregulate T-cell responsiveness. This occurs because CTLA-4 competes with CD28 molecules on T cells for interaction with the costimulatory B7 molecules on the antigen-presenting cell, depriving the T cell of the second signal provided by the costimulatory circuit. Moreover, interaction of CTLA-4 with B7 initiates a signaling pathway that further inactivates T cells by binding to the TCRzchain, thus preventing phosphorylation of this signal transduction molecule (161). CTLA-4 is indeed expressed in high levels by synovium-infiltrating T-lymphocytes (162,163).

It is likely that the physiologic expression of CTLA-4 by activated T cells infiltrating the synovium accounts for a significant part of the decreased T-cell functions seen in RA.

The attenuation of the TCR signaling and T-cell functions, including cytokine production, could also be explained in part by the chronic exposure of the synovial T cells to high levels of TNF, a phenomena that has been shown in a TCR transgenic animal model to regulate T-cell functions (164,165). Asymptomatic synovitis may precede the onset of clinical RA; conceivably, when disease manifests itself, high levels of TNF are already present in the synovium, interfering with T-cell functions. It will be of interest to study T-cell functions of patients treated with anti-TNF agents, with the prediction that their responsiveness may increase.

On the other hand, several factors could explain why T cells are important in disease pathogenesis, despite the small production of IL-2 and IFN-g by the synovium-infiltrating lymphocytes. (a) T cells in the tissues are largely memory phenotype T cells, which elaborate little IL-2. These cells would require little IL-2 to maintain their function because they have high-affinity IL-2 receptors. (b) The presence of many cells with IL-2 receptors may consume detectable IL-2, which perhaps accounts for the divergence between mRNA for IL-2 and the amount of the cytokine detected at the level of protein (166,167). (c) The proportion of immunologically specific T cells in an immune reaction may be on the order of well under 10% of all T cells, thus requiring only small amounts of IL-2 and IFN-g.

Additional evidence for the importance of T cells and their costimulatory interaction with antigen-presenting cells comes from animal models of autoimmune arthritis. Studies of CD28-deficient DBA/1 mice revealed that these mice are highly resistant to CIA and have markedly reduced levels of IgG and IgM anticollagen antibodies (168). Similar effects were seen with the use of anti-CD40 ligand antibodies, which also prevented the development of CIA (169).

In recent years, several new pathways have emerged that regulate T-cell function in the rheumatoid synovium. *In vitro* studies showed that macrophage-derived IL-12, a Th1-driving cytokine, was capable of inducing synovial T cells to produce IFN-g (170). This observation raises the issue of whether monocyte-derived cytokines *in vivo* can override the decreased T-cell responses and impaired signaling. IL-15 was identified in the RA synovium and shown to be capable of activating T cells in the absence of IL-2 and to induce the production of TNF-a (171), a cytokine of major importance in RA, produced by fibroblasts and macrophages. Subsequent work from the same group demonstrated that blocking IL-15 interaction with its receptor prevented the development of autoimmune arthritis in mice (172), further supporting the importance of this molecule in disease pathogenesis.

The cytokine IL-18, also known as IFN-g inducing factor, was identified in the RA synovial membrane and found to be capable or inducing both IFN-g and TNF-a production by synovial infiltrating lymphocytes. Furthermore, administration of IL-18 to mice with CIA increased susceptibility to disease and severity, supporting the *in vitro* findings that this cytokine may have an important role in disease pathogenesis (173).

Some of the CD8+ T cells present in the RA synovial tissue appear to have an antiinflammatory activity that is at least partially mediated by the release of IL-16, downregulating the production of Th1 cytokines by CD4+ T cells (174). CD8+ regulatory T cells also appear to operate through secretion of Th2 cytokines such as IL-4 (175).

Taken together, these findings suggest that the T cell has a major role in the sustained injury phase of RA. Moreover, they indicate that the seemingly lower level of T-cell activation is exactly what would be anticipated in a sustained immune response driven by T-cells.

T-CELL REPERTOIRE

The vast majority of T cells infiltrating the synovium express ab TCRs. Several laboratories have carefully investigated the representation of the TCRs in the tissue in an effort to characterize the biologic nature of the lymphocytic infiltration. One of the questions asked was whether the infiltration was primarily monoclonal or oligoclonal. This was addressed using a variety of methods, and in each study the answer was that the large majority of infiltrating T cells are of polyclonal origin (176,177), suggesting that a nonantigen-specific recruitment process accounts for most tissue T cells. The second question was whether any samples exhibited evidence of the possibility of clonal predominance. Here the answer was affirmative in that in a small proportion of cases, up to 12%, dominant patterns of TCR rearrangements were found (176). Moreover, in another study, two of three samples obtained early in the course of the disease had evidence for dominant Vb rearrangements, but none was found in six patients with more established disease (178). A different approach to this question of identification of antigen-specific T cells was developed using the addition of IL-2 to expand selectively only the T cells that express the IL-2 receptor. Clones derived by this method illustrated a greater degree of oligoclonal rearrangement (179).

Weyand et al. identified $CD4+CD28^-$ expansions in the peripheral blood of RA patients (180). These investigators determined that those cells are CD40 ligand negative, perforin positive, and capable of cytotoxic activity (181). They associated the presence of these cells with extraarticular manifestations (182). Further functional characterization of these cells is certainly awaited, and they remain to be identified in the rheumatoid synovium.

Oligoclonal expansions also have been identified among the synovium-infiltrating CD8 cells (143,183). These CD8 expansions may persist in the synovium for long periods (184), and they have not been functionally characterized.

Wagner et al. identified a tenfold contraction of the TCR repertoire in RA patients compared with healthy controls or other chronic inflammatory diseases (142). This contraction was present in both the CD4+ naive and memory cell compartment and correlated with shorter telomere lengths, suggesting an enhanced cell turnover. Although these data suggest abnormalities in CD4 cells homeostasis, it remains to be better understood how these abnormalities could influence the development of RA.

The suggestion that T cells might have been specifically responding to mycobacterial heat shock proteins led to an examination of the gdTCR repertoire composition because these receptors are used to recognize certain mycobacterial products (185). Only a small proportion of synovial samples exhibit increases in the representation of bearing gdT cells, however (186,187). The cells staining positively for gd chain were located principally in small perivascular aggregates (186). gd T cells may have a regulatory or cytotoxic function. Whereas their role in the rheumatoid synovitis remains mostly unknown, the finding that these cells do not appear to be critical for the development of autoimmune arthritis in mice suggests that they are not a critical component (188).

B Cells and Autoantibody Production, Including Rheumatoid Factors

Although the T cell receives a prominent place in the pathogenesis of RA, it also plays several other critical roles. Indeed, the B cell is likely essential for the development and sustenance of the autoimmune response itself. Although the initial event leading to loss of tolerance might be mediated by a dendritic cell, cognate interactions via the B cells may provide more efficient costimulatory signaling to T cells in the afferent portion of the development of a sustained autoimmune response. In part, this interaction could be due to the concentration of stimulating peptides presented by their MHC molecules that results from their capture of specific antigen by surface immunoglobulin. The importance of B cells can be seen in rodent models of autoimmune arthritis, where the absence of B cells protects animals from disease (189). Second, the B cells produce autoantibodies, notably including RFs, that form immune complexes capable of interacting with monocytes and other cells expressing Fc and complement receptors and with fluid-phase effector systems such as complement. The engagement of these systems results in the production of the proinflammatory cytokines, such as TNF and IL-1 by macrophages, or inflammatory mediators derived from the complement cascade.

The phenotype of the B cells in RA synovial fluid and peripheral blood is distinctive. Although not appreciably increased in number, a large proportion actively secretes immunoglobulin (190,191,192,193 and 194). Synovial fluid and synovial membrane lymphocytes exhibit 10- to 15-fold more plaque-forming cells than blood lymphocytes. A large proportion of B-lymphocytes in RA expresses the CD5 molecule. The elevation of CD5-positive B-cells is greatest in those with high RF levels, and comparable elevations are not found in systemic lupus erythematosus (SLE) (195). B cells in the synovium also exhibit activation molecules (196). For example, among the markers expressed by B cells is CD23, a low-affinity receptor for IgE (FceRII) predominantly expressed by mature B cells. B cells from RA patients have increased expression of this molecule (197,198), emphasizing the activation state of the B cells. Levels of soluble CD23 correlate with disease activity (198). Furthermore, antibodies to CD23 ameliorate murine CIA (199). Additionally, the introduction of the CD23 gene knockout into the CIA-susceptible DBA/1 strain delayed the onset and reduced disease severity while not significantly modifying the magnitude of the B- and T-cell autoimmune response (200). These findings demonstrate that CD23 could have an important role in the regulation of RA through mechanisms that likely involve the regulation of B-cell function or the soluble form of CD23.

Another set of experiments that support a role for B cells came from studies using the K/BxN TCR transgenic mice that develop a spontaneous erosive arthritis (201). Like in CIA, this model depends on cognate T- and B-cell responses (202); however, the antigen here is the glucose-6-phosphate isomerase, a glycolytic pathway enzyme (203). It remains unknown whether these findings also apply to the human disease, but it certainly raises an interesting issue about disease development through mechanisms fundamentally different from an immune response to a joint-specific peptide and the important role of B cells in the response.

As with T cells, the migration of B cells into the synovium is likely mediated by cytokines and chemokines produced by the fibroblast lineage synoviocytes and macrophages, including SDF-1 and IL-6. SDF-1 is also an important B-cell developmental and maturation factor, as revealed by the observation that mice lacking SDF-1 show defects on B-cell lymphopoiesis (204). It is likely that the cytokine and cell receptor interaction in the synovium milieu is highly supportive of B-cell

differentiation, as discussed subsequently. These factors may be of major importance in the development of the pathogenic autoimmune response of RA.

In some synovia, B cells organize into conspicuous classic germinal centers, whereas in others the cytoarchitectural organization is less striking. The expression of RAG genes by synovial lymphocytes (138) suggests that B cells undergo local affinity maturation and that cognate interactions with T-cells are occurring. The presence of RF isotype switch and somatic mutation variants, discussed later, within RA synovial B cells supports the interpretation that the B-cell aggregates within synovial lining are clonally related and exhibit all the features of a germinal center that has received T-cell cognate help. Supporting evidence for involvement of Th functions in RF production includes that blood lymphocytes stimulated by pokeweed mitogen require T-cell help for the production of RFs (205,206 and 207). Other evidence for the participation of the T cells includes the presence of class switching to IgG and IgA isotypes, supported by the finding of the same idiotypes on IgM and IgG RFs (208). Williams et al. showed in a detailed analysis that RF H-chain sequences undergo both somatic mutation and isotype switching (209). Size analysis revealed oligoclonal RF+ populations and identically sized VH-D-JH transcripts of different immunoglobulin isotypes. Sequencing of individual chains demonstrated a clonal relationship between IgM and IgA RF, suggesting that this isotype switch occurred in the synovium. Furthermore, most somatic mutations were found to have occurred after the isotype switch, which suggests that the RA synovial microenvironment sustains somatic mutation and isotype switching in RF-specific B lymphocytes akin to secondary lymphoid organs.

Rheumatoid Factors

GENERAL FEATURES

Several autoantibodies are produced in RA, of which the RFs are the best studied. RFs, a distinctive feature of RA, are autoantibodies of IgM, IgG, or IgA class that react with antigenic epitopes present on the constant region of native IgG molecules. RFs are members of a larger class of antiimmunoglobulins that also include antiidiotypic antibodies directed to regions around the combining site and homoreactant or pepsin agglutinator antibodies directed to cryptic sites revealed by degradation. Although many of the detection systems used to measure RFs involve aggregated or otherwise altered IgG, there is no evidence that RFs have a preferential specificity for determinants on altered IgG molecules. The apparent preference for these indicator systems is due to the multiplicative effect on avidity of the interaction of RF with multiple IgG molecules on one particle or surface. RFs are by far the major autoantibody produced in RA patients but, as discussed later, are not the only autoantibody. In some samples, more than half the serum and joint fluid IgG is found in the form of RF complexes. The sites of RF production include many germinal centers in rheumatoid synovial tissues (210). Up to 50% of the plasma cells in the synovial tissues contain RFs, most of the RF-specific cells are revealed using a plaque-forming cell assay or by staining for IgG-RF rather than IgM-RF (192,211). Many more cells produced IgG-RF than IgM-RF in synovial tissues compared with that produced by the blood.

The presence of serum RF detectable by conventional testing methods is a characteristic feature of RA; however, it does not appear to be an essential element for the disease. Some have used this and the fact that RF may be found in normal persons as a specious argument against the importance of RF in RA. Additional information acquired in several aspects of the study of RA has provided a clearer picture of the important place of autoantibodies such as RF in RA. (a) The RFs in RA differ from those found in normal individuals in that they reflect the presence of a strong antigen-driven immune response, as reflected by the their pattern of somatic mutations away from germ line, high titer, and isotype. (b) RF positivity may precede the development of RA by several years (16,17). (c) RFs appear to be the major subclass of a still larger group of partially cross-reactive autoantibodies, as discussed subsequently. (d) Although indeed some patients with RA test negative for RF, in some instances, this reflects the difficulty of detecting certain forms of RF by conventional tests.

TYPES

Rheumatoid factors may be subdivided into three subgroups: monoclonal RFs equivalent to those arising from a transformed B cell that do not exhibit significant mutations from their germ-line origin. These are mainly of IgM isotype, they utilize a limited number of V-region gene elements, they remain in or near a germ-line sequence, and they form mixed cryoprecipitates with IgG molecules. The disease associations of this group are a variety of B-cell neoplasias, including chronic lymphocytic leukemia, Waldenström macroglobulinemia, other lymphomas that are often extremely low grade, and benign monoclonal proliferations. Some diseases with autoimmune features including Sjögren syndrome are characterized by RFs that fit into this classification. Most, but not all, the RFs in Sjögren's syndrome are in germ-line configuration and are of IgM isotype, indicating little or no role for somatic hypermutation and passage through a germinal center reaction in their generation (212). RFs of this group are often termed *cold reactive RFs* to describe their property of forming cryoprecipitates (213). They are not found in RA.

The second group of RFs is a minor component of the normal immune response to certain antigens or infectious agents. These RFs are somewhat similar to the monoclonal RFs in their characteristics, but they are more polyclonal and rarely form cryoprecipitates. Some of these are polyspecific, whereas others appear to be monospecific for IgG (214). These RFs, along with other antiimmunoglobulins, occur naturally in patients with chronic infections and are particularly well known in subacute bacterial endocarditis (SBE). Williams and Kunkel (215) showed that sustained RF production depends on the continuing presence of the infection and that RF disappears with effective treatment with antibiotics. RFs can be induced by immunization of animals with bacteria (216,217). They are also found in persons with various other chronic infections, such as tuberculosis, leprosy, syphilis, and kala azar. RFs also have been observed to appear transiently in normal military recruits subjected to heavy immunization schedules (218). The variable regions of these RFs undergo considerable hypermutation, but, in contrast to antibodies against exogenous antigens, there is a strong selection against mutations that result in replacement of amino acids in the hypervariable or complementarity-determining regions. Furthermore, there was no increase in the affinity of these RFs with the accumulation of mutations (214,219). This suggests that high-affinity RF variants are tolerized during the hypermutation process, and there is a peripheral mechanism operating on certain autoreactive B cells in normal individuals that, although not deleting or anergizing all autoreactive cells, prevents the generation of high-affinity autoantibodies (219).

The third group of RFs found in RA are polyclonal and are present in all isotypes; these use a variety of V-region gene elements and are considerably removed from germ-line structure. They are characterized by extensive mutation that involves amino acid replacements leading to higher affinity, indicating antigen-driven selection (220,221 and 223). These RFs have a broader pH optimum, reacting well at pH 8 or 9, and do not form mixed cryoprecipitates. They are characterized as “warm reactive.” There is considerable evidence that T-cell help is involved in the production of this variety of RF from B cells that have passed through germinal centers. In particular, the structural relatedness of IgM RF in the synovium of RA patients argues strongly in favor of antigen-driven selection (222,223 and 224).

GENE USAGE OF RF AND IMPLICATIONS FOR AN ANTIGEN-DRIVEN RESPONSE

The findings from molecular analyses of RF-producing clones from RA joint tissues argue persuasively that they use a different repertoire of V genes. Robbins et al. (225) published the first sequence of the heavy and light chains from a RA synovial B-cell clone that had been obtained without EBV transformation and showed that it used the Humhv1f10 Vh1 gene (226). Sequencing a set of clones also derived from rheumatoid synovial material (227), Pascual et al. significantly extended this line of inquiry by identifying two new RF-related V genes, designated GL-SJ2 Vh3 and the A23 Vk2 gene (228,229). New V-region genes encoding IgH chains with RF specificity were also reported by Ermel et al. (222), demonstrating that the repertoire of V-region genes used in the synthesis of immunoglobulin molecules with RF specificity was large. Similar results were obtained by other investigators (230,231). Olee and colleagues also identified the germ-line counterparts of genes used to synthesize IgG RFs. They found up to 16 somatic mutations in a gene encoding a RF heavy chain, which otherwise might encode an element in the physiologic natural antibody repertoire (231). Two groups studied the kappa light-chain recombinants from RA synovium by an extensive sequence analysis (232,233). Numerous somatic replacement mutations were found in the antigen-binding site, many of which contained non-germline-encoded nucleotides (N regions) at the site of V kappa–J kappa joining. The combination of N-region addition and variation in the sites of V kappa–J kappa splicing generated unusually long complementarity-determining region 3 (CDR3) regions and charged amino acids near the V kappa–J kappa splice site. The pattern of somatic mutations supports the interpretation that these synovial plasma cells are the product of antigen-driven selection. The extent of N-region addition raised the additional possibility that these antibodies derive from an unusual set of B cells that have escaped normal regulation. Taken together, these data strongly support the interpretation that an antigen-driven mechanism underlies the RF response in RA. McGee et al. reported that B-lymphocytic clonal expansions in blood were identified by double staining for the B-cell marker CD19 plus either k or l. The fraction of clonally expanded B cells in RA was more than 50% higher than in controls and correlated with RF titer (234).

FORMATION OF COMPLEXES BY RHEUMATOID FACTORS

Franklin et al. and Kunkel et al. first clearly recognized the singular property of RF to complex with autologous IgG in serum (235,236). This was also the earliest demonstration of circulating immune complexes in a disease. The method these investigators used, analytical ultracentrifugation, remains the best method for examining this type of molecular association under conditions that approximate those occurring in the body. IgM RF circulates complexed to five IgG molecules.

At least eight different antigenic sites that are recognized by polyclonal RFs from RA patients have been identified on the Fc portion of the IgG molecule by using reactivity with overlapping peptides obtained from IgG. Some of these are in the hinge region (Cg2), and others are in the C-terminal portion (Cg3) (237,238). Most of these autoantigenic sites are present on each of the four subclasses of human IgG molecules: IgG1, IgG2, IgG3, and IgG4. Certain of the antigenic sites are also present on Fc region of IgG from other species, notably the rabbit and sheep. One variety of IgM RF that recognizes determinants on IgG1, IgG2, and IgG4, but not IgG3 molecules, a pattern designated as *Ga specificity*, binds particularly well to serum IgG. These RFs with Ga specificity cannot be detected in the usual assays unless all serum IgG is removed (239). This variety of RF is termed *hidden RF*. Group A streptococcal protein A apparently also binds to this site (240). That IgG is not necessarily the inciting or the only member of the immunoglobulin family with which RFs react was shown in a study that identified reactivity with b_2 microglobulin (241).

The ultracentrifugation technique also first revealed IgG RF (226). The complexes of IgG and IgG RF found in serum were seen to sediment as a spectrum of complexes ranging between 7S (monomeric IgG) and 19S or greater (³5 IgG or IgG RF molecules). Complexes involving IgG RFs have a distinct molecular conformation that enables them to interact with various molecular receptors of immune effector systems of the body. This can be demonstrated simply by allowing serum containing such complexes to react with preparations of purified IgM RF or C1q in solution or in agarose double-diffusion experiments. The purified IgM RF identifies the presence of immunoreactant IgG complexes in the patient's serum by the formation of a distinct precipitin band that is not given with monomeric IgG. The exact form that the IgG/anti-IgG complexes assume in joint fluid or serum is still not completely defined, but several models are postulated. In one model, the IgG RFs cluster around the several epitopes on the constant region of an IgG molecule. This form would be especially favored if the steric location of the IgG antigenic determinant recognized by the RF was situated symmetrically so that both arms of the RF could combine with determinants on a single IgG molecule. A second arrangement is that of a growing tree with each IgG RF binding either a molecule of IgG or another IgG RF. Pope et al. proposed one of the most interesting patterns of association. They obtained evidence that some IgG RFs form a self-associating complex in which the steric factors of the location and orientation of the antigenic determinant favor a parallel–antiparallel interaction between two IgG RFs in which the antibody serves as the antigen. This complex also would be stabilized by the presence of two binding sites locking two molecules together, which greatly augments the avidity of the complex (242); however, it likely would not bind C1q.

VASCULITIS AND CIRCULATING COMPLEXES

Circulating complexes formed through the action of IgG RFs or IgM RFs are thought to be central to the vasculitic phenomena in the disease (243,244 and 245). The contribution of T cells has not been evaluated by contemporary techniques, however. Pernis et al. and Conn et al. demonstrated deposition of immunoglobulin, RF and complement molecules in the vessel walls of patients with necrotizing vasculitis (243,245). Mongan et al. (244) reported that necrotizing vasculitis is found primarily in patients with extremely high RF titers as well as significantly lowered hemolytic complement titers. Zubler et al. found that serum of patients with these features reacted with a sensitive C1q binding test for immune complexes (246). IgG-RF has been highly associated with the pathologic process in RA, both in synovial fluids and in necrotizing vasculitis (192,247).

RHEUMATOID FACTOR COMPLEXES AS PROINFLAMMATORY IMMUNOREACTANTS

A pathogenic role for IgG complexes containing IgG RF is supported by markedly diminished levels of complement proteins and total hemolytic complement activity in the synovial fluid as shown by several investigators (248,249 and 250). The joint fluid IgG complexes have been implicated as the basis for intraarticular complement activation (251). The levels of IgG RFs are usually much greater in the joint fluid than in the serum. This is evident first at the level of B-cell precursors that are the antecedents of RF-producing cells. A variety of physical and immunologic measurements also support the presence of elevated levels of IgG RF in the joint fluid. IgG in the form of complexes also accounts for a remarkably large amount of the IgG in joint fluid, in the range of several milligrams per milliliter (251,252 and 253). Joint fluid complexes are considerably larger and react both with C1q and RFs, whereas the smaller complexes in serum reacted only with IgM RFs (251,253). As little as two or three IgG molecules were sufficient to confer demonstrable reactivity with IgM RFs, but at least five or six IgG molecules had to be present in complexes that reacted with C1q (252,254). Elevated turnover of both IgG and the C4 complement component in individuals with RA support the participation of complexes containing RFs in initiating inflammation. This enhanced turnover correlates with elevated titers of RFs and is especially high in those with extraarticular disease (255,256). Taken together, considerable evidence suggests that a proportion of the immune complexes involving IgM and IgG RF forming in RA fix and activate complement and are processed by phagocytic and other systems with considerable inflammatory consequences.

Deposition of immunoglobulins and complement in the matrix of the synovium and their phagocytosis by neutrophils (257,256,257,258 and 259) has been shown. Immunoglobulin deposits were absent from cartilaginous areas covered with pannus but could readily be detected on the free surface (260). These observations were used to suggest that immunoglobulin deposits on cartilage represent antigen persistence in immune complexes initiating pannus formation (261).

Other Autoantibodies and the Potential Relation to Rheumatoid Factors

Other autoantibodies have been described in RA, and certain of these may provide clues to understanding the nature of RF. Among the best known are IgG antibodies to native collagen II that have been described in the serum of 15% to 27% (262,263) and in the synovial fluid of 52% (264) of RA patients. Cook et al. described a reduction in the rate of anticollagen antibodies after 1 year of follow-up, and this reduction correlated with the development of radiographic erosive changes (263). Evidence from rodent models of autoimmune arthritis, where disease is induced with type II collagen and adjuvant injections, showed that IgG anti-collagen antibodies are required for the development of the disease and also can induce transient disease in passive transfer experiments (265,266 and 267). Epitope mapping analyses have identified the immunodominant and critical domains of the collagen molecule regulating rodent anticollagen antibody production. It remains unknown whether these anticollagen antibodies have a pathogenic role in human disease.

Autoantibodies to cytoskeletal elements—such as the cytokeratins, vimentin, and a perinuclear body containing filaggrin in buccal mucosal cells are found in RA (268,269). Their identification adds a new dimension to the study of the pathogenesis of RA because they may be particularly important for integrating some of the RF-negative cases into pathogenic schemes involving the B-cell arm of autoimmune recognition. Antiperinuclear factor (APF) has been reported to be highly specific for RA compared with other arthropathies and to be positive in 28 of 33 (85%) RA cases with extremely low or negative RF, reducing the number of “seronegative” RA from 33 of 119 to 5 of 119 (270). A recent metaanalysis that included 939 patients with RA, 1,539 with other rheumatic diseases, and 375 from healthy controls or patients with unclassified rheumatic diseases calculated a sensitivity of 75% and a specificity of 93% for APF positivity (271). Kessel et al. pointed out that antikeratinocyte antibody (AKA) positivity could be useful in distinguishing RA from hepatitis C–associated polyarthritis, where the rate of positivity in association with positive RF was only 8% (272). Another recent study analyzed the role of APF, AKA, and anti-RA33 in early onset RA. Among RF-negative patients, 51.7% were positive for AKA, APF, or anti-RA33 antibodies. Positivity of AKA, APF, and anti-RA33 usually persisted throughout follow-up, whereas in this series RF was lost by 58% of patients with early, RF-positive disease (273). AKA has been reported as an independent risk factor for the development of radiographic damage in RA (274). An enzyme-linked immunosorbent assay (ELISA) test for the measurement of antifilaggrin antibodies(AFA) using filaggrin purified from human skin as an antigen was applied to 306 patients with recent-onset inflammatory joint diseases (275). The APF and AFA results on those with peripheral polyarthritis or oligoarthritis were in good agreement, whereas AKA and AFA only partially overlapped. The AFA test detected 10 of 22 of the RF-negative erosive cases, particularly those with a large number of erosive joints, supplementing RF in the prediction of erosiveness. Similar results were obtained by Vincent et al., who performed a detailed characterization of the serologic distinction between epitopes recognized by AKA and AFA sera (276). Perinuclear staining pattern of antineutrophil cytoplasmic antibodies (p-ANCA) also was described in one third of Felty syndrome patients (277). Sa, another novel peptide, 50 to 55 kd autoantigen, present in placenta and spleen by Western blotting, elicits autoantibodies, predominantly of the IgG isotype. They are found in 27% of RF-negative and 50% of RF-positive RA patients but at low or negative levels in RF-positive individuals with diseases other than RA. The specificity for RA was 98.9%, positive predictive value 96.7%, and negative predictive value 69.8% (278,279,280 and 281). Anti-Sa was not associated with either RF or with anti-A2/RA33. Anti-RA33 was present in 21 RA sera, only eight of which also contained anti-Sa; 51% of RA patients were positive for at least one of these two autoantibodies, and 18 of these patients were RF negative.

Antibodies to the main epitope recognized by the RANA antibodies, the glycine/alanine repeat sequence on the EBV p62 nuclear antigen-1 (EBNA-1), are elevated to about fourfold greater titers in RA sera than in controls. They cross-react with epitopes on keratin, actin, and collagen (119), raising the possibility that this molecular mimicry might contribute to the breakdown of tolerance and autoimmunity in RA.

Antibodies to calpastatin (282), p64 (283), and cyclic citrullinated peptide (284) have been described. The last two in particular appear to be highly specific for RA in limited studies.

It has been known for many years that RFs react with epitopes on rabbit IgG, usually with analogous structures also found on human IgG. Fang et al. conducted a study of a subpopulation of RFs from RA patients who reacted exclusively with rabbit but not human IgG. They observed that certain of these anti-rabbit IgG RF also had specificity for other mammalian antigens, including cytoskeletal proteins and intracellular proteins found in HeLa cells as well as for antigen present in an extract prepared from the cell wall of group A streptococci (285). This finding expanded the understanding of the meaning of RF in RA by emphasizing the potential relationship of RF to other autoantibodies and also by emphasizing that autologous reactivity with IgG is not an essential feature. These non-Ig antigens, along with others described in this section (e.g., the spectrum of AKA, APF, anti-RA33 autoantibodies), raise the possibility that the antigenic drive in RA consists of one or several molecules that are distinct from IgFc regions but that share conformational epitopes capable of initiating spreading reactions via a B-cell mechanism.

ARTHRITIS, SYNOVIAL HYPERPLASIA, AND JOINT DESTRUCTION

The hyperplastic response of the intimal synovial membrane to the autoimmune response of T cells and B cells that accumulate in the subintima establishes the locus for much of what will become the joint inflammation of RA. This intimal hyperplastic response also mediates the destruction of joint tissues that is the dominant clinical characteristic of the disease. The remarkable degree of hyperplasia exhibited by the intimal synovial lining cells is presumably driven, at least in part, by paracrine factors such as IL-1 and TNF elaborated by the inflammatory autoimmune response. This section first reviews the characteristics of the normal synovial membrane. Our viewpoint is that much of the pathology of RA derives from the expansion of normal biologic processes of the joint by the consequences of the autoimmune response. The section then discusses the cellular abnormalities seen in the synovial membrane in RA. The distinctive phenotype of fibroblast lineage synoviocytes cultured from RA leads to a presentation of gene-discovery studies directed at understanding this phenotype and gaining insight into the nature of synovitis and the biology of the normal joint. One of the major consequences of the gene-discovery effort was the unexpected finding that fibroblast lineage intimal synoviocytes, and

presumably those engaged in destruction of cartilage and bone, were nearly indistinguishable from mesenchymal stem cells. Thus, among the hyperplastic synoviocytes of the pannus are cells that are the embryologic precursors of the cells they seek to destroy. Additional insights into the molecular basis of cell–cell and cell–matrix interactions underlying synovitis provided by the gene discovery effort are discussed also. The genetic susceptibility and other potential factors involved in the regulation of the fibroblast lineage synoviocyte biology are also within the scope of this chapter. Experimental animal models of arthritis have contributed greatly to our increased understanding of the genetic regulation and pathogenesis of RA, and findings originated from such studies are incorporated herein.

Relevant Features of the Normal Joint

The joint is a functionally unique structure primarily formed from mesenchymal cells. The normal synovial membrane is lined by a layer of interspersed specialized cells that is one to three cells thick (Table 37.1, Fig. 37.5). About two thirds of the specialized cells lining the joint cavity are fibroblast lineage intimal synoviocytes and belong to the fibroblast lineage (19,286,287). Through their unique and increasingly defined pattern of gene expression, the fibroblast lineage intimal synoviocytes appear to be differentiated to perform a series of functions critical to the biologic function of the normal joint. This phenotype also appears to confer the potential for a special role in fostering the development and synovial localization of the autoimmune response underlying RA and to participate in joint destruction. The remainder of the lining is composed of monocyte lineage intimal synoviocytes that are derived from the CD14-positive branch of the monocyte lineage (288). The two types of intimal synoviocytes originally were named *type B* and *type A*, respectively according to their appearance in electron microscopy (289). The progenitors of the monocyte lineage intimal synoviocyte enter the intima after leaving blood vessels and differentiate into their mature form in response to guidance clues and interactions apparently provided by the fibroblast lineage intimal synoviocytes. The molecules that are responsible for this critical phase of joint histogenesis are beginning to be identified. The function and especially the interactions of these two cell types are of special importance in understanding both the biology of the normal joint and the inflammation of RA.

Surface specialization for
Synovial fluid face
Extracellular matrix face of subintima
Receptors for
1. Homotypic (fibroblast-fibroblast)
2. Heterotypic (fibroblast-monocyte/macrophage)
Synthesis of components of the synovial fluid and
factors for cartilage nutrition and function
Histogenic functions
Guidance clues to monocyte entrance
Immune surveillance
Matrix remodeling
Metalloproteinases and other proteinases
Synthesis of matrix components

TABLE 37.1. Fibroblast Lineage Intimal Synoviocyte Functions

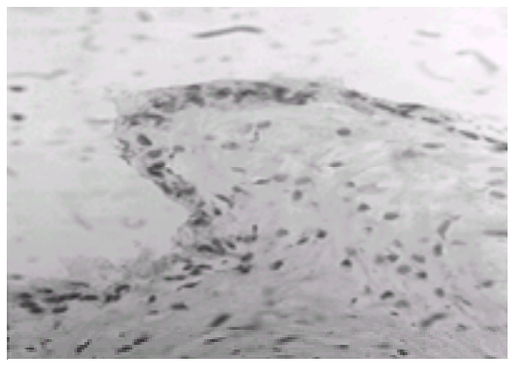

Figure 37.5. Noninflammatory osteoarthritis synovial membrane showing two or three layers of lining cells stained for stromal cell–derived factor-1 (SDF-1). (See Color Figure 37.5.)

The two varieties of intimal synoviocytes together have five major functions, including (a) the production of extracellular matrix fibrous and ground substance components that integrate the intima with the subintima; (b) expression of cell surface receptors specialized for interactions with the subjacent extracellular matrix fibrous components (expression of receptors and counterreceptors that mediate the close cell–cell interactions responsible for the patterning of the intima); (c) synthesis of components of the synovial fluid, notably hyaluronate and factors for the nutrition of the chondrocytes and the avascular cartilage, and elaboration of the molecules that provide lubrication for cartilage motion; (d) matrix remodeling through the production of metalloproteinases and other proteinases; (e) immune surveillance of the joint cavity and removal of cartilage debris resulting from weight bearing and other joint stresses. (19).

Several features distinguish the normal fibroblast lineage intimal synoviocyte from typical fibroblasts, including the following (Table 37.1):

1. Polarization unusual for a typical connective tissue fibroblast in which the receptors for interaction with collagen and other fibrous elements of the subintimal matrix are disposed only on the abluminal surface of the cell while the luminal side interacts with the synovial fluid.
2. Extensive cell–cell interactions. The fibroblast lineage intimal synoviocyte exhibits, in addition to the homotypic cell–cell interaction with each other, a receptor-mediated heterotypic interaction with monocyte lineage intimal synoviocytes. In electron microscopic studies, tight junctions or desmosomes, characteristic of epithelial cells, are not seen, suggesting that homotypic and heterotypic cellular interactions during the continued histogenesis of the synovial lining are perhaps entirely receptor mediated. Evidence for the presence of various receptor/counterreceptor interactions that could mediate these interactions is reviewed subsequently. Additionally, matrix components like collagen VI have been implicated in maintaining cells attached to each other and to the matrix (290).
3. The fibroblast lineage intimal synoviocytes appear responsible for the localization and guidance clues that result in the entrance of monocytes in the intima and their subsequent differentiation into monocyte lineage intimal synoviocytes. These and other features of the fibroblast lineage intimal synoviocyte appear to confer the potential for a role in both the afferent and effector pathways of the pathogenesis of RA, as discussed (19). Several comprehensive reviews of the synoviocyte and synovitis provide additional information (19,287,291).

Beneath the intimal lining layer lies a thin zone of vascular connective tissue, the *subintima.*There is no basement membrane separating the intima and the subintima. In contrast to the fibroblast lineage intimal synoviocyte, the subintimal synoviocytes appear to be more typical connective tissue fibroblasts. Monocyte lineage cells are not conspicuous. The subintima may contain variable numbers of adipocytes.

During fetal development, cavitation occurs within the primitive mesenchyme along planes destined to become the articular surfaces of synovial joints. These planes are delimited by cells that are the anlage of the intima. Evidence suggesting that joint cavitation is dependent on the behavior of primitive fibroblasts that are the precursors of synoviocytes or adjacent developing chondrocytes has been presented (292). Macrophages enter the site of the future joint at the periphery of the joint interzones but are not located near to where cavity formation occurs.

Clues about the Synoviocyte from Studies on the Distinctive Phenotype of Cultured Rheumatoid Arthritis Fibroblast Lineage Synoviocytes

It has long been recognized that synoviocytes obtained from synovial tissue of patients with RA do not revert to an entirely typical fibroblast lineage morphology and behavior (289,293,294). They rather maintain a complex distinctive phenotype that includes fibroblast lineage cells with varying degrees of “stellate” morphology, enhanced growth, increased glucose consumption, altered adherence behavior, constitutive overproduction of metalloproteinases, and the elaboration of proinflammatory cytokines (295,296,297 and 298) as well as loss of contact inhibition (293). This phenotype is infrequently found in similarly cultured synoviocytes obtained from normal synovia or osteoarthritis synovia that lack lining cell hyperplasia and any inflammatory cell infiltration. In addition, cell lines exhibiting elements of this distinctive phenotype are not uniquely specific for RA, as they are also demonstrable in cultures initiated from a number of different entities characterized by chronic inflammation and synovial hyperplasia, including psoriatic arthritis (298,299). Cells freshly isolated from a rheumatoid synovium exhibiting the striking stellate or dendritic morphology strongly express HLA-DR, presumably reflecting the effect of paracrine factors (297,300). Their fibroblast nature has been established by their lack of the property of enhanced endocytosis or phagocytosis; their lack of expression of CD14, Fc receptors, or the leukocyte common antigen CD45 (301); and their

expression of a number of genes from vimentin to type I collagen, characteristic of fibroblasts (19). When preparations of these cells are placed in culture, the preponderance of these cells lose expression of HLA-DR, but the complex stellate morphology may remain in varying proportions along with the distinctive phenotype.

SYNOVIAL GENE-DISCOVERY EFFORTS

The pattern of gene expression in lines of cultured RA synoviocytes has been characterized in a series of studies (20,297,298 and 299,302,303). To identify the genes responsible for the distinctive phenotype of the cultured synoviocytes obtained from a RA patient, a gene discovery approach has been taken that is based on identifying genes similarly and differently expressed compared with a line derived from a selected osteoarthritis sample. One recent approach (20) was based on the construction of representational difference libraries (304,305). It involved a cloning procedure with polymerase chain reaction (PCR) amplification of cDNA to generate simplified representations of the expressed genes from RA and osteoarthritis (OA) synovia, followed by repeated modified subtractive steps using the OA sample and subsequent sequencing to identify the genes. Several genes were identified, many of which were constitutively expressed at high levels in RA fibroblast lineage synoviocyte lines as demonstrated by Northern analysis (Table 37.2). Included among these are genes encoding matrix components: collagens, biglycan, laminin, fibronectin, crystallin, and proteoglycans; proteinases and inhibitors, including matrix metalloproteinases (MMPs) and cathepsins; genes involved in cell–cell, cell–matrix interactions; receptors, such as MAC-2BP, vascular cellular adhesion molecules-1 (VCAM-1); cytokines, chemokines such as SDF-1; growth factors; genes reflecting the operation of activation pathways, oncogenes, transcription factors; and others with still unclear functions in the synovium (19,20). The identification of SDF-1 was confirmed by Zvaifler et al. (306). We interpret these findings to imply that the genes found constitutively overexpressed in the RA fibroblast lineage synoviocyte culture are expressed at high levels in fibroblast lineage intimal synoviocytes and are primary markers of the phenotype of this lineage. Moreover, identification of additional genes has been reported, including various embryonic growth factors, from the wingless (wnt) and frizzled (fz) families, implicated in cell-fate determination in both bone marrow progenitors and limb-bud mesenchyme (307). Each of these groups similarly interpreted their results to suggest that the unusual phenotypic properties of RA fibroblasts in culture are attributable to their replacement with primitive fibroblast lineage synoviocytes with characteristics of immature bone marrow and mesenchymal cells. These genes can mediate chemotaxis of T- and B-lymphocytes and monocytes into the synovial membrane and participate in further cell differentiation. Sen et al. (307) provided additional evidence in support of the above concept by transfecting *wnt5a* into normal fibroblasts, which induced increased amounts of IL-8, IL-6, and IL-15 protein secretion, similarly to the levels produced by cultured fibroblast lineage synoviocytes from RA.

Genes preferentially expressed in RA synovial cultures: intimal origin	Genes expressed in both RA and OA synovial fibroblast cultures: intimal and subintimal origin
Biglycan	Adrenomedullin
IFN-induced 56 kd	α subunit of Gs GTP binding protein
IFN-induced 71 kd 2'5'-oligoadenylate synthetase	α-B-crystallin
IGF-BP5	B94 protein
Lumican	β subunit of prolyl-4-hydroxylase
Mac2-binding protein	Candidate sulphatase
ML2122	Cathepsin B
SDF-1α	Collagen α 1 type III
Semaphorin 3C/E	Collagenase IV
SLIT-3	Complement C1r
VCAM-1	Complement C1s
	Complement factor B
	DNA-binding protein TAXREB107
	Elongation factor 2
	Epithelin
	Extracellular protein (SI-5)
	HLA-E heavy chain
	IFN γ IEF SSP 5111
	Manganese superoxide dismutase
	Milk-fat globule protein
	Muscle fatty-acid binding protein
	NM8 protein
	Osteoblast specific factor 2

HLA-E, human leukocyte antigen-E; IFN, interferon; IGF-BP5, insulinlike growth factor binding protein-5; OA, osteoarthritis; RA, rheumatoid arthritis; SDF-1α, stromal cell-derived factor 1α; VCAM-1, vascular cell adhesion molecule-1.

TABLE 37.2. Genes Identified Through a Subtraction Method Differently Expressed in RA and OA Fibroblast Lineage Synoviocyte Cultures and Correlation with Their Possible Lineage Origin

Clues about Fibroblast Lineage Synoviocyte Biology from the Constitutive Production of SDF-1 by Some Synoviocytes

The identification of the high level of constitutive expression of SDF-1 by RA compared with low levels in OA synoviocytes was of special interest. First, SDF-1 is one of the most efficacious chemokines in inducing T-cell and monocyte migration (308). Second, SDF-1 is an important B cell developmental and maturation factor, as revealed by the observation that mice lacking SDF-1 show defects on B-cell lymphopoiesis (309).

MESENCHYMAL STEM CELLS AND FEATURES OF THE FIBROBLAST LINEAGE SYNOVIOCYTE

The unique pattern of expression of SDF-1 by RA synoviocyte lines is similar to that seen in mesenchymal stem cells. This suggests that some fibroblast lineage synoviocytes represent a form of less differentiated cells, closer to, if not indistinguishable from, the mesenchymal stem cell than to a well-differentiated fibroblast. An overall scheme of differentiation in the fibroblast lineage (310) proposes a pluripotent stem cell termed the *mesenchymal stem cell* or the *marrow stromal cell*, and a series of committed progenitors for each of the differentiated fibroblast lineages that constitute this connective tissue cell family. These include the connective tissue fibroblasts in various tissues and more specialized cells, such as osteoblasts and osteocytes, chondroblasts and chondrocytes, smooth-muscle cells, adipocytes, neuroglial astrocytes (311), and the fibroblast lineage intimal synoviocytes of the joint. Marrow stromal cells are readily isolated from bone marrow and maintain a stable phenotype in culture (312,313). Marrow stromal cells express SDF-1 and phenotypic markers like vimentin, tenascin, the integrin subunits, a_5b_1, a_vb_3, and a_vb_5 epithelin, SH2, SH3, intercellular adhesion molecule-1 (ICAM-1), VCAM-1, Thy-1, CD40, and CD44H (314,315,316 and 317).

The expression of SDF-1 and other genes in RA synoviocytes, to be discussed subsequently, indicates that the mesenchymal stem cell of the bone marrow closely resembles the fibroblast lineage intimal synoviocyte (19,20,306), although the two populations might not be identical in that myelopoiesis is not an overt finding in the joint. Nevertheless, the possibility that a particular population of synoviocytes might share with marrow stromal cells the ability to differentiate into osteoblasts and adipocytes, as is the case for the marrow stem cells (318), remains an important unanswered question in synovial biology.

GENES LIKELY CONSTITUTIVELY EXPRESSED IN FIBROBLAST LINEAGE INTIMAL SYNOVIOCYTES

The specialized functions of the fibroblast lineage intimal synoviocytes are mediated by either quantitative differences in the expression of genes also found on other members of the fibroblast lineage or the qualitative expression of genes unique to the synoviocyte sublineage. The genes identified by the subtraction library method as differentially expressed in RA synoviocyte cultures are candidates for comprising the fibroblast lineage intimal synoviocyte phenotype (20). These include differentially expressed chemokines, such as SDF-1, connective tissue matrix components like biglycan and lumican, adhesion molecules like VCAM-1 (Fig. 37.6), and other molecules of a less clear function in the synovial tissue, such as SLIT-3, and semaphorin 3C, MAC2-binding protein, insulinlike growth factor binding protein-5 (IGFBP-5), IFN-inducible 56-kd protein, and IFN-induced 71-kd 2'5' oligoadenylate synthetase (Table 37.3). Additionally, one gene that was not homologous to any known gene was overexpressed in RA synovial fibroblast lineage cultures (ML2115).

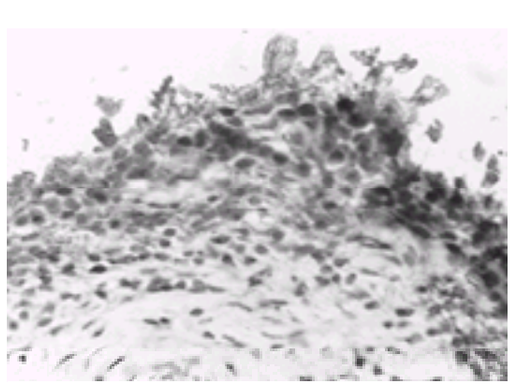

Figure 37.6. Rheumatoid arthritis. The synovial lining hyperplasia stained for vascular cell adhesion molecule-1 (VCAM-1).

TABLE 37.3. Genes Expressed by Fibroblast Lineage Synoviocytes[a]

Edwards and colleagues identified VCAM-1 as being expressed by normal fibroblast lineage intimal synoviocytes (292,319), and this observation supports the interpretation that the remaining genes found by the subtraction method are characteristic of fibroblast lineage intimal synoviocytes. In addition, several other genes were identified as being selectively or more highly expressed by fibroblast lineage intimal synoviocytes, either from staining patterns in normal or diseased synovial membranes or from cultured cells. These include other chemokines, such as IL-8, RANTES, MCP-1 (20,150,320,321), cytokines like TNF-a, IL-1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) (98,99,322,323), IL-11 (324,325), metalloproteinases and other proteinases (94,326,327), adhesion molecules like ICAM-1 (328,329,330,331 and 332), integrins (333) and CD44 (331,334,335), and costimulatory molecules like CD40 (336) (Table 37.3).

GENES EXPRESSED BOTH IN FIBROBLAST LINEAGE INTIMAL AND SUBINTIMAL SYNOVIOCYTES

Numerous genes were similarly expressed in the RA and OA synovial fibroblast cultures and likely are genes expressed by both cultured fibroblast lineage intimal and subintimal synoviocytes. Among these genes are some involved in cellular and matrix turnover, such as collagenase IV, genes involved in the inflammatory response like manganese superoxide dismutase, complement factor B, IFN-g, IEF SSP 5111, HLA-E heavy chain, and other genes with unknown function in the synovium, for example, NMB protein, aB crystallin, B94 protein, and muscle fatty-acid–binding protein. Other genes were shown to be either similarly expressed in synovial fibroblasts from patients with a variety of diseases or specifically expressed in both intimal and subintimal layer by *in situ* hybridization or staining with monoclonal antibodies (Table 37.2 and Table 37.3).

RELEVANCE OF THE DISTINCTIVE PHENOTYPE OF RA SYNOVIOCYTE CULTURES TO SYNOVIAL BIOLOGY

Based on the gene discovery findings, there are several possible explanations for the distinctive phenotype and function of these RA synoviocytes in long-term cultures (Table 37.4). Each has a different implication in terms of whether the genes found to be overexpressed in these cultures are identifiable in the normal synovium. First is the lineage hypothesis, where there is an increased number of normal fibroblast lineage intimal synoviocytes with mesenchymal stem cell characteristics, expressing normal amounts of transcripts per cell; however, because this distinct cell population is overrepresented, the absolute pattern of gene expression is increased (19). The distinctive phenotype observed in these cultures would be the normal phenotype of the fibroblast lineage intimal synoviocyte found in the normal joints in all individuals (20). The differences in culture phenotype between inflammatory and noninflammatory synovitis would simply reflect the increased proportion of fibroblast lineage intimal synoviocytes compared with subintimal synoviocytes in the starting culture material obtained from a joint with intimal hyperplasia. In support of this hypothesis is the pattern of gene expression seen in synovial fibroblast clones generated from RA and OA: High levels of SDF-1 and bone morphogenic protein receptors (BMPR) that characterize mesenchymal stem cells were seen more often in clones generated from RA than from OA synovial fibroblast cell cultures. Yet several clones originated from OA had a high level of SDF-1 and BMPR gene expression, which was interpreted as clones representing the normal number of intimal mesenchymal stem cell–like synoviocytes (R. Winchester, unpublished observations). This explanation possibility remains the most likely one. The second hypothesis is the overexpression phenotype in which the starting phenotype of the individual's prearthritis intimal synoviocytes is due to genetically determined increased expression of a gene that predisposes to immunologically mediated arthritis. In the third hypothesis, the phenotype could be a consequence of a disease-specific sustained modulation in gene expression in the intimal and subintimal fibroblast lineage cells of the joint, where the long exposure of synovial fibroblasts to increased amounts of proinflammatory cytokines would lead to a persistent state of cell activation with increased cytokine and oncogene expression (298,299,302,337). Although this hypothesis cannot be excluded, it is unlikely that cells would maintain a cytokine–cytokine receptor dependent phenotype in the absence of that cytokine. The fourth hypothesis suggests that cells could be primarily “transformed,” as suggested by Trabandt and colleagues (338) where the disease of RA results from an immune response against the agent responsible for the transformation, a possibility for which there is little support.

Normal intimal cell phenotype
Differences represent different percentages of intimal versus subintimal cells in the synovial tissue
Normal intimal cell phenotype is dependent on genetic polymorphisms in arthritis susceptibility genes
Disease-specific sustained modulation in gene expression
Local paracrine regulation
Phenotypic “imprinting”
Transformed cells
Secondary to unidentified viral infection

TABLE 37.4. Four Possible Explanations for the Distinct Phenotype of Rheumatoid Arthritis Cultured Synovial Fibroblasts

GENOMIC DISSECTION OF THE FIBROBLAST LINEAGE INTIMAL SYNOVIOCYTES PHENOTYPE

Several susceptibility loci have been identified both in rodents and humans, as discussed already. Although it is not known which genes account for those susceptibility loci, several map to genomic regions that contain some of the genes discussed in this chapter that are differentially expressed in synovial cells from RA patients (Table 37.5). Therefore, some of these genes are candidate susceptibility genes and may be involved in the regulation of the synovial fibroblast function. We hypothesize that the inheritance of certain alleles may facilitate transcription, inducing increased expression of certain genes in tissues such as the synovium. Additionally, some of those genes may be involved in the regulation of disease severity as well (8,9,13,339,340).

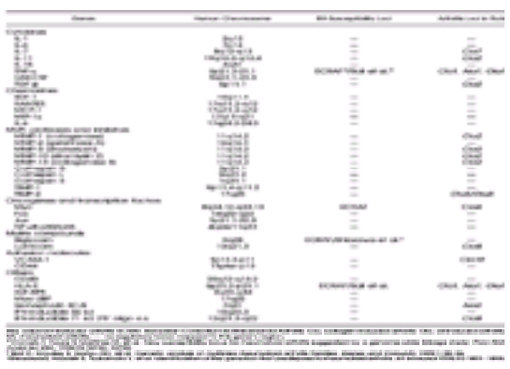

TABLE 37.5. Genes Differentially Expressed in Cultured Rheumatoid Fibroblastoid Intimal Cells, Their Chromosomal Location, Their Relationship with Susceptibility Loci Identified in Linkage Studies in Rheumatoid Arthritis and With Homologous Loci Regulating Experimental Arthritis in Rats

Role of Molecules Identified in Fibroblast Lineage Synoviocytes Involved in the Proinflammatory Response and Intensification of Synovitis

CHEMOKINES, CYTOKINES, AND GROWTH FACTORS

Chemokines

In addition to SDF-1 (20), numerous other chemokines have been identified as likely to be overexpressed by the fibroblast lineage intimal synoviocytes, including IL-8 (341), GRO-a (342), MCP-1 (320,341), and MIP-1a (152). Expression of chemokines and their receptors has been demonstrated to have a critical role in the regulation of the attachment of leukocytes and endothelial cells and in their passage into the tissues (308,343). Among chemokines, SDF-1 is one of the most efficacious in T-cell and monocyte migration (308). Both CD4+ and CD8+ cells, as well as CD45RA+ naive and, less effectively, CD45RO+ memory T-lymphocyte subsets in peripheral blood are subject to SDF-1 chemoattractive effects (344). Similarly, monocyte-lineage dendritic cells acquire CXCR4 on induction with GM-CSF and IL-4 (345). Several antichemokine strategies targeting RANTES (153) and MCP-1 (346) have been used in animal models of arthritis with good results and may prove promising strategies for clinical studies.

Cytokines and Growth Factors

There is an increased expression of several cytokines, including TNFa, IL-1, IL-6, IL-11, IL-15, GM-CSF, transforming growth factor (TGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) by fibroblast lineage synoviocytes (94,98,99,323,324 and 325,347,348,349,350,351 and 352). These cytokines importantly influence the activation state of the endothelium by affecting the expression of particular genes. The migration of a given inflammatory cell into the synovium is not a random event but one determined by the prior immunologic history of the particular cell as well as that of the endothelium. The increased production of certain cytokines like IL-1 and TNF-a activates the endothelium and initiates overexpression of certain adhesion molecules enhancing directed cellular egress. Accordingly, the activation status of the endothelium in concert with chemokine production could greatly facilitate the localization of inflammatory and autoimmune cells to the synovial membrane, further perpetuating the disease process and tissue injury. The hyperplastic RA synovium, with its increased number of fibroblast lineage synoviocytes, appears to have a critical role in the chemotaxis of monocytes, T-, and B-lymphocytes into the synovial membrane, as evidenced by their increased production of proinflammatory molecules involved in the activation of endothelial cells like TNF, IL-1, and IL-6 as well as chemokines like IL-8 (341), RANTES (150,151), GRO-a (342), MCP-1 (320,341), MIP-1a (353), MIP-1b (354), IP-10 (149), and SDF-1 (20) (Table 37.3).

MOLECULES INVOLVED IN CELL–CELL INTERACTIONS AND OTHERS OF UNCLEAR FUNCTION IDENTIFIED IN FIBROBLAST INTIMAL

Several molecules expressed by fibroblast lineage intimal synoviocytes appear to be candidates for mediating cell–cell interactions involved in the histogenesis of the normal synovium, including monocyte lineage–fibroblast lineage and fibroblast lineage–fibroblast lineage synoviocyte interactions. Some of these genes have a well-defined role in cell–cell interactions, and others have the potential to act as cell interaction receptor–ligand systems but also have other actions.

Among the well-recognized adhesion molecules and receptors differentially expressed in the rheumatoid fibroblast lineage intimal synoviocytes are VCAM-1 and MAC-2BP, also termed *90k tumor associated protein*. VCAM-1 was described previously as markedly increased on RA synoviocytes (292,355), and it binds circulating monocytes and lymphocytes expressing the a_4b_1 [very late antigen-4 (VLA-4)] integrin. Additionally, VCAM-1 may have a role in the formation of germinal centers in the rheumatoid inflammatory synovium. Based on these data, it appears that normal fibroblast lineage intimal synoviocyte cells can support the development of germinal centers, B-cell migration, and affinity maturation. Furthermore, several additional molecules produced by the synoviocyte can interact to facilitate other aspects of B-cell development, such as IL-6. MAC-2BP binds to the macrophage-associated lectin Mac-2 (galectin-3) (356,357) and is increased in the serum of cancer and human immunodeficiency virus (HIV)–positive patients. MAC-2BP also binds to the monocyte CD14 structure in the presence of LPS and LPS-binding protein (358). Binding of MAC-2BP to these receptors induces monocyte lineage cells to secrete IL-1 (359).

The overexpression of the semaphorin 3C, a human homolog of mouse semaphorin E by synovial fibroblasts (20,360), is intriguing because the semaphorins are a family of transmembrane signaling and secreted guidance glycoprotein molecules that are implicated in directing axonal extension and operate broadly in neuronal patterning (361). Neuropilin, the receptor for semaphorin, is expressed on vascular endothelial cells, and neuropilin expression is upregulated by TNF-a and is implicated in angiogenesis as a coreceptor of vascular endothelial growth factor (VEGF).

Molecules Involved in Cell–Matrix Interaction Expressed by Fibroblast Lineage Intimal Synoviocytes

Several matrix component genes exhibited a pattern of expression suggesting that they are constitutively produced by fibroblast lineage intimal synoviocytes. Lumican was identified as likely to be constitutively expressed by fibroblast lineage intimal synoviocytes (20). Although lumican's role in the synovium is not understood, its role in corneal transparency (362) and in the inhibition of macrophages adhesion to intact corneal keratan sulfate proteoglycans are of interest. Biglycan, another gene likely to be constitutively expressed by fibroblast lineage intimal synoviocytes, is a dermatan sulfate-proteoglycan. It is both induced by TGF-b and binds TGF-b (363), suggesting that it may downregulate TGF-b activity by sequestering this growth factor in the extracellular matrix.

Hyaluronan, which is an abundant constituent of the extracellular matrix and is especially increased in the synovial fluid, may modulate local chemokine production through the interaction between its low-molecular-weight forms (fragments) with CD44 (see later) (364).

Fibroblast lineage intimal synoviocytes strongly express IGFBP-5 (20), an important regulator of fibroblast growth that increases IGF-1 binding to the fibroblast membrane by attaching to the extracellular matrix proteins, including types III and IV collagen, laminin, and fibronectin (365). The increased expression of IGFBP-5 seen in scleroderma skin fibroblast (366) and alveolar rhabdomyosarcoma cell lines (367) suggests that this gene is involved in the regulation of mesenchymal cellular functions, including proliferation and perhaps the hyperplasia seen in RA synovium.

INITIATION OF HYPERPLASIA AND DEVELOPMENT OF SUSTAINED ARTHRITIS

In addition to the likely role of the fibroblast lineage intimal synoviocyte in facilitating the afferent limb of the development of the autoimmune response underlying synovitis, the intima also plays a major part in the loss of function and joint destruction that characterize fully developed RA. A feature of the rheumatoid synovium is the marked hyperplasia of the lining layer and the apparent invasion and destruction of cartilage and other joint structures by the mesenchymally derived fibro-blasts and the bone marrow derived monocyte lineage cells (Fig. 37.6). The changes in the lining during hyperplasia include a massive increase in the number of fibroblast lineage intimal synoviocytes and an altered cell–cell relationship with the monocyte lineage synoviocytes.

In RA, it is unknown whether initiation of the autoimmune response and its localization to the joint occur in the setting of entirely normal intima or whether minor degrees of nonspecific hyperplasia could play a role in localizing an immune response into the joint through the repertoire of immunologically relevant molecules expressed by these cells. Hyperplasia could be initiated by a nonspecific minor traumatic event or even driven by a local immune response to a common pathogen, and the constitutive production of chemokines might provide a nonantigen-specific mechanism for localizing potential pathogenic immune responses to the joint. In other words, the production of such chemoattractant molecules would be part of the normal function of the fibroblast lineage intimal synoviocytes and would have increased transcription when either activated or subjected to an inflammatory “imprinting” or when the number of cells increases. The unusual behavior of fibroblast lineage intimal synoviocytes in culture may reflect this behavior.

Hyperplasia appears to be an intrinsic response of intimal synoviocytes to injury and healing. The synovial membrane undergoes this striking change in its form and in its pattern of gene expression, transforming from a nutritive tissue into one that is the central agent of joint destruction. The destruction occurs through expression of enhanced levels of degradative enzymes and through secretion of cytokines that can act to alter the pattern of gene expression in the chondrocyte or osteocyte. This alteration in the synovium involves a massive influx of monocyte-lineage cells and extensive neovascularization as well as marked hyperplasia of the intimal synoviocytes, which is likely mediated, in part, by the genes described already. Three sets of biologic events are evident: (a) the intrinsic biology of the fibroblast lineage intimal synoviocyte, where increased cell number simply is reflected as increased local concentration of mediators and cell surface molecules; (b) the pathways of mutual interaction of fibroblast lineage and monocyte lineage intimal synoviocytes; and (c) paracrine influences of the products of the autoimmune response on the intimal synoviocytes. It is possible that the loss of normal cell-matrix signals due to hyperplasia and its replacement by more extensive cell-cell receptor interactions result in reverse signaling that leads to a perpetuation of the hyperplasia.

Several cytokines produced in the synovium are candidates for mediating the paracrine effect that results in hyperplasia. IL-1 induces the expression of more IL-1a and IL-1b, in a positive feedback loop (368). IL-1 also induces transcriptional activation of protein kinase C and, by a separate pathway, induces the synthesis of prostaglandin E2 (PGE_2) (369). Fibroblast lineage intimal synoviocyte is one of the sources of increased amounts of this cytokine (370) in that macrophages also produce them. These two pathways initiated by IL-1 also converge to regulate the transcriptional activation of stromelysin. IL-1 induces fibroblast lineage synovial cell

lines to increase IL-6 gene expression by an incompletely defined pathway that is suppressed by corticosteroids (351). Similarly, IL-8 expression is induced by the addition of IL-1 or TNFa (371), although to much lower levels than those elaborated by synovial monocyte lineage cells from RA samples. IL-1 induces the expression of GM-CSF mRNA with a maximum at 4 hours (349). IL-1 also induces the production of fibronectin and types I and III collagen (372).

Fibroblast lineage intimal and subintimal synoviocytes and synovial monocytes and macrophages produce TNF-a, and among several functions, TNF-a is capable of inducing fibroblast lineage intimal synoviocytes cellular proliferation, MMPs (349), and cathepsin production (373), enzymes involved in tissue degradation (see below). As with IL-1, the addition of TNF-a to fibroblast lineage cell cultures induces the expression of GM-CSF (349). Several studies documented the importance of TNF in the development of arthritis, including a TNF-transgenic mouse that develops chronic arthritis (374), and the significant improvement of disease with agents targeting TNF (375,376 and 377); see the section on therapy.

Interleukin-6, a cytokine with effects on B-cell differentiation, is constitutively expressed in synovial fibroblasts obtained from RA (322,323). This cytokine appears critical to the development of arthritis in mice since gene-targeted mutation prevents disease (378). IL-6, particularly in the presence of soluble IL-6 receptor, induces synovial fibroblast proliferation and IL-1 production (379). IL-6 upregulates osteoclast activity and may mediate the development of bone loss and erosions in RA. On the other hand, IL-6 is a potent inducer of tissue inhibitor of metalloproteinase-1 (TIMP-1) (380,381), an inhibitor of MMPs, raising the complexity of its functions as potentially protissue or antitissue damaging, perhaps dependent on the other synovial environment conditions.

Hyperplasia could be explained by abnormalities in the regulation of the normal cell turnover, with increased cellular proliferation or decreased cell death. Mitotic figures have been reported to be uncommon in sections of the RA synovium, and therefore abnormalities in apoptosis regulatory pathways were hypothesized. Early studies described an increased number of apoptotic cells in the rheumatoid synovium (382,383). Additional studies described somatic mutations of p53 tumor suppressor gene in RA fibroblast lineage synoviocytes (384), and the inactivation of p53 in synovial fibroblasts was capable of changing the proliferation rate of the cells (385). Still, it remains unclear what percentage of fibroblast lineage synoviocytes have such mutations and how relevant are those mutations, if rare, in the regulation of synovium hyperplasia. Fas is expressed by these cells, and anti-Fas antibodies are capable of inducing apoptosis of the fibroblast lineage synoviocytes, indicating that this pathway is preserved in RA (383). TGF-b, which is highly expressed in the RA synovium, decreases Fas expression and could contribute to a decreased rate of cell death (386). Anti-Fas antibodies have been used successfully to treat murine autoimmune arthritis in the HTLV-I Tax-transgenic mouse, a spontaneous model of arthritis that shares several similarities with RA. It is conceivable that either somatic mutations in critical cell-cycle regulatory molecules or an antiapoptotic effect from cytokines or other factors present in the RA synovium could contribute to the synovial hyperplasia.

Fibroblasts from hyperplastic RA synovial tissue have increased expression of transcription factors like NFkB (387) and AP-1 (388), which regulate the transcription of several cytokines, including TNF and IL-1 as well as cell proliferation and programmed cell death. Recent studies by Miagkov et al. using the streptococcal cell wall–induced arthritis (SCWIA) and PIA, two well-established models used to gain insight into the pathogenesis of RA in Lewis and DA rats, respectively, described the expression of NF-kB as coinciding with the onset of arthritis. They also noted overexpression of NF-kB-protected synovial fibroblasts from TNF and FasL-induced cell death (389). Additionally, the same study concluded that NF-kB inhibition increased the rate of synovial fibroblast apoptosis and protected animals from developing arthritis, establishing an additional link between NF-kB expression and hyperplasia. It has been demonstrated that AP-1 regulates IL-1 transcription in the RA synovium (388,390) and also that AP-1 inhibition prevents joint destruction in CIA in mice (391). Transfection of synovial fibroblasts with jun D inhibited the proliferation and production of proinflammatory cytokines and MMP by RA synovial cells, mainly as a result of the inhibition of their transcription via downregulation of the AP-1 transcription factor (392).

Monocytoid Infiltration

The biology of the normal synovial lining includes a specific cytoarchitectural relationship between monocyte lineage cells and fibroblasts. In RA, there is an abundance of monocytoid lineage cells occupying sites rather diffusely distributed through the subsynovial tissues or locally enriched along the outer layer of the synovial lining. These cells exhibit varying and often marked degrees of differentiation and activation. It is likely that some or all of these monocytes are activated by lymphokines and that, when activated, they release degradative enzymes, monokines, and other factors.

Monocytes and macrophages are a major source of IL-1, IL-6, and TNF, cytokines involved in the synthesis of MMPs, the principal enzymes involved in cartilage degradation. Also, these cytokines are involved in angiogenesis, endothelial cell activation, and upregulation of adhesion molecules, which facilitate the migration of leukocytes out of the vessels and into the synovium. In fact, therapeutic strategies aiming to antagonize IL-1 with IL-1 receptor antagonist has been effective in clinical trials, and anti-TNF-a strategies have obtained approval from the Food and Drug Administration (FDA) because of its high efficacy (see section on therapy). Furthermore, mice with a disrupted IL-6 gene are either resistant to (378) or develop milder and delayed-onset disease (393) in CIA, making this molecule another potentially good target for drug development. Additionally, a recent study using a combined strategy of subtraction and semiquantitative reverse transcriptase (RT)-PCR identified several known cytokines genes, including IL-1, TNF, and IL-6, chemokines, other genes, and yet unknown genes that were differentially expressed in the activated peripheral blood monocytes from RA patients (394). The better characterization of these known and yet unknown genes is likely to contribute to a clearer understanding of the role of monocytes in the pathogenesis of RA.

Several Fc receptors promote phagocytosis and the release of proinflammatory mediators from monocytes and macrophages. The Fcg receptor IIB, a gene capable of suppressing responses from macrophages, B cells, and mast cells has been shown to be critical in resistance to autoimmune arthritis, CIA, in mice (395). The introduction of the Fcg-RIIB gene knockout into the CIA-resistant strain C57BL/6 ($H\text{-}2^b$) rendered these mice highly susceptible to this erosive arthritis and with increased levels of anticollagen antibodies, similarly to those seen in a highly susceptible strain, the DBA/1. It was not clear from the study whether this increased liability to disease is accounted specifically by one single-cell lineage or whether it involves all different cells that express this gene. Again, this demonstrates the importance of regulatory genes in preventing the development of an autoimmune response or its localization to the target organ or tissue.

Neutrophil

The synovial fluid contains an abundance of neutrophils in varying stages of activation. There is abundant evidence of fixation and activation of complement and immune complexes as well as various proinflammatory cytokines capable of inducing and modulating this response. Neutrophils are largely absent from the synovial tissues, but their capability to produce a number of proinflammatory molecules, such as prostaglandins, nitric oxide, and oxygen radicals among others, suggests that they are indeed involved in the joint destructive processes. The extent of this exudative reaction varies considerably among the joints, between individuals, and in the course of the disease.

Blood neutrophils are found in a state of heightened activation. There is an increased proportion of morphologically polarized cells that correlates with enhanced expression of the CD11b/CD18 (CR3) integrin (396). Similarly, using different methodology, activated neutrophils were demonstrated in synovial fluid effusions (397,398). It remains to be considered an alternative mechanism if cytokines derived from other cells responding to the proinflammatory state in RA also activate neutrophils. Support for this pathway was provided by finding elevated IL-8 levels in synovial fluid (399). Adhesion of neutrophils to inflamed endothelium and their subsequent diapedesis and trafficking to the joint space are also characteristic events in the rheumatoid joint. This is a stepwise process (400) initiated by retardation of free-flowing neutrophils by E and L selectins, which induces the cells to roll slowly along the endothelium. Because the circulating neutrophils in the blood of patients with RA are activated, this process is facilitated compared with that in normal subjects.

Evidence to suggest an important role for neutrophils in arthritis also comes from mice that lack GM-CSF and studies of Felty's syndrome patients. GM-CSF knockout mice are resistant to CIA, despite evidence for autoimmune responses to collagen (401), demonstrating that, in addition to the autoimmune response, processes involved in the localization of this response to the synovium are also critical. Patients with Felty's syndrome, a form of RA that has neutropenia and mild or no arthritis (see later), reportedly have flareups of arthritis that develop as the neutropenia resolves.

The chemokine IL-8, derived from monocytes responding to the proinflammatory state in RA, is another pathway for activating neutrophils (402,403). Support for this latter pathway is provided by the finding of elevated levels of IL-8 in synovial fluid (399), which correlates directly with the number of infiltrating neutrophils (404). Additionally, yet unidentified factors produced by synovial fibroblasts have been demonstrated to maintain the viability and to augment the function of cultured neutrophils (405).

Mast Cells

Mast cells are a conspicuous component in the rheumatoid synovium (406) and are also found in the synovial fluid. The extent of the mast cell infiltration parallels the degree of lymphocytic infiltration (407). The overall mean synovial mast cell density can be as high as 40.3 cells/mm^2, with regional densities of 60.6 and 34.2 mast cells/mm^2 in the superficial and deeper synovial layers, respectively (408). Gotis-Graham et al. found that the mast cell triptase (MCT)–only positive subset predominates, outnumbering MCT and chymase positive (MCTC) by 3:1 (408). These same researchers also found a significant correlation between the histologic inflammation index and the MCT density. Stem cell factor (SCF) produced by fibroblast lineage synoviocytes induces MC chemotaxis. Furthermore, TNF-a, which is abundant in the rheumatoid synovium, augments SCF expression in fibroblast lineage synoviocytes (409).

The rheumatoid synovial fluid produces mast cell growth factors (157). RA, but not OA, synovial mast cells express significant amounts of C5aR (CD88) (410) and

release histamine in response to anti-IgE (410, 411) and to rHuC5a (410). Mast cell degranulation induced artificially in rheumatoid synovial explant cultures consistently resulted in an increased production of PGE_2 and had variable effects on the quantification of released MMPs, suggesting that mast cells may contribute to the regulation of specific MMPs (412). Studies with cultured fibroblast lineage synoviocytes treated with histamine further corroborated the preceding findings by inducing increased amounts of MMP-1, a response that was mediated by H1 receptors (413) and suggesting that mast cells may participate in the regulation of the proinflammatory processes that ultimately lead to cartilage and joint destruction.

Angiogenesis

Angiogenesis is a prominent feature of the rheumatoid synovium and has been the subject of numerous reviews (414). Several factors produced by the synovium and synovium-infiltrating cells induce angiogenesis, including TNFa, IL-1, IL-6, IL-8, IL-15, GRO-a, PECAM, VEGF, and integrins like a_vb_4 and a_vb_3. Several other angiogenic molecules are produced by the rheumatoid synovium. PDGF is structurally related to VEGF and is also an angiogenic factor produced by the rheumatoid synovium. bFGF and aFGF have been identified in the rheumatoid synovium and are potent angiogenic factors. bFGF is more specifically produced by fibroblast lineage synoviocytes (354). In addition, bFGF receptor is also expressed in high amounts in T-cell-rich areas of the rheumatoid synovium (415). IGF-1 is present in the RA synovial fluid and induces endothelial proliferation and chemotaxis (416).

Antiangiogenic strategies have been developed, and one that antagonized a_vb_3 was highly effective in the treatment of a rabbit experimental model of arthritis (417). It will be interesting to see the efficacy and safety of this and other new antiangiogenic agents that are going into clinical trials, at this moment primarily in cancer, in arthritis research.

TISSUE INJURY MEDIATED BY FIBROBLAST LINEAGE INTIMAL SYNOVIOCYTES

The transition of the synovial membrane in RA from a nutritive tissue into one that is the central agent of joint destruction is one of the most troublesome events in the disease. The biologic significance of this transition is heightened by recent observations that suggest the intimal synoviocytes responsible for the destruction of bone and cartilage are closely related to the mesenchymal stem cells, the progenitors of the same osteocytes and chondrocytes. The tissue destruction is mediated in part through expression of enhanced levels of degradative enzymes, capable of altering matrix structure and, likely, through secretion of cytokines that act to alter the pattern of gene expression in the chondrocyte or osteocyte, leading to tissue disruption.

Cartilage injury likely proceeds by two distinct mechanisms.The first is an indirect mechanism in which cytokines released by the synovial lining cells and infiltrating mononuclear cells activate chondrocytes to a pattern of gene expression that results in remodeling and degradation of the cartilage matrix; for example, IL-1 stimulates chondrocytes to release degradative enzymes (418,419). The second is a direct mechanism in which metalloproteinases and other enzymes released by the synovial lining cells and perhaps the infiltrating macrophages directly act to digest the matrix (129, 420). The junction between the hyperplastic synovium and the cartilage appears to be the principal early site of tissue damage and erosive changes. Assessment of the rate and character of cartilage injury has been determined by measuring the fine structure of the products of proteoglycan fragmentation. The glycosaminoglycan-rich region of the core protein predominates during the early phase of cartilage injury before there is significant damage evident on conventional radiographs (421). Later, when frank radiographic changes are evident, the joint fluid contains an abundance of hyaluron-binding domains and lesser amounts of the glycosaminoglycan-rich region of the core protein.

Immune Processes and their Consequences

The different elements present in the synovium interact with each other at different levels. It was first postulated by Krane and Dayer that the lymphocytic response initiates the cascade of immune interactions and cytokines by acting directly on the target fibroblast cells and indirectly on them by activating monocytelike cells to release additional cytokines. As discussed previously, the T-cell compartment does not secrete overwhelming amounts of lymphokines; however, how much cytokine is enough cytokine to sustain the pattern of activation and injury is an unanswered question. Considerable attention has been directed to the major role played by cells in the monocyte compartment in mediating aspects of the cytokine-driven process leading to tissue destruction (422). An important role for the monocyte in producing angiogenic factors has been postulated that emphasizes the multiple feedback loops on which the immune response of RA is built (97). Still more recently, the pattern of gene expression in the fibroblast lineage synoviocyte was recognized to be persistently modulated, either reflecting exposure to inductive components in the inflammatory milieu or due to intrinsic characteristics of this cell lineage. As discussed already, the hyperplastic synovium in RA would lead to an overall increased expression of certain genes involved in chemotaxis, cell activation (cytokines), and synthesis of degradative enzymes (Table 37.3) (19,298,423).

Role of Cell–Matrix Interaction

CD44, one of the critical adhesion molecules for leukocyte extravasation, is also a hyaluronan binding protein. Hyaluronan is an abundant constituent of the extracellular matrix and is especially increased in the synovial fluid. Both high- and low- (fragments) molecular-weight forms bind to CD44. In alveolar macrophages, lower-molecular-weight hyaluronan fragments induce the production of chemokines such as IL-8 and MIP-1a through its receptor CD44; the high-molecular-weight fragments inhibit chemokine production (364). CD44 is predominantly expressed by fibroblast lineage intimal synoviocytes (334,335). Although this pathway has not been extensively studied in fibroblast lineage synoviocytes, one could envision similar effects in the synovium. It is also conceivable that a similar concept may apply to other matrix components. For instance, if intact and large matrix components predominate in the synovium, representing absence of injury, a chemokine/cytokine inhibitory signal would predominate. On the other hand, when traumatic or inflammatory injury occurs, signaling through small hyaluronan fragments/CD44 and maybe through biglycan, lumican, or other component fragments and other receptors would activate a proinflammatory response to remove cellular debris or fight infection. Furthermore, blocking antibodies targeting CD44 prevent the development of arthritis in a murine experimental model (424), further supporting a major role for this molecule in disease pathogenesis.

Proteinases, Proteinase Inhibitors, and Cartilage Injury

MATRIX METALLOPROTEINASES

This class of enzymes consists of collagenase, stromelysin, and gelatinase, which, in concert, can attack all elements of connective tissue, thus participating in physiologic remodeling or pathologic destruction. All are synthesized as proenzymes and are activated by proteolytic cleavage. These enzymes, their transcription regulation, cellular sources, and role in tissue destruction were discussed in part in the preceding section. These molecules are particularly interesting in regard to the mechanism of synovitis because, first, they are induced from low basal levels by a variety of cytokines present in the rheumatoid synovium (IL-1, TNF, and oncostatin M), and they are also constitutively expressed by the lining cells. Second, the cells that produce them are centrally involved in rheumatoid synovitis, namely monocytes, neutrophils, fibroblasts, chondrocytes, and endothelial cells. There is an active effort in pharmaceutical companies to develop MMP antagonists.

Collagenases

Collagenases cleave fibrillar (undenatured) collagens types I, II, and III at one specific site in their triple helical domain. Along with other metalloproteinases, they digest type VII and X collagens. Collagenases are elaborated by mesenchymal cells and by neutrophils and monocytes. Neutrophils store collagenase in granules. The mesenchymal cell variety of collagenase has been strongly implicated in synovitis by the finding that its mRNA is expressed at high levels in the synovial lining (425). The identification of abundant collagenase at the protein level in the vicinity of erosions, but not in equivalent abundance in other regions of the synovium, suggests that it may play a special role at these sites (426).

Stromelysins

The primary action of stromelysin is to cleave proteoglycan core and link proteins, fibronectin, elastin, and procollagens I, II, and III, thereby mediating the remodeling of most of the matrix components other than collagen. Stromelysin also participates in collagenase activation (427). Stromelysin mRNA is strongly expressed in RA fibroblast lineage intimal synoviocytes cells (423,425). Immunohistochemical staining reveals that stromelysin protein is present in fibroblasts and endothelial cells (369) as well as in monocyte lineage lining cells using *in situ* probing (327). Using *in situ* hybridization, collagenase mRNA was colocalized with that for stromelysin, suggesting that the production of these two metalloproteinases is coordinated (327,425). The fact that disruption of the stromelysin 1 (MMP-3) gene did not protect mice from developing cartilage destruction in murine CIA suggests that redundant or compensatory functions exist among MMPs or between MMPs and other genes (428).

Gelatinase

Gelatinase cleaves denatured collagens types I, II, and III and digests native types IV, V, VII, X, and XI. Levels of gelatinase mRNA vary considerably from sample to sample of synovial lining but are commonly elevated in both OA and RA.

Cathepsins

Cathepsins are cysteine proteinases that are produced by the rheumatoid synovium and induced by TNF-a (373). Cathepsin L is one of the major Ras-induced proteins in Ras-transformed cells and is expressed in half the rheumatoid synovia, being localized to the fibroblast lineage intimal synoviocytes (429). Cathepsin B was identified both in fibroblast lineage synoviocytes from OA and RA, likely representing gene expression by both the intimal and subintimal fibroblast lineage synoviocytes (20).

Tissue Metalloproteinase Inhibitors

The activated metalloproteinases bind stoichiometrically to a_2-macroglobulin in the plasma, but their major regulation after activation is through the two tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2 (430,431). These two homologous molecules are secreted in a highly regulated manner by cells elaborating metalloproteinases. The TIMPs also stoichiometrically bind to the metalloproteinases (430). It appears that the ratio of synthesis of TIMP to specific metalloproteinase is a critical index of the potential of a tissue to mediate matrix remodeling. In cultured fibroblast lineage synoviocytes from OA patients, there is a much higher average ratio of TIMP to stromelysin than is found in RA (423). Although IL-6 is thought to be a proinflammatory cytokine critical for the development of autoimmune arthritis in rodents (378), it does not appear to induce MMP expression directly (432). On the contrary, IL-6 can be a potent inducer of TIMP-1 (380,381). Although oncostatin M can induce MMP expression, particularly in the presence of IL-1 (433), it can also induce TIMP-1 expression much as LIF does (381,434), further demonstrating the complexities of the molecular interactions in different tissue environment conditions.

OTHER FEATURES OF RHEUMATOID ARTHRITIS

Pregnancy-associated Remission

DISEASE BEHAVIOR DURING PREGNANCY AND POSTPARTUM

Another phenomenon relating to the female RA patient is the occurrence of significant and occasionally striking improvement or remissions in disease activity during pregnancy (435,436 and 437). Whereas pregnancy-associated improvement usually appears in the first trimester, the most striking decrease in disease activity occurs in the third trimester. Recently, in a study by Barrett et al. (438), the largest prospective study of pregnancy in RA done so far, no consistent pattern could be identified. These researchers found that 16% of 95 RA patients achieved remission during pregnancy, two thirds reported significant improvement in swelling and pain, but 16% to 20% reported either no changes or disease worsening. RF-negative women were more likely to achieve remission than RF-positive women (28% versus 10%). Swollen and tender joint counts during pregnancy were in general low (counts of only 2).

A related observation is that there is a reduction in the incidence of RA during pregnancy, which is compensated by a numerically greater incidence of disease appearing in the 3 months postpartum (439). Interestingly, prepartum RF positivity appears to be a risk factor for the onset of RA in the postpartum period, as suggested by Iijima et al. (440). These investigators followed 410 healthy pregnant subjects prospectively and found that two patients, 22% of the RF positive and none of the RF negative patients, developed RA in the postpartum period.

Besides the increased risk for disease onset in the postpartum period, RA patients who experienced improvement in disease activity during pregnancy commonly notice a flareup in the postpartum period. The same study described by Barrett et al. (438) also followed up on pregnant RA patients through the postpartum period. The health assessment questionnaire (HAQ) scores, validated questionnaires that are used to measure health status, did not differ between the last trimester and the first and sixth month postpartum; however, joint counts were significantly higher in the postpartum period. At 6 months postpartum 66% and 77% of the women reported worsening of their joint swelling and pain, respectively; most of that deterioration occurred by the first month (438).

IMMUNOLOGIC AND HORMONAL MECHANISMS IN DISEASE IMPROVEMENT DURING PREGNANCY

Numerous mechanisms are likely involved in the disease improvement seen in some RA patients, but it remains unclear why some patients improve and others do not. Possible mechanisms include (a) changes in the production of different cytokines with a shift toward the predominance of Th2 cytokines; (b) an immunosuppressive effect in cellular immune responses mediated by estrogens; (c) a pregnancy-induced down-regulation of the synovial production of certain proinflammatory cytokines, growth factors, and proteinases; and (d) HLA differences between mother and child that would contribute to a downregulation of the immune responses through yet uncharacterized mechanisms. Although there is evidence to support a role for all four of these mechanistic processes, none has been thoroughly studied or understood.

The Th2 cytokines, particularly IL-4, increase as pregnancy progresses (441). Additionally, there is constitutive production of Th2 cytokines (IL-4, IL-5, and IL-10) in the maternal–fetal interface (442), and the human placenta at term expresses high levels of IL-10 (443). A shift in the cytokine balance to Th2 occurs during pregnancy and is probably essential for the pregnancy to proceed, as suggested by murine studies in which the administration of Th1 cytokines induced fetal resorption [for review, see Elenkov et al. (444)]. IL-2 production by peripheral whole blood from pregnant RA patients is decreased, more so in the third trimester, and soluble TNF receptors p55 and p75, which bind and interfere with TNF function, were increased, again most significantly in the third trimester, whereas TNF-a and IL-1 b remained unchanged (445). Thus, the suppression of Th1 cytokines, which is essential for fetal survival, may be a central factor in the immunomodulatory function of pregnancy in the disease activity of patients with RA, shifting the immune response from Th1 to Th2.

Estrogen production increases during pregnancy, and its immunoinhibitory functions (see preceding discussion on gonadal hormones) may be involved in this cytokine shift. Conceivably, these functions operate through cytokine-independent pathways as well. In mice with CIA, estrogen given postpartum prevents the normally seen postpartum flareup of RA (446). Individual variability on the magnitude of the increase of the estrogen levels during pregnancy or the patient's immune system's capacity to produce Th2 cytokines are possible variables that could predict disease improvement or remission during pregnancy.

A third possibility was raised in a study by Hart and Reno (447), who studied the pattern of expression of 17 genes in the synovium of pregnant rabbits and controls. They found alterations in several of these genes, suggesting that pregnancy can affect the biology of the normal synovium and likely of the rheumatoid synovium as well. Although these researchers studied a few genes, a broader analysis of other synovial gene products would contribute to clarifying the spectrum of changes induced by pregnancy. Whether estrogens or nonsynovial cytokine changes induced by pregnancy account for the synovial gene expression changes remains to be determined. The basis of this difference in the effect of pregnancy was studied by Nelson and colleagues, who noted that pregnant individuals who do not experience a remission were more likely to share HLA alleles with the husband and the fetus, especially those at the HLA-DQ locus (448,449).

The fourth and particularly intriguing mechanism was proposed by Nelson et al. (448,449 and 450). Because an immune response to paternally inherited fetal HLA occurs during normal pregnancy and RA has a known HLA class II association, the hypothesis that maternal–fetal disparity in HLA alloantigens might be associated with the pregnancy-induced remission of RA was tested. Maternal–fetal disparity in alleles of HLA- DRB1, DQA, and DQB occurred in 26 of 34 pregnancies characterized by remission or improvement (76%) compared with 3 of 12 pregnancies characterized by active arthritis (25%) (OR, 9.7; $p = 0.003$). The difference between the two groups was most marked for alleles of HLA-DQA (450). van der Horst-Bruinsma et al. also found that DQA1 and DQB1 incompatibility between mother and child contributes to a favorable effect on the course of RA and may postpone the risk of RA onset during pregnancy (451). Identification of the regulatory mechanism may be challenging. For instance, in another study, simple maternal exposure during pregnancy to either fetally inherited paternal HLA-DR1 and DR4 genes or to paternal DR genes similar to their own did not appear to contribute to postpartum maternal susceptibility of RA (452). These findings suggest the possibility that immunologic mechanisms involved in maintaining the immunologically privileged status of the fetal “allograft” influence processes essential to the presence of the inflammation of RA. Clearly, delineating the mechanisms involved in the pregnancy-induced remission would provide a new lead to the therapy of this disease.

PROLACTIN AND LACTATION

The onset of RA or a flareup during the postpartum could also point to a pathogenic role of hormones produced during the lactation period. The relative risk accounted for by breastfeeding in RA susceptibility and disease severity remains controversial (453). Barrett et al. (438), in a prospective study, compared disease activity during pregnancy and at 6 months postpartum among 49 nonbreastfeeders, 38 first-time breastfeeders, and 50 repeat breastfeeders. After adjustment for several possible confounding factors, including treatment, they found that first-time breastfeeders had increased disease activity at 6 months postpartum. Although there were limitations with the self-reported strategy used, this study provided prospective evidence in support of the previous clinical impression.

It is not clear why RA patients would experience disease exacerbation during the postpartum period. Hormonal changes, including the production of prolactin, certainly are among the most obvious possibilities. In fact, even male patients with RA have higher serum prolactin levels than normal controls (454) or other chronic nonautoimmune inflammatory diseases (455). High prolactin levels significantly correlated with the duration of RA and with functional stage according to the Steinbrocker classification, but they were not correlated with the low levels of androgens observed in male patients with RA (454). In mice, administration of prolactin

during the period of induction of CIA makes the arthritis worse, but treatment at later stages has no effect (456). Furthermore, administration of the prolactin inhibitor bromocriptine to arthritic mice in the postpartum period induced a 50% reduction in disease severity compared with the clinical exacerbation of disease seen in untreated animals (457). Conceivably, prolactin could operate as an end-stage proinflammatory mechanism for accumulating events that achieve the climax in the postpartum period.

Among other immunoregulatory effects, prolactin increases nitric oxide and TNF synthesis by neutrophils (458), enhances natural killer cell function, and can increase IL-2 and IFN-g production by lymphocytes (459). Prolactin receptors are expressed in primary lymphoid organs, such as in bone marrow and thymus. In peripheral organs, all B cells and macrophages and 70% of both CD4+ and CD8+ T cells are prolactin receptor positive, further supporting the concept that prolactin may regulate the development and function of the immune responses. On the other hand, proinflammatory cytokines like TNF-a and IFN-g can decrease the pituitary production of prolactin (460), again pointing to the complexity of networks involved in the neuroimmune regulation.

Previous Use of Oral Contraceptives

A related question is whether oral contraceptive use diminishes the incidence of RA. This possibility has received considerable attention since an initial study suggested it (461), and the question of its generality and significance has been the subject of reports with differing conclusions. Brennan et al., in a review of the literature, concluded that only current use of oral contraceptive is protective for the development of RA (462). A carefully done metanalysis of 15 studies on oral contraceptives and RA found no conclusive evidence of a protective effect of oral contraceptives on the risk of developing RA (463).

Neuroendocrine Abnormalities

HYPOTHALAMIC–PITUITARY–ADRENAL AXIS

Although RA patients have normal cortisol and adrenocorticotropic (ACTH) levels, if one considers the stress associated with this chronic disease, this would be interpreted as inappropriately normal. This suggests a blunted hypothalamic–pituitary–adrenal (HPA) axis responsiveness. The regulation of ACTH levels is affected by IL-6 levels and other cytokines. Additionally, cortisol clearly affects the immune response, and its impaired production is likely involved in the regulation of disease activity (464).

CATECHOLAMINES

Catecholamines are involved in the normal stress response likely present in chronic diseases. *In vitro* studies have shown that Th1, but not Th2, T-cell clones express b_2-adrenergic receptors (465). On stimulation with b_2-adrenergic drugs, Th1 clones showed an increase in intracellular cyclic adenosine monophosphate (cAMP) and inhibition of the production of IFN-g, whereas IL-4 synthesis by Th2 clones was unchanged (465). Additionally, b_2 agonists inhibit IL-12 production by human monocytes and dendritic cells (466). These data suggested that increased catecholamine synthesis or the use of b-adrenergic agonists could be beneficial in the treatment of RA, a disease thought to involve Th1 cytokines in its pathogenesis. Malfait et al., in fact showed that daily administration of salbutamol was effective in the treatment of established CIA in mice, decreased joint damage, and reduced IL-12 and TNF-a release by peritoneal macrophages in a dose-dependent manner (467). Additionally, salbutamol treatment also decreased TNF release by synovial cells from arthritic mice, and decreased the CII-specific IFN-g production and proliferation of draining lymph node cells. *In vivo*, salbutamol specifically blocked mast cell degranulation in joint tissues. It will be interesting to see whether this strategy will prove beneficial as an adjuvant to the treatment of RA.

CLINICAL FEATURES AND DIAGNOSIS

Disease Presentation and Articular Manifestations

The onset of RA may vary from acute to one of an insidious character in which low-grade fever, stiffness, fatigue, joint pain, and swelling progress over weeks to months. The course of RA also varies from patient to patient. The classic additive pattern consists of an accumulation of newly involved joints, with inflammation persisting in those that were first involved. A small percentage of patients exhibit a monocyclic course in which, after a period of arthritis, the disease goes into complete remission. Most commonly, however, the disease has a chronic pattern, alternating periods of improvement with periods of exacerbations ultimately causing joint erosions, malalignments, and deformities (Fig. 37.7). In 10% to 20% of cases, RA may present as palindromic rheumatism with recurrent and self-limited (days) episodes of nonerosive monoarthritis/oligoarthritis (468).

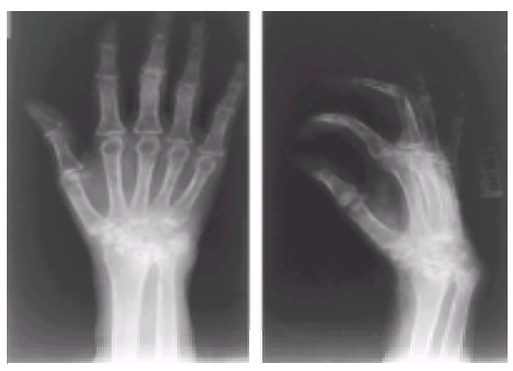

Figure 37.7. Hand radiographs of a patient with rheumatoid arthritis revealing extensive erosive changes in the wrist, carpal joints, proximal interphalangeal joints, and metacarpophalangeal joints.

Arthritis in RA occurs in a characteristic pattern with pain and swelling of the small joints of the hands and feet, particularly the proximal interphalangeal (PIP), metacarpophalangeal (MCP), and metatarsophalangeal (MTP) joints. In addition, the arthritic process commonly involves wrists, elbows, shoulders, knees, ankles, temporomandibular joints, cervical spine, and, to a lesser degree, the hip joints. The pattern of arthritis typically involves the joints on both sides of the body in a symmetric distribution.

Whereas most joints are affected, certain joints like the distal inter-phalangeal (DIP), sacroiliac, thoracic, and lumbar spine joints are rarely involved in RA. Similarly, enthesitis, the inflammation of the bone insertion site of the tendons typically seen in the spondyloarthropathies, is not present in RA.

Extraarticular Manifestations

Rheumatoid arthritis is a systemic disease that may involve organs other than the joints. The extraarticular manifestations range from anemia of chronic diseases, subcutaneous nodules, and ocular involvement to necrotizing vasculitis and Felty's syndrome. Extraarticular disease correlates with a higher risk for erosive changes, disability, and decreased life expectancy with death from a variety of causes (469,470). Titers of RFs are usually elevated, and circulating immune complexes involving IgG RF may be identified in association with decreased complement levels (244,469). The presence of antinuclear antibodies and eosinophilia also correlate with increased risk for extraarticular disease. In the following paragraphs, we review some aspects of the extraarticular manifestations in RA. The reader is referred to rheumatology textbooks for a more complete review of extraarticular manisfestations and laboratory abnormalities of RA.

SUBCUTANEOUS NODULES

Rheumatoid nodules develop in 20% of patients with RA and are a unique feature of the disease. Typically, the rheumatoid nodule is found at pressure areas in the subcutaneous tissue overlying bone, such as the olecranon and digits, but they also occur in the synovium, tendons, myocardium, pericardium, lung, central nervous system, and ocular sclerae. At the earliest stage, the nodule is characterized by a perivascular round cell infiltration in the terminal vascular bed with proliferation of fibroblasts and monocytoid cells. At its advanced and characteristic stage, the nodule is dominated by a central necrobiotic mass, which is essentially devoid of blood vessels. Surrounding this are palisades of activated cells that express monocyte lineage markers and MHC class II molecules (471,472). At the periphery is a sparse inflammatory infiltrate composed of T cells with a CD4/CD8 ratio like that of the peripheral blood.

FELTY'S SYNDROME

Felty's syndrome typically occurs much later in the course of RA, sometimes after the joint disease activity has become somewhat quiescent. Neutropenia often associated with thrombocytopenia along with splenomegaly in a patient with RA constitute the syndrome. As mentioned, vasculitis also may be seen in association with Felty's syndrome. Nonvasculitic chronic leg ulcers also have been described. The neutropenia has been related to high levels of circulating immune complexes as well as to specific antineutrophil antibodies (473). Coating of neutrophils or platelets by complexes or antibodies may facilitate their clearance by the reticuloendothelial system. The response to splenectomy is variable, and the procedure is not routinely recommended. In addition to methotrexate and other cytotoxic agents, the recent use of granulocyte colony-stimulating factor (G-CSF) and GM-CSF has been helpful in treating neutropenia (474,475). Interestingly, there have been descriptions of reactivation of previously quiescent arthritis in RA patients in association with the improvement of the neutrophil counts (476). This suggests that, whereas some of the extraarticular manifestations of Felty's syndrome are neutrophil independent and occur despite neutropenia, neutrophils, or the granulocyte-stimulating factors may have a significant role in the activity of the joint disease in RA, as discussed in the pathogenesis section of this chapter.

Diagnostic Criteria

The American College of Rheumatology (ACR), formerly the American Rheumatism Association, published the revised criteria for the diagnosis of RA in 1988 (477). These criteria were established in an attempt to create a uniform nomenclature and disease definition, thus facilitating comparison of studies done in different research institutions. In reality, these criteria are widely disseminated among physicians and are used for diagnosis. The criteria are listed in Table 37.6. A person who fulfills four of these seven criteria is said to have RA, with criteria 1 through 4 present for at least 6 weeks to rule out viral arthritides. The criteria set has a sensitivity of 91.2% and a specificity of 89.3% for RA (477).

TABLE 37.6. 1987 Revised Criteria for the Classification of Rheumatoid Arthritis[a]

The combined use of hand and feet radiographs increases the likelihood of identifying erosive changes at earlier stages, as demonstrated by Paimela et al. (478). These researchers described MTP joint erosive changes in 23% of patients in the first year of illness, but hand involvement was found in only 11%, of whom half also had foot involvement (478).

Studies of recent-onset synovitis cohorts show that most RA patients meet the ACR criteria at an early stage after the disease onset and that it is uncommon that an undifferentiated oligoarthritis, particularly in patients who are RF negative, will progress to meet the criteria for RA (479,480 and 481).

NEW THERAPEUTIC STRATEGIES

The treatment of RA involves several aspects in a multidisciplinary approach that is critical for the achievement of good disease control. Although the ultimate goal of therapy is disease remission, this goal is not commonly achieved. The management goals are to control disease activity, alleviate pain, maintain function for activities of daily living and work, maximize the quality of life, and slow the rate of joint damage. The ACR published guidelines for the management of RA (482). It is beyond the scope of this chapter to review extensively the drugs used in the treatment of RA. For that, the reader is referred to textbooks of rheumatology. This section provides a concise summary of new drugs and recent advances and strategies in the pharmacologic therapy of RA.

Leflunomide

This is a new FDA-approved drug that interferes with dihydroorotate dehydrogenase, an enzyme critical for *de novo* synthesis of pyrimidines. It interferes with T-cell proliferation. Clinical trials demonstrated that it is superior to placebo and similar to sulfasalazine in disease control (483). Also, leflunomide was as effective in disease control and in delaying radiographic disease progression as methotrexate (484). Although long-term safety data are not available, this new drug appears to be quite promising.

Anti-TNF Agents

Strategies aiming to antagonize TNF, both with antibodies to TNF or with the use of a TNF receptor fusion protein (etenarcept), have been shown to be highly efficacious in disease control (375,377,485) with overall good safety profiles, and about 70% of patients achieve significant clinical improvements. These agents achieve clinical response in a few weeks, earlier than other DMARDs. Etanercept is administered as two subcutaneous injections per week, and anti-TNF antibodies intravenously.

Combination Therapy

Combination therapy has been proposed and used for cases that do not respond to a single agent, emphasizing that early intervention and the achievement of disease control improves quality of living, correlates with the maintenance of better function and reduces mortality. Recent studies are also trying to identify early markers for poor response to single-drug therapy, like, for example, the presence of certain shared-epitope-containing alleles (486), and advances in pharmacogenomics should also facilitate this task in the near future.

Several studies have emerged during recent years addressing strategies to treat patients that do not respond to one or multiple DMARDs. The first combination drug trial by McCarty and Carrera (487) showed that the combination of cyclophosphamide, azathioprine, and hydroxychloroquine was highly efficacious but had a high rate of side effects. Among the most important combination trials published after that is the triple-drug regimen described by O'Dell et al., which included methotrexate, sulfasalazine, and hydroxychloroquine (488). The triple-drug combination was more effective than either methotrexate alone or a combination of sulfasalazine and hydroxychloroquine. In another study that enrolled patients with severe RA and partial response to methotrexate, the combination of cyclosporine and methotrexate achieved significant clinical improvement (489). In a third study, Dougados et al. studied recent-onset RA and could not find a significant difference in clinical disease control or radiographic changes in a study that compared sulfasalazine and methotrexate alone or in combination (490). Boers et al. used an alternative strategy in recent-onset RA: These investigators used methotrexate in combination with sulfasalazine and incorporated high-dose prednisone (60 mg daily) therapy for the first few weeks, with a rapid taper to 7.5 mg daily, and compared this regimen to sulfasalazine alone (491). After 28 weeks of follow-up, the intensive/combination therapy group had achieved a statistically significant clinical improvement, with significantly less radiographic damage.

Anti-TNF agents were used in combination regimens. Both the combination of anti-TNF antibodies (492) or etenarcept with MTX (493) achieved significantly better disease control than methotrexate alone. The combination of leflunomide and methotrexate also may become a good alternative once better studied (494).

Although the use of combination therapy in recent-onset disease is still a controversial issue, it is a widely used strategy for severe and refractory disease. The evidence in support of a better disease outcome, quality of living and function, and improved survival associated with clinical response to DMARDs highlights the importance of a dynamic approach to the treatment of RA, trying to maximize clinical improvement and balancing risks of potential drug toxicities.

New Directions for Therapy

Currently, great activity is ongoing in the field of therapy of RA using novel agents that have the potential for specific immunomodulation. These agents include the use of a variety of cytokines like IFN-b, IL-10, and IL-4 or cytokine receptor antagonists such as the IL-1 receptor antagonist. A controlled trial of IL-1 receptor antagonist showed significant improvement in disease activity (495) and reduced joint damage (496).

Other agents include monoclonal antibodies directed against surface structures on T-cells, such as CD4, or toxin-conjugated cytokines, such as those to IL-2, which have the potential of inactivating or extirpating cells in a particular lineage or state of activation (497,498). Anti-T-cell strategies based on anti-CD4 antibodies (499) and IL2-diphtheria toxin fusion protein (500) have provided disappointing results in patients with a difficult-to-treat disease, raising the issue that, although T cells may be critical to the onset of RA, T-cell-independent pathways may be more important during the chronic stages of disease in a subset of patients. At the level of attempting to achieve greater therapeutic finesse, agents are being developed that are directed to the MHC class II molecule or TCRs that are putatively involved in the disease, although here again the rationale for their use is not unambiguous.

Attention is also being given to the possibility of inhibiting the egress of leukocytes into the synovium by blocking antibodies to adhesion molecules (501,502) or antichemokine agents and to antiangiogenic agents (417). Although plasmapheresis was previously ineffective in RA, apheresis with the Prosorba column was recently shown to be an efficacious treatment of RA in patients with active disease who have failed other treatments (503). Interestingly, the patients showed only clinical improvement weeks after completion of the therapy, and there were no significant changes in the levels of immunoglobulins of RFs, leaving no clear idea as to how these columns exert their effect. We are seeing a number of new strategies being used in experimental animal models of arthritis that are likely to go into clinical trial in the near future, including antichemokine agents, as discussed already.

Gene therapy is being actively studied in autoimmune arthritis. Studies using gene therapy with IL-4 and IL-10 into the joints of animals with autoimmune arthritis have been performed, and the reports appear promising (504,505). Another actively used strategy is DNA vaccination, where a blocking immune response against a particular protein is generated. This latter strategy has been used to induce an immune response against certain chemokines expressed in high amounts in both arthritic joints from rats and RA patients. Repeated administration of the constructs encoding MCP-1, MIP-1, or RANTES inhibited the development of adjuvant-induced arthritis, and when each vaccine was administered after the onset of disease, it inhibited progression (506). In another study, vaccination with naked DNA encoding for mycobacterial heat shock protein 65 also protected rats from developing adjuvant-induced arthritis (507), and it certainly will be interesting to see these strategies going into human clinical studies.

CONCLUSION

There is still a lot to be learned and understood about the pathophysiology and the genetic regulation of RA; however, patients already are benefiting from new strategies using combination drug regimens and new therapeutic agents recently approved by the FDA for the treatment of RA. Early use of DMARDs has been shown to modify the disease outcome, quality of life, and survival. The rapid biotechnological and genetic developments most certainly will transfer into better tools for early diagnosis, prognostication, as well as to even better, more specific, and less toxic therapies.

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38 JUVENILE RHEUMATOID ARTHRITIS

Alexei A. Grom, M.D., and David N. Glass, M.D.

Juvenile rheumatoid arthritis (JRA) is a chronic idiopathic inflammatory disorder primarily involving joints. The diversity of its clinical symptoms, both at onset and during the course of the disease, and the lack of specific laboratory tests can make the diagnosis problematic. Compounding these difficulties, the diagnostic criteria used in different countries are not the same. Some differences in terminology also exist. The term *juvenile rheumatoid arthritis* is broadly used in the United States and in Canada. In Europe, the term *juvenile chronic arthritis* was in general use until recently. It is being superseded by the umbrella term *juvenile idiopathic arthritis.*

INITIAL DESCRIPTION AND CLASSIFICATION

The first clinical report of juvenile-onset arthritis was supplied in 1864 by Cornil, who described a 29-year-old woman with chronic inflammatory arthritis from the age of 12 years (1). Still's classic presentation of 22 cases appeared in 1897, accompanied by the suggestions that JRA may have different origin from adult rheumatoid arthritis (RA) and may include more than one disease (2). In 1937, Colver presented results of a long-term follow-up study of 63 patients, whose outcomes were relatively good compared with those of patients with adult RA (3).

Attempts to classify disease subtypes have been made since the 1970s (4,5,6,7 and 8). In the Americas, the most frequently used criteria are those of the American College of Rheumatology; these criteria were first published in 1972, and they were then revised in 1977 and again in 1986 (Table 38.1). In the United States, classification has been based on the type of onset, which is determined by clinical manifestations during the first 6 months of the disease. The three major types of onset are pauciarticular (four or fewer joints involved at onset), polyarticular (five or more joints), and systemic (9).

1. Age at onset <16 yr
2. Arthritis in one or more joints, defined as swelling or effusion, or presence of two or more of the following signs: limitation of motion, tenderness or pain on motion, and increased heat
3. Symptoms persistent for 6 wk or longer
4. Type of onset during the first 6 mo classified as
   A. Systemic disease: arthritis with intermittent fever
   B. Polyarthritis: five joints or more
   C. Oligoarthritis (pauciarticular form): four joints or fewer
5. Exclusion of other forms of juvenile arthritis

TABLE 38.1. American College of Rheumatology Criteria for Diagnosis of Juvenile Rheumatoid Arthritis

In 1997 in Durban, South Africa, the criteria for classification of childhood arthritis were reviewed at the meeting of the Pediatric Standing Committee of the International League of Associations for Rheumatology. The classification was expanded to include seven types of the disease: systemic arthritis, oligoarthritis persistent, oligoarthritis extended, polyarticular arthritis rheumatoid factor (RF)–positive, polyarticular arthritis RF-negative, enthesitis arthritis, and psoriatic arthritis (Table 38.2). This new classification is now widely being used in most European countries, with the umbrella term *juvenile idiopathic arthritis* to indicate disease of childhood onset primarily characterized by arthritis persisting for at least 6 weeks and currently having no known cause (8).

Systemic
Polyarthritis (RF positive)
Polyarthritis (RF negative)
Oligoarthritis persistent
Oligoarthritis extended
Enthesitis-related arthritis
Psoriatic arthritis
Other arthritis: fits no other category or fits more than one category

RF, rheumatoid factor.
From Petty RE, Southwood TR, Baum J, et al. Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban, 1997. *J Rheumatol* 1998;25:1991–1994; with permission.

TABLE 38.2. Durban Classification of Childhood Arthritis

CLINICAL DESCRIPTION BASED ON ONSET TYPE

The *pauciarticular* type is divided into two subtypes (9). *Early-onset pauciarticular JRA* (EOPA-JRA or type 1) occurs mostly in girls less than 6 years of age. Antinuclear antibody (ANA) positivity is a characteristic finding in this group of children with JRA. The large weight-bearing joints are usually the primary sites of the inflammatory activity, and an asymmetric pattern of joint involvement is common. Severe destructive changes are the exception rather than the rule in this type of the disease, although some cartilage erosions may appear. As the disease progresses, it converts to polyarticular disease in as many as 20% of patients (10,11). EOPA-JRA is accompanied in 10% to 20% of patients by chronic anterior uveitis (iridocyclitis), which can lead to loss of vision (12,13). The uveitis is a chronic, nongranulomatous inflammation affecting the iris and ancillary body. The posterior uveal tract is rarely involved. The eye disease may present later than arthritis, and in at least half of the children, it is asymptomatic at early stages. In many instances, it can be detected only by slit-lamp examination. Therefore, it is important that affected children undergo regular slit-lamp examination every 3 months when they are young, with the interval between examinations extending to not more than 1 year as their age increases. The probability that a patient will develop eye disease diminishes markedly after 5 years from the onset of the arthritis. The overall morbidity in EOPA-JRA may be determined more by the outcome of the eye disease than by the outcome of the arthritis.

Late-onset pauciarticular JRA (type 2) occurs more often in boys. Involvement of the spine along with the large joints of the lower extremities is typical. Patients often carry the human leukocyte antigen (HLA)-B27 gene, which is a marker for the development of enthesopathy and even ankylosing spondylitis in some (14). In this form of JRA, the eye disease, also uveitis, is more acute and tends to be self-limiting, in contrast to the ocular effects of EOPA-JRA.

The *polyarticular* type is divided into two subtypes on the basis of the presence or absence of RF. In the group positive for RF, which is relatively small, late onset of the disease is more common, and the course of the disease resembles that of adult RA. RF-negative disease is frequently less severe and may begin at any time during childhood. Some systemic manifestations, such as fever spikes and lymphadenopathy, may be present in both subtypes, but the joint disease usually overshadows the more general symptoms. Although the large joints such as the knees, wrists, and ankles are commonly involved, symmetric inflammation of the smaller, more distal joints is more characteristic. In a significant proportion of patients in this group, the metacarpophalangeal and the proximal interphalangeal joints are involved (15). The spine, especially the cervical region, may be affected. Hip joints are frequently involved at later stages of the disease. The arthritis is typically destructive. Early stages of the disease are characterized by soft tissue swelling and juxtaarticular osteoporosis. Later changes include destruction of the cartilage and bone and eventually bony ankylosis. Subcutaneous nodules (not typical of JRA in general) are common in patients with polyarticular disease. Similar to those in adult with RA, rheumatoid nodules usually appear in areas of pressure such as on elbows and heels and along vertebrae.

The *systemic* form of JRA also appears to be heterogeneous. Some of the patients in this group have relatively mild arthritis with prominent extraarticular features, such as spiking fevers, hepatosplenomegaly, and vasculitis (16). The typical fleeting pink macular rash, pleurisy, and pericarditis (as a part of generalized serositis) are common. Generalized enlargement of lymph nodes, especially in the axilla, is also typical. The joint involvement, like the rash, may be more marked at the time of the temperature elevation and sometimes may be entirely absent when fever is gone. Other patients with the systemic form develop severe, progressive arthritis with an early appearance of erosive changes in the cartilage. This more severe illness is likely to be associated with the HLA-DR4 gene in some populations. These patients can have the worst outcome for joint disease of any group of patients with JRA. Patients with systemic onset may have marked polymorphonuclear leukocytosis and thrombocytosis. RF and ANA are generally negative in this group of patients.

ACUTE COMPLICATIONS

Vasculitis

Rheumatoid vasculitis is uncommon in JRA and occurs most often in the older children with RF-positive polyarthritis (9,16,17). This devastating, widespread, small to medium-sized vessel involvement must be distinguished from benign digital vasculitis, which is more frequent, and it may occasionally be associated with vascular calcification that is seen on radiographs along the course of the digital arteries (18).

Macrophage Activation Syndrome

The macrophage activation syndrome is a rare but serious complication of systemic JRA associated with considerable morbidity and death (19,20). It is a clinical syndrome caused by the excessive activation and proliferation of well-differentiated macrophages. Massive release of proinflammatory cytokines during macrophage activation appears to be the major pathogenic mechanism. The macrophage activation syndrome can be clinically differentiated from a typical exacerbation of systemic JRA by several features that become apparent early in the course of this syndrome. Patients with an exacerbation of systemic JRA usually have a spiking daily (quotidian) or twice-daily (double quotidian) fever, evanescent rash, generalized lymphadenopathy, and hepatosplenomegaly. Marked polymorphonuclear leukocytosis, thrombocytosis, and increased erythrocyte sedimentation rate with hyperfibrinogenemia are common features that reflect the activity of inflammation. Mild elevation of liver enzyme activity is also common. In contrast, persistent, unremitting fever (which is different from the quotidian fever of systemic JRA), mental status changes, and moderately elevated liver enzyme activity, accompanied by a sharp fall in erythrocyte sedimentation rate in association with hypofibrinogenemia, relative leukopenia, thrombocytopenia, prolonged prothrombin time, and easy bleeding, should raise the suspicion of macrophage activation syndrome. The diagnosis is confirmed by demonstration of hemophagocytic histiocytes in bone marrow and lymph nodes.

LONG-TERM COMPLICATIONS

Growth Retardation

The chronic inflammatory process in children with JRA causes problems relating to growth. These may be localized, such as when the disease is associated with epiphyseal overgrowth and premature epiphyseal closure (9). A clinically apparent consequence is the finding of unequal leg length; this finding is particularly typical in EOPA-JRA. Another characteristic localized growth problem is mandibular hypoplasia (21). Chronic inflammation also has an effect on overall growth. These children are frequently smaller than expected, and this effect is compounded by the use of corticosteroid therapy (22,23).

Amyloidosis

Amyloidosis has been described in as many as 50% of patients with systemic-onset JRA in European populations (24,25,26 and 27). It is a much less common finding in the North American populations (28). One European study demonstrated a genetic association between the development of amyloidosis and certain isoforms of amyloid P component (29). However, the finding that patients with JRA who are of European origin and living in the United States develop amyloiodosis only on rare occasions suggests an environmental rather than a genetic cause of this complication. Attempts to associate amyloidosis with major histocompatibility complex (MHC) genes have not been successful (30).

EPIDEMIOLOGY

Overall Incidence and Prevalence

The lack of standard diagnostic criteria has complicated epidemiologic studies as well. Probably, the most accurate study applied the revised American College of Rheumatology classification criteria to the unique database of the Rochester Epidemiology Program Project: in 1983, this study reported an incidence for JRA of 13.9 cases per 100,000 in a year (31). However, the follow-up study, using the same database, noted some decrease in the JRA incidence in the 1990s (32). Results of other population studies are presented in Table 38.3.

Country	Incidence	Reference
Finland	9.2/100,000	174
Finland	19.6/100,000	175
Sweden	10.9/100,000	176
Canada	5.3/100,000	46
United Kingdom	10.0/100,000	177

TABLE 38.3. Incidence of Juvenile Rheumatoid Arthritis in Different Countries

In the Rochester study, the prevalence rate for JRA was 94.3 patients per 100,000 children on January 1, 1980, and 86.1 on January 1, 1990 (32). Based on the extrapolation of these numbers to the entire United States population younger than 16 years of age, there must be 70,000 to 100,000 cases of JRA (active and inactive) in the United States. However, the Rochester study included a predominantly white population. Because the incidence of JRA appears to be lower in the African-American and Asian populations (as discussed in the next paragraph), the real prevalence must be much lower (33). In fact, Gewanter and Baum estimated that, in 1986, only 15,000 to 36,000 children had JRA in the United States (34).

Ethnic Differences

Some evidence indicates that JRA is less frequent in black South African (35) and Chinese populations. Increased frequency in a Native American population has also been reported (36). There are also some differences in disease onset types among ethnic groups (37). It appears that African-American children are more likely to have disease with polyarticular onset compared with white children, in whom the pauciarticular onset type is most common (38). In contrast, in the Japanese population, systemic disease is most common, with relatively fewer cases of pauciarticular JRA (39). Genetic factors (see later) are likely to contribute to these differences.

Age of Onset

Sullivan et al. reported a peak age at onset of 1 to 3 years, with girls dominant in this age group (40). A second, less prominent peak age at 8 to 10 years included more boys with pauciarticular JRA than were present in other groups. In a study by Schaller, the mean age of onset of systemic disease was 5.7 years, of polyarticular disease was 6.5 years, and of a pauciarticular disease was 4.3 years in girls and 10.2 years in boys (41).

Gender Differences

Although equal sex ratios among Indian and black South African patients have been reported (35), most published female-to-male ratios range from 2:1 to 3:1. These numbers depend on the age of onset of the children and the type of the disease (42). For example, EOPA-JRA affecting children less than 8 years old may have a female preponderance as high as 8:1, especially if iridocyclitis is present (12,43,44). In contrast, late-onset HLA-B27–related disease occurs more commonly in boys (12,45). Systemic-onset JRA shows little or no discrimination between the sexes.

Secular and Seasonal Variations

Substantial variation in disease occurrence over extended (secular trends) and shorter (seasonal) periods has been found for JRA in some populations (32,46), but not in others. For instance, a report from the United Kingdom demonstrated the presence of an influenza virus epidemic before an “outbreak” of JRA (47). The strongest seasonal variations have been observed with systemic-onset JRA (48,49), although this appears to be true only in some geographic regions.

PATHOGENESIS

At least some familial predisposition to the disease, characteristic histopathologic features of inflamed synovium, HLA associations, T-cell clonality, and the presence of autoantibodies point to an autoimmune pathogenesis of JRA with involvement of genetic and environmental factors.

Genetic Factors

The level of risk to family members of a JRA proband appears to be increased, although the increase is modest (50,51). Patterns of inheritance consistent with mendelian or monogenic inheritance have not been observed in this disease. Extensive kindreds with multiple affected family members in several generations, as seen in other rheumatic and nonrheumatic autoimmune diseases, are not evident either. In a series drawn from more than 3,000 European patients with juvenile chronic arthritis, Clemens et al. were able to identify only 12 affected sib pairs (52). The JRA Affected Sib Pairs Research Registry in Cincinnati, Ohio, estimates the total number of affected sib pairs at 300 in the United States population of more than 250,000,000 (53). Anecdotal evidence suggests that families with JRA probands have an additional, and perhaps stronger, risk of having other, nonrheumatic autoimmune diseases (54,55).

PREDISPOSING MAJOR HISTOCOMPATIBILITY COMPLEX GENES

Multiple genetic associations involving predominantly the MHC locus suggest a complex genetic trait and also reinforce the idea that JRA is as heterogeneous genetically as it is clinically (51,56). Patients with EOPA-JRA constitute the subtype with the most distinctive HLA associations. Furthermore, in 71 registered affected sib pairs who were the subject of an analysis by Moroldo et al., a high sib-sib concordance of pauciarticular onset disease was noted, a finding suggesting a stronger genetic component in this form of the disease (57). Associations with class II HLA-DR8 (58,59), HLA-DR11 (60,61 and 62), HLA-DR13 (63), and HLA-DPw2 (64,65) specificities have been reported. An HLA-A2 class I association has been found consistently as well (66). Two HLA class II specificities, HLA-DR4 and HLA-DR7, are significantly reduced in frequency, and therefore they appear to be protective (62). DNA sequence-dependent methods have defined more precisely the HLA alleles associated with pauciarticular JRA. It appears that disease susceptibility is associated with particular alleles of each serologically or cellularly defined specificity, for instance, HLA-DRB1*0801 of DR8 and HLA-DPB1*0201 of DPw2 (56).

HUMAN LEUKOCYTE ANTIGEN AND EARLY-ONSET PAUCIARTICULAR JUVENILE RHEUMATOID ARTHRITIS OUTCOME

Determination of HLA specificities using DNA-based methodologies has also better defined the genetic markers of disease outcome in EOPE-JRA. Haplotypes carrying HLA-DRB*0801, HLA-DRB* 1301, and HLA-DP*0201 alleles appear to predispose to pauciarticular JRA in general. In contrast, HLA-DRB1104* haplotypes predispose to eye disease (67); HLA-DQA*0101 haplotypes predispose to a polyarticular, erosive disease outcome, but they are protective against the development of eye disease (68). The HLA-DRB*0101 split of HLA-DR1 is another allele present on HLA HLA-DQA*0101 haplotypes that has been shown to be associated with a polyarticular course. Whether the HLA-DQA*0101 allele or, as suggested by Ploski and colleagues (69), the HLA-DRB1*0101 allele is a better marker for the haplotype is uncertain, but data are compatible with either possibility.

Polygenic Inheritance

The finding that HLA genes from four loci (HLA-A, HLA-DR, HLA-DQ, and HLA-DP) are involved in inherited predisposition to pauciarticular JRA raises the possibility that these genes make up a potential susceptibility haplotype. However, the absence of linkage disequilibrium among the specific HLA genes involved suggests otherwise and provides evidence indicating independent genetic effects at three loci, that is, that EOPA-JRA is truly a polygenic disease. This is illustrated in Table 38.4, which demonstrates the odds ratio obtained by the cumulative addition of risk factors. Such interactions have been demonstrated in other populations (70).

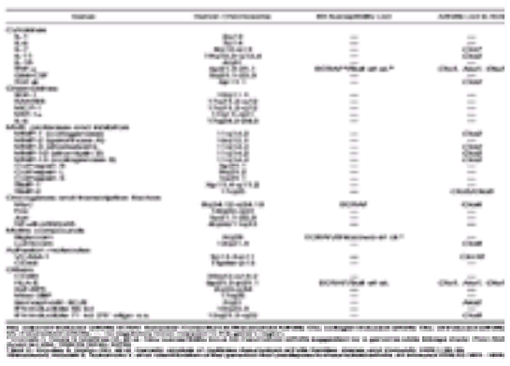


TABLE 38.4. Polygenic Risk in Early-Onset Pauciarticular Juvenile Rheumatoid Arthritis[a]

In a significant proportion of patients with EOPA-JRA, especially those with uveitis, evidence indicates that the development of the disease is associated with the presence of two susceptibility alleles of either HLA-DR or HLA-DQ loci. For example, patients who are heterozygous for HLA-DR5/HLA-DR8 have a particularly increased risk for developing eye disease (71). An HLA-DP gene and a class I gene, HLA-A2, contribute as well, appearing as independent risk factors. Class II gene homozygosity is not increased in patients with pauciarticular JRA, a finding suggesting that a dosage effect of any individual gene does not increase risk in patients (71). MHC associations with other clinical forms of JRA are weaker. As previously mentioned, late-onset pauciarticular JRA has been associated with HLA-B27 (14,45). In polyarticular onset, RF-negative JRA, susceptibility is associated with HLA-DPw3 (72). RF-positive polyarticular JRA has the same HLA-DR4 associations as does adult RA, that is, with HLA-DRB1*0401 and HLA-DRB1*0101 (73). HLA-DR4 associations with the systemic form of the disease have been found in northern, but not southern, European populations (56).

JUVENILE RHEUMATOID ARTHRITIS AND THE HLA-DR1/DR4 SHARED EPITOPE HYPOTHESIS

RA is associated with the presence of the DNA sequence common to HLA-DR1 and some HLA-DR4 specificities (74,75). However, the shared epitope hypothesis applied to RA is not useful for pauciarticular JRA in which HLA-DR4 is protective, and HLA-DR1 haplotypes predispose to a polyarticular outcome. Patients with pauciarticular JRA thus show a dissociation in the effects of the haplotypes involved in the hypothesis: one predisposes and others are protective. Patients with the seronegative polyarticular JRA do not carry the appropriate epitopes. Older patients with JRA with polyarticular disease who are immunoglobulin M (IgM)–RF positive and who have the childhood equivalent of adult RA are the only JRA group in whom this shared epitope hypothesis may hold, but this group accounts for fewer than 10% of all children with JRA.

AGE-SPECIFIC EFFECTS OF MAJOR HISTOCOMPATIBILITY COMPLEX ALLELES

Newer evidence indicates that the genes operative in JRA appear to have a “window-of-effect” during which time they may contribute risk of disease, but they may be neutral or even protective at other times (76). This finding appears to be particularly true in the pauciarticular groups, in which HLA-related risks clearly differ with age. For instance, 50% of the children carrying at least one of the susceptibility MHC alleles have disease onset before their third birthday, a finding suggesting that the period of susceptibility to EOPA-JRA is limited to the first years of life. In contrast, HLA-B27 and HLA-DR4 appear to be associated with the protection against EOPA-JRA early in life, but with an increased risk of other forms of JRA later in childhood (Fig 38.1).

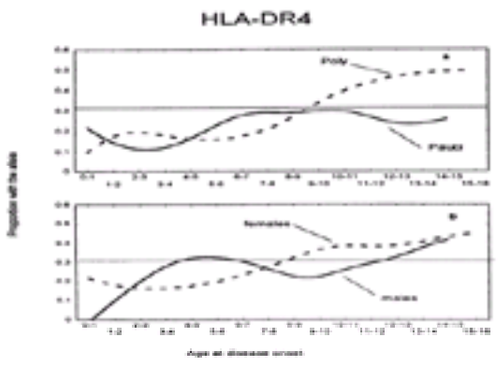
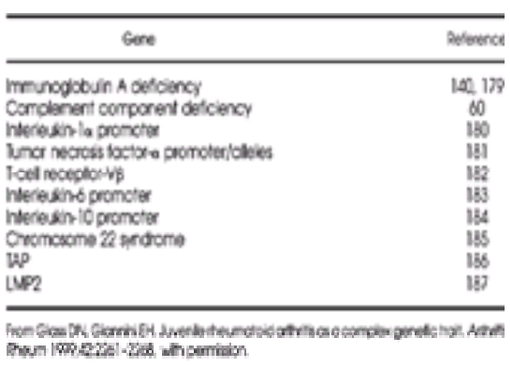


Figure 38.1. Proportion of the study population by disease-onset type **(A)** and by sex **(B),** in each age-at-onset category with the HLA-DR4 allele. The *horizontal line* shows the frequency of the allele in the control group. Pauci , pauciarticular; poly, polyarticular. (From Murray KJ, Moroldo MB, Donnelly P, et al. Age-specific effects of juvenile rheumatoid arthritis–associated HLA alleles. *Arthritis Rheum* 1999;42:1843–1853, with permission.)

PREDISPOSING NON–MAJOR HISTOCOMPATIBILITY COMPLEX GENES

The spectrum of non-MHC genes associated with JRA is expanding rapidly. However, their contribution appears to be less significant than that of MHC genes, and their effects may not be specific to JRA. Table 38.5 lists the non-MHC genes or chromosome regions that have thus far been reported to be associated with JRA. In general, the odds ratios are low, and distinguishing between founder effects (the incomplete mixing of genetically disparate populations) and associations that relate to pathogenesis has been difficult.



Gene	Reference
Immunoglobulin A deficiency	140, 179
Complement component deficiency	60
Interleukin-1α promoter	180
Tumor necrosis factor-α promoter/alleles	181
T-cell receptor-Vβ	182
Interleukin-6 promoter	183
Interleukin-10 promoter	184
Chromosome 22 syndrome	185
TAP	186
LMP2	187

From Gross DN, Giannini EH. Juvenile rheumatoid arthritis as a complex genetic trait. Arthritis Rheum 1999;42:2261–2268, with permission.

TABLE 38.5. Nonmajor Histocompatibility Complex Genes Associated with Juvenile Rheumatoid Arthritis

In summary, the occurrence of the disease and of its phenotype is likely to be determined by complex genetic traits with genetic interplay between MHC and non-MHC predisposing alleles (77). Newer methods are available to study the entire genome and thus potentially to locate all the specific chromosomal regions involved in each type of JRA. Implicit in this concept is the likelihood that the genes involved are weakly penetrant, although in most instances they are necessary for expression of the disease and its phenotype (53).

Environmental Factors

Although at least some genetic component is evident in all clinical forms of JRA, the environmental component appears to be stronger for some of them (78). The

infectious origin of JRA has been suspected for a long time (79). However, classic epidemiologic studies have not generally shown clustering of JRA that would imply a definite infectious origin, although some seasonality in systemic-onset disease has been reported (48,49). Documented long-term trends for the incidence of JRA to change over time (32,46) may also signify an environmental or infectious rather than a genetic effect.

Several potential pathogens have been implicated but have not yet been shown to be definitely causal. Rubella virus has been implicated for some time (80,81), and a few persons who receive rubella immunizations develop arthritis similar to that of pauciarticular JRA. Sequence similarities have been shown between HLA-DRB1*0801, DRB1*11, and DPB1*0201, all associated with pauciarticular JRA, and an Epstein-Barr virus protein (82), a finding supporting the hypothesis about the role for molecular mimicry in this form of JRA. Chlamydia has been identified in the joints of some children with arthritis, although the significance of this in the context of JRA is uncertain (83). The clinical presentation of chlamydial arthritis can resemble that of JRA, including the development of iridocyclitis. In a novel study from South Wales, Australia, a relationship was shown between influenza A infection in pregnancy and the subsequent development of polyarticular JRA in later childhood (47). HLA-B27–associated forms of JRA can be triggered by bacterial infections (84), as is the case with HLA-B27–associated disease in adults. Consistent with this finding, an immunodominant epitope, Gro EL of *Escherichia coli* heat shock proteins (hsps), is one target of immune responsiveness in HLA-B27–positive JRA (85). In two studies of long-term trends in the incidence of JRA in Canada and in Rochester, Minnesota, several peaks were noted (32,33,34,35,36,37,38,39,40,41,42,43,44,45 and 46). In Canada, these peaks were concurrent with the increases in confirmed *Mycoplasma pneumoniae* infections, a finding suggesting a possible role for this pathogen as well (46).

PATHOBIOLOGY

The major clinical manifestation of JRA is persistent joint swelling that results from accumulation of synovial fluid and synovial lining thickening.

Synovial Fluid

The volume of synovial fluid in affected joints is increased in JRA, with viscosity decreased mainly because of reduced concentrations of hyaluronic acid. Synovial fluid contains various inflammatory cells, including neutrophils, plasma cells, dendritic cells, and a high proportion of T cells expressing markers of activation (86). These cells are most likely to extravasate from inflamed synovial lining. Mediators of inflammation such as cytokines and cleavage products of the complement system are also abundant (87).

Synovial Tissue

One of the hallmarks of the pathology of both RA and JRA is the tumorlike expansion of inflamed synovial tissue, or pannus, which causes much of the joint damage in this disease (88,89). With the progression of the disease, pannus spreads over the synovial space and adheres to intraarticular cartilage. It is in the areas of pannus-cartilage junction that the cartilage eventually degrades. Such expansion results from the proliferation of synoviocytes and from invasion of the synovial tissue by inflammatory cells recruited from the peripheral circulation. Mitotic figures are rarely seen in synovial histopathologic examination, a finding suggesting that ongoing recruitment of inflammatory cells from peripheral circulation is a major pathway of pannus expansion (90). Pannus growth is also supported by extensive formation of new blood vessels, which provide not only a source of nutrients for growing pannus, but also an increased access for inflammatory cells to infiltrate the synovium and thus further to promote its growth.

Little is known about the major angiogenic factors driving new blood vessel formation in JRA synovium. One study showed strong correlation between increased serum levels of vascular endothelial growth factor and disease activity in polyarticular JRA (91).

Composition of Synovial Tissue Cellular Infiltrate

Initial light and electron microscopic studies of the synovium in JRA revealed prominent infiltration with lymphocytes, plasma cells, macrophages, and proliferation of fibroblast- and macrophagelike sy-noviocytes. Infiltration with inflammatory cells does not appear to be homogeneous: lightly infiltrated areas alternate with areas of heavy cell aggregates (88).

Aggregates are usually seen around or near blood vessels and are composed of clusters of $CD4^+$ cells surrounded by a mantle of mixed CD4 and $CD8^+$ cells and B lymphocytes. $CD68^+$ cells are numerous and are typically found in the cell aggregates. Such a histopathologic pattern in the inflammatory response in JRA resembles that of classic delayed-type hypersensitivity reactions (92). In general, tissues from patients with the polyarticular form of JRA appear to have larger mononuclear cell foci as compared with patients with pauciarticular JRA. However, the tissues cannot be reliably distinguished on this basis (93).

Another prominent feature is the increase in numbers of dendritic cells within the synovial membrane and fluid. Dendritic cells from the synovial compartment are larger than most of those from peripheral blood and have a more extensive Golgi complex (88). They are often found in close juxtaposition with T lymphocytes (94). A distinctive feature of synovial dendritic cells is their high level of HLA-DQ and HLA-DR expression. Such dendritic cells are particularly potent antigen-presenting cells (95). Inasmuch as their only known function is to present antigens, it is reasonable to believe that antigen presentation is taking place in inflamed synovium. This notion is supported by the demonstration that dendritic cells from joints of some patients with JRA stimulate autologous lymphocytes in an autologous mixed lymphocyte reaction (96).

Most of the T cells infiltrating synovium are of a memory phenotype. T-cell activation levels vary among different areas in synovium. Most interleukin-2R–positive ($IL\text{-}2R^+$) cells were found in the “mantle” surrounding the aggregates or in interaggregate areas (93). DNA-synthesizing T blasts represent no more than 5% of all synovial compartment mononuclear cells (97), a finding suggesting that most of these cells are recruited from peripheral circulation. Among radiolabeled blast cells, $CD4^+$ lymphocytes are the dominant cell population (97).

Mechanisms of Recruitment of Inflammatory Cells into Synovium

Mononuclear cell aggregates are found predominantly around blood vessels, particularly postcapillary venules, which have distinct morphologic features (88). They are lined up by tall, metabolically active endothelial cells that express high levels of adhesion molecules, features similar to those observed in venules of lymphoid organs. Some of the postcapillary venules in lymphoid organs are specialized to facilitate the transendothelial migration of circulating lymphocytes (90,98). It appears that high endothelial venules may play a similar role in JRA synovium as well. Thus, Oen et al. demonstrated that $CD4^+$, $HLA\text{-}DR^+$ lymphocytes from peripheral blood of children with JRA adhere to human umbilical vein endothelial cells activated by tumor necrosis factor-a (TNF-a) (99). Given the abundance of TNF-a in JRA synovial tissues associated with high levels of the p55 TNF receptor on the endothelial cells (100), this cytokine is likely to play a pivotal role in the enhancement of inflammatory cell trafficking into inflamed synovium. IL-15 is another cytokine that has been shown to enhance the transendothelial migration of lymphocytes to RA synovium (101). Using RNase protection assay, Scola et al. showed that at the mRNA level, IL-15 appears to be one of the most highly expressed cytokines in JRA synovium as well (102). It also has been shown that very late activation antigen (VLA-1, a surface protein from the integrin family of adhesion molecules that mediates leukocyte adhesion to matrix proteins) is overexpressed on lymphocytes from patients with JRA (103). The recruitment of proinflammatory cells into synovium does not appear to be random. Several studies suggested a possible role for certain chemokines in the selection of the predominantly Th1 lymphocytes (see further). Such recruitment may be both antigen specific as well as antigen nonspecific (104).

T-Cell Contribution to the Disease

T-cell receptor (TCR) ab T lymphocytes are the predominant cell population in the JRA synovium and exhibit phenotypic and functional characteristic of cells that have undergone prior activation *in vivo.* These characteristic include expression of IL-2 receptors (CD25) (105), early activation antigen CD69, CD45RO (memory phenotype), VLA-1 (103), and HLA class II antigens (86,106).

A hypothesis that such activation could be induced by an autoantigen located in the inflamed joints prompted several groups of investigators to look for clonally expanded synovial T-cell populations, an approach that had been successfully used in experimental allergic encephalomyelitis (107). In this model, a known autoantigen (i.e., myelin basic protein) was shown to stimulate expansion of T cells with receptor specificity for this particular antigen. Furthermore, clonally expanded T cells used a limited number of Vb segments in their TCRs. Investigators anticipated that if detected in the synovium of patients with JRA, such cell populations could provide a clue to the identification of the antigens driving the immune response in JRA. The results of these studies, however, were contradictory. Initially, it was shown that clonal expansion of ab T cells in both $CD4^+$ and $CD8^+$ populations could be detected in the synovium of most patients with JRA (108,109,110 and 111). In addition, identical clones with the same receptor specificity were shown to be present in multiple joints of the same person and to persist over a long period (109,111). Some of these clones could be detected in peripheral blood as well, although preferential accumulation of these clonally expanded populations in inflamed synovial tissues was evident (109,110). These findings were thought to be consistent with the concept of persistent antigenic stimulation of T cells in the synovial compartment of patients with JRA. However, the discovery of similar clonally expanded $CD8^+$ populations in peripheral blood of healthy adults (usually older than 35 years of age) (112) made

some investigators question this concept.

Further studies were focused on the analysis of the TCR CDR3 region, the site of specific interaction of the TCR with unique peptide-HLA complexes. Investigators anticipated that expansion of structurally identical clones would indicate an ongoing antigen-driven immune response in the joint. Because the CDR3 region is encoded by the V-(D)-J segments, several studies were carried out to determine whether oligoclonal T-cell populations preferentially used particular Va- or Vb-chain TCR segments. Initially, Sioud et al. detected significantly fewer Vb families in fresh T cells from the synovial fluid than from the peripheral blood (113). These differences were more obvious when IL-2R$^+$ T cells were isolated (108). Restricted usage of Va segments by synovial fluid T cells was noted in this study as well. In a more comprehensive study of synovial fluid samples obtained from 36 patients with different forms of JRA, Thompson et al. showed that most patients with JRA had multiple synovial T-cell clones using different TCR Vb segments (110). However, some differences in patterns of TCR Vb use among clinical subtypes of JRA were noted, a finding suggesting that different antigens may be involved in different clinical forms of JRA. Subsequent extensive DNA sequencing efforts revealed that despite a wide diversity of CDR3 motifs, certain similarities among multiple synovial T-cell clones derived from an individual patient could be found (114). These data provided an indirect support for the concept that the persistent synovial inflammation in JRA may indeed be antigen driven. However, the wide variety of both Va and Vb segments, and the CDR3 motifs used by clonally expanded T-cell populations, precluded their use as potential targets for specific immunotherapy.

gd T CELLS

A clonal analysis of JRA synovial T cells responding to IL-2 revealed the presence of a distinct T-cell population with CD3$^+$ CD4$^-$ CD8$^-$ phenotype (105). Further investigation demonstrated that these cells had gd (instead of ab) TCRs. Subsequent immunohistochemical studies showed that gd T cells comprised about 5% to 10% of T cells infiltrating the JRA synovial membrane (115,116). In most cases, gd T cells are clustered in lymphoid follicularlike structures (116). In approximately 30% of patients with JRA, proportions of gd T cells were found to be increased in synovial fluid relative to peripheral blood (117). Many synovial gd T cells express CD25, CD69, and HLA-DR (115). Clonal expansion of synovial gd T cells has been demonstrated in several studies.

Although the specificity and function of human gd T cells are unknown, at least some of these cells appear to respond to mycobacterial antigens and stress proteins (in particular, hsp 65) (118,119). Synovial fluid T cells from patients with JRA have been shown to respond selectively to mycobacterial antigens and hsps (85). At low levels, hsp is constitutively expressed in practically all organisms, and increased synthesis can be induced by a variety of stress conditions including inflammation. A high degree of homology exists between bacterial and mammalian hsps. During infection, the synthesis of hsp is elevated on both microorganism and host cells. If cross-reactivity occurs between these hsps, it can be inferred that the immune response to hsp would be potentially autoreactive. Using monoclonal antibodies that recognize human hsp 65, high levels of hsp expression were demonstrated within synovial lining cells in JRA synovial tissue (120). Close juxtaposition of gd T lymphocytes and cells expressing hsp has been shown in histochemical studies of multiple sclerosis (121), but whether such events play a pathogenic role in JRA remains to be determined. In fact, one study demonstrated some correlation between the high immune responses to hsp and a relatively benign course of arthritis (122). In another study, a synovial fluid T-cell clone responsive to hsp secreted a Th2 cytokine IL-4 that may have antiinflammatory effects in JRA (123).

T-CELL–DERIVED CYTOKINES

In RA, despite heavy T-cell infiltration, T-cell–derived cytokines are usually detectable only with highly sensitive techniques such as polymerase chain reaction. In contrast, in JRA, T cells appear to be more active (93). Th1 cytokines such as interferon-g or TNF-b are found to be present in most of the synovial tissue and fluid samples (87,124). In one study, TNF-b expression correlated with the occurrence of lymphocytic aggregates (100). Th2 cytokines such as IL-4 are also found occasionally, mainly in tissue and fluid samples obtained from patients with more restricted disease (124). In another study, the *in vitro* production of cytokines by T-cell clones derived from synovial fluids of pauciarticular JRA were Th1/Th0 (125). These findings are consistent with the concept that Th1 cytokine response that has been implicated in orchestrating cell-mediated immune responses may promote disease in JRA (104). IL-10 is another Th2 cytokine that has been consistently found in JRA tissues, although its pathogenic significance is not entirely clear (124).

Investigators have suggested that T cells infiltrating the inflamed synovium in children with JRA are of a skewed cytokine phenotype, perhaps as a result of selective recruitment of cells expressing chemokine receptors characteristic of Th1 cells. This hypothesis has been supported by two studies that demonstrated enrichment of synovial fluid lymphocytes for T cells expressing CCR5 and CXCR3, markers for Th1 phenotype (126,127). Another study showed enrichment of JRA synovial tissues for chemokines capable of stimulating CCR5 and CXCR3 receptors, that is, IP-10 and RANTES (regulated on activation normal T cell expressed and secreted) (102). Another chemokine capable of upregulating interferon-g production, I-309, was found to be abundant in this study as well.

Natural Killer Cells

Natural killer (NK) cells in JRA have been assessed in several studies, with contradictory results. Odum et al. found no significant differences in the distribution of cells bearing CD16$^+$, a surface marker for cytotoxic T lymphocytes (103). However, some functional studies indicated decreased NK numbers (128), and an inverse correlation between disease activity and NK cell content was noted.

Macrophage-Fibroblast Contribution to the Disease

Increased numbers of macrophages and dendritic cells and proliferation of fibroblastlike and macrophagelike synoviocytes are typical fea-tures of JRA synovium (88). The abundance of cytokines secreted by these cells (e.g., IL-1, IL-6, IL-12, and TNF-a) provides further evidence for the major role of these cells in the perpetuation of the synovial inflammation.

Martini et al., in 1986, noted high spontaneous production of IL-1 by mononuclear cells derived from peripheral blood of JRA patients (129). In another study, levels of IL-6 expression in the sera of patients with systemic JRA were shown to correlate with the extent of joint involvement (130). Serum IL-12 levels have been shown to be elevated in patients with JRA (131). In 1991, Eberhard et al. detected TNF-a, TNF-b, and IL-6 in synovial fluids of a large proportion of JRA patients (87).

Prompted by the demonstration of the pivotal role of TNF-a in the inflammatory cascade in RA (132), analysis of TNF expression and the distribution of the TNF receptors in JRA synovial tissues provided further evidence for the role of both TNF-a and TNF-b in the amplification of the synovial inflammation in JRA as well (100). In most of the synovial tissue samples, cells staining for TNF-a were found within dense cellular aggregates and in the “mantle” of cells surrounding the aggregates. The pattern of distribution of TNF-a staining was similar to the pattern observed with CD68 staining. The level of expression of TNF-a correlated closely with the degree of inflammatory infiltration of synovia.

High levels of TNF expression were associated with wide distribution of TNF receptors in most tissues. For instance, staining with the antibodies specific for the p55 and p75 TNF receptors revealed that cells with diverse morphology expressed TNF receptors on their surface. Higher numbers of positive cells were observed in the areas of cellular aggregates adjacent to synovial venules. The most intense staining, however, was observed on the endothelial cells lining the synovial blood vessels (100). Previously, Oen et al. had shown that activated lymphocytes from peripheral blood of children with JRA adhered to vascular endothelial cells stimulated with TNF-a *in vitro* (99). *In vivo,* this would lead to the extravasation of such cells and their accumulation at the site of inflammation. These observations suggest that one of the mechanisms by which TNF-a amplifies inflammation in JRA is its ability to promote trafficking of the inflammatory cells from peripheral circulation into synovium.

Silverman et al., in 1987, demonstrated that supernatants of mononuclear cells obtained from patients with systemic or polyarticular JRA caused elevated proteoglycan release when they were cultured with articular cartilage (133). Key et al. showed that supernatants of monocytes from JRA patients were able to cause bone resorption (134). Although neither of the studies looked at the TNF-a activity in the examined samples, it is likely that the described effects, at least in part, were caused by this cytokine.

The proinflammatory role of TNF-a in the rheumatoid disease does not appear to be limited to synovium. TNF-a is a major proinflammatory cytokine with both local and systemic effects (135). At low concentrations, it acts locally, mostly as a paracrine and autocrine regulator of leukocytes and vascular endothelial cells. These actions contribute to the accumulation of the inflammatory cells at local site of inflammation. When production of TNF-a is increased significantly, it is released into peripheral circulation and has numerous systemic effects (135). For instance, TNF-a acts on hypothalamic regulatory systems to induce fever. It also stimulates hepatocytes to produce certain proteins that are known to constitute the acute-phase response. When TNF-a is produced at high levels over a long period, it also suppresses bone marrow and may cause metabolic alterations leading to cachexia. Although similar clinical symptoms are often observed in JRA, particularly in the systemic form, it is still not clear to which extent they can be attributed to TNF-a. An increasingly beneficial clinical experience with anti TNF-a treatment strategies, however, does provide evidence for the critical role of TNF-a in the developments of the systemic features of JRA as well.

B-Cell Contribution to the Disease

Although T cells are usually more numerous in inflamed synovial tissue, B-cell activity appears to be enhanced in both peripheral blood and synovial compartment. Lindblad et al. described synovial tissue specimens from patients with JRA with significantly increased numbers of immunoglobulin-synthesizing cells (94). These cells tended to be focally aggregated. Functional studies produced similar results. With the use of reverse hemolytic plaque assay, Tsokos et al. demonstrated increased

numbers of B cells spontaneously producing immunoglobulins in the peripheral blood of patients with JRA (136,137). As a consequence of increased B-cell activity, hyperglobulinemia, circulating immune complexes, ANAs, and RFs are common findings in many patients with JRA.

Serum Immunoglobulins

The serum levels of immunoglobulins are elevated in many patients with JRA (138). In the studies conducted by Cassidy et al., 37% of patients with JRA exhibited hyperglobulinemia in at least one immunoglobulin class, and this correlated with the activity of the disease (139). There were also some differences in immunoglobulin levels among different clinical subgroups. The degree of immunoglobulin elevation was greater in patients with polyarticular and systemic disease than in those with pauciarticular JRA. Elevated IgA levels were associated with the appearance of cartilage erosions. Conversely, hypoglobulinemia has also been associated with JRA (140,141), and a few investigators have described a selective decrease in serum IgA concentrations in some patients (139,142).

Rheumatoid Factor

RFs, the autoantibodies directed against the Fc region of human IgG, are found in the serum and synovial fluid of most patients with RA. RF can only rarely be detected in patients with JRA by the classic Rose-Waller reaction or latex fixation method, although the probability of finding it increases with the age and the duration of the disease. Allen and Kunkel demonstrated a so-called hidden RF defined as IgM 19S antiglobulin (143). This antiglobulin is “hidden” because of its occupied binding sites. Gel filtration at acid pH dissociates IgM from IgG and allows IgM RF to fix complement in hemolytic assay. Moore et al. found that hidden RFs correlated with clinically active disease in 67% of patients with JRA (144,145).

Antinuclear Antibodies

The reported incidence of ANAs in patients with JRA varies from 4% to 88%, depending on the clinical subtype and the laboratory technique used (13). Thus, ANAs are uncommon in systemic-onset disease, whereas more than half of children with pauciarticular JRA have ANAs in serum. Children who are ANA positive carry an especially high risk of chronic anterior uveitis (13,146). Rahim et al. demonstrated local synthesis of ANA in the eyes of patients with chronic iridocyclitis. ANAs are occasionally found in patients with polyarticular disease, mostly in those who are RF positive (147).

The full specificity of these antibodies remain to be determined. Some studies indicated that ANAs in most patients are directed to a ribonucleoprotein that requires both RNA and protein components for antigenic integrity (148). Szer et al. concluded that ANA specificity profiles were highly individual and did not appear to correlate with disease subtype or activity (149). Several studies found that antibodies to many antigens typical for other rheumatic diseases, such as the extractable nuclear protein, centromere proteins, scleroderma Scl-70, Ro, La, and double-stranded DNA, are generally absent in JRA. More recently, the antibodies to the 45-kd DEK nuclear antigen, a putative oncoprotein, have been associated with the pauciarticular type of JRA, particularly in patients with a history of iridocyclitis (150). However, the presence of these antibodies does not appear to be limited to JRA (151).

Other Autoantibodies

Anticollagen (152), antiretinal (153,154 and 155), and anti–T-cell antibodies (156,157) have been described, but their relevance to the pathogenesis of JRA remains to be determined. Investigators suggested in one study that anti–T-cell antibodies intensify immunoregulatory disturbances in autoimmune disease (158).

Circulating Immune Complexes and Complement Activation

Abnormal antibody production and probably defects in clearance function of the reticuloendothelial system may result in an increased concentration of circulating immune complexes in serum. Attempts at estimating this increase have yielded inconsistent results. With the help of C1q binding assay, Rossen et al. demonstrated that 22% of patients with JRA have elevated levels of circulating immune complexes, and these investigators found that the concentration of circulating immune complexes correlated with the severity of the disease (159). The polyethylene glycol precipitation method used by Balogh et al. detected circulating immune complexes in the serum of only 1 of 13 patients (160). Using 4 different techniques, Moore et al. were able to demonstrate elevated levels of circulating immune complexes in 79% of patients with JRA by at least a single method (161). Findings in synovial fluid have also been inconsistent. Using the C1q binding assay, Miller et al. found elevated levels of circulating immune complexes in some patients, but the results varied substantially among patients (162). Martin and Pachman, using the Raji method, found normal levels of circulating immune complexes in synovial fluids of patients with pauciarticular JRA (163).

Despite these conflicting results, many investigators think that deposition of circulating immune complexes in synovial compartments may result in complement system activation, which, in turn, causes tissue damage. Indeed, some evidence to support this hypothesis has been obtained. Using a qualitative assay for C3c and C3d, Miller et al. demonstrated these products in the serum of 7 of 10 patients with active systemic disease, in 16 of 29 patients with active polyarthritis, in 7 of 20 with active pauciarthritis, and in only 2 of 20 with inactive disease (164). However, the same study failed to find C3c and C3d in synovial fluid. In contrast, in 1986, Miller et al. demonstrated elevated levels of C3d in some patients with JRA (162). In the same year, Miller et al. characterized systemic and, to a lesser degree, polyarticular subgroups by elevations of both C4d/C4 and C3d/C3 ratios, whereas only the C3d level was increased in pauciarticular disease (165).

Mollnes and Paus contrasted the complement activation cascade (C1 to C5), which participates in inflammatory reactions mostly by releasing anaphylotoxins with the terminal cascade, which results in the formation of the cytolytic macromolecular complex (166). The entire cascade was shown to be involved in the activation process both in peripheral blood and in the synovial compartment. Furthermore, weak deposits of C3b and C3dg, or terminal complement complex were found in the synovial tissue of a few patients with JRA. Neither these studies nor studies of RF revealed any associations between clinical activity and the degree of complement activation. On the contrary, the level of C-reactive protein correlated strongly with the degree of complement activation. The main conclusion drawn from this study was that mechanisms that have other than specific immunologic causes (such as C-reactive protein) may be responsible for complement activation in JRA.

Several studies have shown that the red blood cell C3b receptor may be involved in the impaired clearance of circulating immune complexes (167), but no significant deviations in the expression of this receptor have been found in patients with JRA (168). A general impression is that circulating immune complexes may contribute to the perpetuation of chronic inflammation in JRA, but they do not play a major role in its pathogenesis.

TREATMENT

Traditionally treatment starts with nonsteroidal antiinflammatory drugs. If these drugs prove ineffective, treatment progresses to more aggressive “advanced” pharmacotherapy that includes slow-acting disease-modifying medications (169). Oral or subcutaneously administered methotrexate is clearly gaining favor as a second-line drug for the treatment of JRA. The systemic administration of prednisone and its analogs is limited to short-term use in severe instances of systemic-onset JRA. Cyclosporine has been shown to be effective in treatment of macrophage activation syndrome complicating this form of the disease as well (19). The hepatotoxic effect of salicylate therapy is not uncommon in patients with systemic onset of the disease (170). Intraarticular injections of steroids are commonly used in pauciarticular disease. However, these treatments have had limited effectiveness in modifying the disease processes, perhaps with the exception of methotrexate.

Although several biologic agents have been tried in JRA, only treatment strategies directed against TNF-a have been shown to be effective (171). Two types of TNF inhibitors have been developed: one is a humanized monoclonal antibody (infliximab) that binds TNF-a and selectively blocks its activity. Another TNF inhibitor, etanercept, is a genetically engineered fusion protein consisting of two identical chains of the recombinant extracellular human TNF receptor p75 monomer fused with the Fc domain of human IgG1. It effectively binds both TNF-a and TNF-b and inhibits their activity (172). The ability of etanercept to block both TNF-a and TNF-b may be particularly relevant to JRA, because, in contrast to RA synovium, in which TNF-b is rarely detectable, both cytokines have been shown to be expressed in JRA synovial tissues. A clinical trial of etanercept in patients with polyarticular disease demonstrated a substantial, rapid and sustained clinical response in 76% of patients (171). These results can likely extrapolated to other clinical forms of JRA as well.

PROGNOSIS

In 1991, 31% to 55% of patients with JRA had been reported to have active arthritis 10 years after disease onset (10). Joint space narrowing and cartilage destruction developed in approximately 25% of patients with pauciarticular onset, 50% of patients with polyarticular onset, and 50% of patients with systemic onset. As a result, 9% to 48% of patients with JRA develop marked disability 5 to 15 years after onset of the disease (173), and 30% to 50% of patients with JRA begin their adult lives with active arthritis (10).

The limited effectiveness of existing therapies encouraged many pediatric rheumatologists to consider the earlier use of aggressive treatment in some patients. The introduction of methotrexate as a main second-line medication in the late 1990s and its use early in the course of the disease have seemed to improve the overall prognosis significantly. It remains to be determined to what extent the course of the disease can by altered by TNF blockers. Some evidence indicates, however, that in

adult RA etanercept may significantly slow the progression of cartilage destruction and thus may improve the overall outcome of the disease.

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39 SYSTEMIC LUPUS ERYTHEMATOSUS

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Systemic lupus erythematosus (SLE) is an idiopathic systemic autoimmune disease that is unique among autoimmune illnesses in its breadth of clinical manifestations, in the variability of disease progression, and in the nature of its underlying immunologic abnormalities. SLE potentially involves every organ system. Although it predominantly affects women of child-bearing age, younger and elderly persons also may be affected. The extent of clinical heterogeneity is reflected in the fact that the diagnosis of lupus is used both for the patient with relatively benign skin and joint conditions and for the patient with fulminant, life-threatening disease affecting the central nervous system (CNS), lungs, gastrointestinal tract, and kidneys. This diversity of clinical manifestations demands cognizance on the part of physicians in recognizing this disease in its common and less common presentations and in anticipating potential complications. Judicious therapy requires careful consideration of the extent, severity, and pace of disease.

It has been nearly 50 years since the observation was made that antibodies against normal cellular constituents could be found in the sera of patients with lupus (1,2 and 3). The hallmark of lupus remains the presence of serum antibodies directed against nuclear antigens (antinuclear antibodies, or ANAs). Despite significant advances in describing the specificity and pathogenicity of certain of these autoantibodies, the questions of why they arise and what their role is in disease pathogenesis remain largely unanswered. Development of effective and specific treatment for most of the manifestations of lupus has been impeded by limited understanding of the mechanisms that underlie disease. Specifically, the development of more effective therapies will require an understanding of why tolerance to self-antigens is lost, which genes are responsible for the marked familial predisposition to disease and how these genes interact to cause disease, and the mechanisms whereby these factors lead to chronic tissue injury. This chapter reviews the epidemiology and clinical manifestations of lupus and concludes with a discussion of the current understanding of the underlying immunologic abnormalities that predispose to lupus.

It is interesting to note that convincing evidence for the first description of lupus cannot be found until the 19th century, raising the question of whether lupus is a new disease. Given the variability in disease manifestations, however, it is difficult to determine when the first case of SLE was recognized and reported. Cazenave and Schedel are generally credited with the first description of disease in 1833 (4), when they described cutaneous involvement of the face characteristic of lupus and noted a marked preponderance of women with this condition. Hebra in 1846 described the classic malar rash of acute cutaneous lupus and, along with Kaposi, first described the systemic nature of the disease in the late 19th century (5). The modern study of lupus began in 1948, when Hargraves et al. observed that the sera of lupus patients was capable of inducing the phagocytosis of nucleated cells *ex vivo* (6). This phenomenon (the lupus erythematosus, or LE cell) remained poorly understood until 1957, when Friou, Holman and Kunkel described the binding of factors in lupus sera to the nucleus (1,2). Two years later, the serum binding factor was identified as an antibody to nucleoprotein (3). These serologic abnormalities are unique to lupus and have served as diagnostic criteria formulated by the Rheumatism Association in 1971 (now the American College of Rheumatology, or ACR). They remain invaluable clinical tools in the evaluation of patients with connective tissue disease (7). Importantly, these observations spurred interest in investigating the role of the immune system in the causation of lupus.

EPIDEMIOLOGY

The demographics of SLE are striking: There is a markedly skewed distribution of disease with respect to sex, age, and race (8). SLE is much more frequently found in women, with a female-to-male ratio of 9:1 in adults (9,10). The female-to-male ratio is much smaller in children and in elderly persons, dropping to 2:1. Although SLE can occur at any age, the peak incidence is generally seen between the second and fourth decades of life (9).

The reported prevalence of SLE in the general population of the United States is 40 to 50 cases per 100,000 (11), with an incidence between 1.8 to 7.6 cases per 100,000 people per year (9). Lupus is more common in Asians, African Americans, African Caribbeans, and Hispanic Americans than in whites (11,12 and 13). In addition, disease is more severe among Hispanics and African Americans. Interestingly, lupus is reported to be uncommon in Africa (14), although reporting bias makes definitive conclusions uncertain. The reasons for these ethnic differences remain unknown, prompting investigation into identifying genetic and environmental factors that predispose to systemic autoimmunity.

Genetics

Lupus is a disease with strong genetic predisposition. This is most clearly demonstrated by the high concordance rate (25%–50%) for lupus in monozygotic twins (15,16). The risk of developing lupus in dizygotic twins and in first-degree relatives of lupus patients is also substantial, with a relative risk of 20 (16). Although the genes predisposing to lupus are unknown, various genetic associations with disease have been reported. These include the genes coding for the major histocompatibility complex (MHC), the complement system, proteins involved in programmed cell death (*apoptosis*), and cytokines (17,18).

Of these, the MHC locus has been particularly well studied. There is an increased incidence of different haplotypes encompassing MHC class I, II, and III genes in SLE patients of different ethnic backgrounds; this difference may be due in part to linkage disequilibrium (17,18 and 19). Most of these haplotypes carry human leukocyte antigen (HLA)-DR2 or DR3 (17,18 and 19). Disease associations extend to particular subtypes of the MHC class II DR2 allele: DRB1*1501 is commonly seen in white and Asian persons with lupus, whereas DRB1*1503 is seen in African-American lupus patients. HLA-DR3 (usually associated with a C4A null gene; see later discussion) is seen in lupus patients of European descent. In general, these HLA alleles have a strong association with certain autoantibodies. For example, an association between anti-Ro antibody and DR2/DQw1 has been reported (19), raising the possibility that only certain MHC class II alleles are capable of presenting self-antigen to autoreactive T cells. This appears to be a critical step because activation of autoreactive T cells and subsequent T-cell help to autoantigen-specific B cells are crucial to the production of autoantibodies (to be discussed in the section on immunopathogenesis).

Deficiencies of genes encoding the early components of complement, including C1q, C4, and C2, are strongly associated with SLE (18). Most strikingly, about 90% of patients with C1q or complete C4 deficiency have SLE (20). Similarly, defects in any of the four C4 genes (two genes each for C4A and C4B) are associated with lupus, especially the C4A null deficiency (21). The C4 null allele is the most commonly found complement deficiency seen in lupus, followed by C2 deficiencies. C1s or C1r deficiencies are rare, but these persons again present with SLE. The results of these human studies are consistent with the observation that mice genetically engineered to lack one of the early complement components develop a lupuslike syndrome (22). One hypothesis is that normal physiologic processes such as apoptosis produce a steady stream of self-antigens that must be removed, with the aid of the complement system, to maintain immunologic tolerance. Failure of these or other immune complex removal mechanisms results in excessive stimulation of the adaptive immune system, which triggers systemic autoimmunity.

Associations between allelic polymorphisms of FcgRII, FcgRIII genes, and SLE have been reported in several ethnic groups (23,24 and 25). The FcgRII receptor binds immunoglobulin G (IgG) bound to antigen and is expressed on the surface of phagocytic cells. It differs in its affinity for IgG, depending on the isotype: IgG1 has the highest affinity, followed by IgG3 and IgG2. Ligation of the receptor with IgG bound to antigen results in the uptake of the immune complex and activation of the phagocytic cell. There are two common alleles of FcgRII, with either high (histidine at position 131, H131) or low (arginine 131, R131) affinity for IgG2 and IgG3. An increased frequency of homozygous R131 and a decreased frequency of homozygous H131 have been reported in African-American lupus patients, particularly those with nephritis (23,25). These alterations may result in a decreased ability to remove immune complexes from the serum, thereby promoting their deposition in tissues such as the kidney. This association between Fc receptor polymorphisms and lupus was not found in white, Afro-Caribbean, and Chinese patients with SLE (26). A strong association between alleles of the Fc receptor of natural killer (NK) cells and monocytes/macrophages (FcgRIIIa) having lower affinity for IgG1 and SLE has been reported as well (27).

Low levels of serum opsonins or their receptors also may lead to a decreased ability to remove immune complexes from the circulation. Allelic polymorphisms that result in low levels of serum mannose-binding protein, which can recognize carbohydrate on the surface of bacteria and activate complement, are associated with SLE in patients from different ethnic backgrounds, particularly African Americans (28,29 and 30).

Apoptosis may be important in the initiation and propagation of systemic autoimmunity as a mechanism to generate self-antigens and, as a consequence, immune complexes (to be discussed in the section on immunopathogenesis). The discovery in mice of a mutation in a single gene (*Fas* or CD95 mutation), which results in defective apoptosis of cells, including lymphocytes (31), spurred investigation into whether apoptosis is aberrant in human disease and in murine models of lupus. Individuals with homozygous *Fas* deficiency have been identified (32). They develop lymphadenopathy, splenomegaly, and autoantibodies but do not develop the end-organ disease characteristic of lupus (32). Most lupus patients do not have defective expression of CD95 or its ligand, CD95L: Only one (heterozygous) mutation in *Fas* ligand was found in 75 SLE patients (33). Similarly, a comparison of mRNA expression of Fas, *FasL,* and *bcl-2*, a molecule promoting cell survival, did not find significant differences between lupus patients and controls (34). Homozygous *Fas* deficiency in mice (the *lpr/lpr*mutation) accelerates autoimmune disease but is not necessary or sufficient for the development of systemic autoimmune disease (35). Normal healthy, nonautoimmune mice bred to carry the *lpr/lpr*mutations do not develop significant autoimmune disease (35).

Case-control studies looking for polymorphisms of cytokine and cytokine receptor genes and SLE have yet to identify any major disease associations. An increased frequency of a polymorphism of the interleukin-1 receptor antagonist (IL-1Ra) was reported in one study of white and Japanese SLE patients with discoid lesions and photosensitivity (36,37). Similarly, no major associations between the genes coding for the T-cell receptor (TCR) and disease have been uncovered. One study showed no relation between polymorphisms of TCR Va and Vb chains and SLE (38), suggesting that the germ-line T-cell repertoire is not significantly different in individuals with lupus compared with healthy individuals.

Major advances have been made in understanding the genetics of murine lupus and will be discussed fully in the section on immunopathogenesis. These studies have shown conclusively that loci on murine chromosomes 1, 4, and 7 acting in concert are sufficient to induce the development of systemic autoimmunity and immune complex–mediated glomerulonephritis closely resembling human lupus (39,40). These studies prompted searches for disease-associated loci in syntenic regions of the human genome. Indeed, a telomeric region on 1 (1q41–42) has been associated with lupus in a study of 54 dizygotic sibling pairs concordant for lupus (41). Other groups have not been able to reproduce this genetic association (42), however, and the interested reader should review the editorial that accompanied one of the negative reports (43). Rapid advances in the understanding of the mechanisms by which loci and, ultimately, genes in murine models induce autoimmunity will direct human studies toward the corresponding pathways.

Environmental Factors

A great amount of effort has been spent in the last 30 years trying to elucidate the causes of lupus. No causative factors have been identified, but several environmental triggers of lupus have been established. Ultraviolet (UV) light exposure, especially UVB, can cause flares of systemic lupus, involving both the skin (acute cutaneous lupus) and internal organs. Different types of medications, ranging from antihypertensives to anticonvulsants to antibiotics, have been definitively associated with the development of ANAs and lupuslike disease. It should be noted that the syndrome of drug-induced lupus differs from (idiopathic) SLE in many important respects, including the types of autoantibodies produced and the range of clinical manifestations, which is discussed in the section on drug-induced lupus. Therefore, one must be cautious in extrapolating any mechanisms implicated in causing drug-induced lupus to the study of idiopathic SLE.

One possible molecular mechanism to explain why some lupus patients are photosensitive and how UVB exposure leads to the development of cutaneous lesions was suggested by *in vitro* studies of apoptotic cells. Certain nuclear and cytoplasmic autoantigens specific to lupus (e.g., Ro antigen) are translocated to the cell surface after UVB exposure of keratinocytes from normal subjects (44). What happens next is unclear: Presumably, autoantigens on the cell surface are now “exposed” to the immune system and then can drive an adaptive immune response or be bound by circulating autoantibodies with triggering of an inflammatory cascade. Supporting this notion are data demonstrating that (a) autoantibodies (anti-Ro, anti-La, and anti-RNP) bind to UVB-exposed keratinocytes (45) and presumably injure the cells; and (b) photosensitivity in white SLE patients is associated with an anti-Ro response (46). Not all autoantigens (chromatin, for example) are expressed on the surface of apoptotic cells, however, and therefore there must be other mechanisms that drive the humoral response against these antigens in lupus. In addition, one must explain how this normal physiologic process (apoptosis) leads to disease in patients with lupus but not in healthy individuals. UV light and drugs inducing lupuslike syndromes also can alter cell function by decreasing DNA methylation (thereby increasing gene expression) and enhancing the expression of integrins, such as leukocyte factor antigen-1 (LFA-1) on lymphocytes (47), thereby potentially altering T-cell antigen-presenting cell (APC) interaction.

There is weaker correlation to disease for certain viral infections, diet (e.g., high-fat and high-caloric diets), certain cosmetics (hair dyes and lipstick), and organic solvents (trichloroethene) (10). In addition, psychological factors, such as stress, depression, and fatigue, may exacerbate the manifestations of lupus in some patients. How these putative factors “trigger” disease remains poorly understood. Given the clinical heterogeneity of lupus, it is not surprising that no “smoking gun” has been identified. Patients who have an insidious onset of disease are likely to have different triggers than patients with a hyperacute presentation. In addition, there are likely many different environmental factors that can initiate disease in a genetically susceptible person.

The marked female preponderance of SLE during the reproductive ages has historically driven research into the role of sex hormones in SLE. Estrogen stimulates B cells, T cells, and macrophages *in vitro* to enhance cytokine release and increase the expression of MHC and endothelial adhesion molecules (48). In New Zealand black and white (NZBxNZW) mice, lupuslike disease occurs earlier and is more severe in female mice; chemical or surgical castration improves the disease (49). Decreased levels of androgens, including dehydroepiandrosterone (DHEA), are seen in female lupus patients (50), in addition to abnormal estrogen metabolism. Estrogen use in postmenopausal women is associated with a small increase in risk for developing lupus (51). One must bear in mind, however, that the uneven sex distribution may be due to the absence of gene(s) expressed on the Y chromosome. In some murine models of lupus (BXSB), disease is linked to a locus (*Yaa*) on the Y chromosome (52) whose gene product contributes to B-cell hyperactivity (53).

Finally, viral infections, particularly Epstein-Barr virus (EBV) infection, are possible etiologic agents in lupus. The evidence is circumstantial: Some serologic studies demonstrated increased antibody titers against EBV antigens in SLE patients (54). Polyclonal B-cell activation occurs in lupus, and the observed increases in titer may be attributable to this phenomenon. EBV does infect B cells through the B-cell complement receptor (CD21) and therefore may play a role in increasing B-cell activation in the disease. The data suggesting molecular mimicry between viral and self-antigens are limited to the observation that the Epstein-Barr nuclear antigen II (EBNA II) of EBV and the Sm autoantigen in SLE share partial sequence homology (55). Taken together, however, there is no solid evidence to date supporting a direct causal relationship between an infectious agent and SLE.

CLINICAL MANIFESTATIONS

Systemic lupus erythetmatosus can affect the skin, joints, kidneys, lungs, nervous system, serosal cavities (pleural, pericardial, and peritoneal), hematopoietic system or other organs of the body (Table 39.1) (56,57). Despite great diversity in the clinical manifestations of lupus, certain common clinical features, when taken together, form a pattern of disease unique to lupus. This group of clinical features, in conjunction with characteristic immunologic abnormalities, forms the core of the current diagnostic criteria of the American College of Rheumatology (Table 39.2). Although these criteria were developed for the appropriate classification of patients for clinical studies, they are useful in clinical practice in the evaluation of all patients with systemic rheumatic diseases.

Clinical Signs	%
Fever	84
Arthritis/arthralgias	92
Myalgias	48
Skin involvement	72
Photosensitivity	33
Malar rash	57
Raynaud's phenomenon	18
Psychosis	12
Seizure disorder	14
Pericarditis	31
Pleurisy	45
Pleural effusion	30
Abnormal urinalysis	46
Nephrotic syndrome	23

Adapted from Dubois et al. Clinical manifestations of systemic lupus erythematosus. JAMA 1964;190:104–111, with permission.

TABLE 39.1. Cumulative Frequency (%) of Clinical Manifestations in Systemic Lupus Erythematosus

[illegible]	[illegible]

TABLE 39.2. The Revised Criteria for the Diagnosis of Systemic Lupus Erythematosus[a]

Constitutional Symptoms

Nonspecific systemic symptoms, such as malaise, fatigue, fevers, anorexia, and weight loss, are common in SLE patients. The most common presentation of lupus is a mixture of these constitutional complaints with skin, musculoskeletal, and mild hematologic disease. The pattern of organ involvement during the first few years of illness is usually maintained subsequently (58,59). Fatigue occurs in about 80% of SLE patients. Fever is seen in more than 50% of patients, particularly in those with active disease (60). High fevers (>103°F) may accompany acute severe disease, but concurrent infection always should be suspected in a lupus patient with fever. The presence of shaking chills, leukocytosis (especially in the absence of corticosteroid therapy), elevated C-reactive protein (CRP), and normal complement levels are helpful in distinguishing bacterial infection from a lupus flare.

Systemic symptoms usually are accompanied by other organ involvement; therefore, treatment is directed by the extent and severity of the affected organs in an individual patient. The sense of fatigue and malaise often improves with rest and nonsteroidal antiinflammatory drugs (NSAIDs).

Cutaneous Manifestations

The skin and mucous membranes are major targets of the autoimmune response in lupus, affecting over 80% of patients (57) at some point during the course of illness. The dermatologic manifestations of lupus are extremely diverse, ranging from acute, transient facial rashes in adults to bullous disease, to chronic, scarring, and disfiguring rashes of the head and neck. Despite this clinical diversity, most of these rashes share three features in common, and these features offer insight into the immunopathogenesis of disease: (a) they are brought on or are exacerbated by exposure to sunlight (UVB much more than UVA), (b) IgG and complement are found in a characteristic location in the skin, and (c) small blood vessels in the dermis (arterioles, capillaries, and postcapillary venules) are targeted by the inflammatory response. A discussion of these common attributes as they relate to cutaneous lupus is the focus of this section.

Acute cutaneous lupus erythematosus is an erythematous rash of the face that occurs in more than half of patients with SLE. It is a transient, nonscarring erythematous maculopapular rash that can affect the entire face or can be limited to the cheeks (*malar rash*) (Fig. 39.1). Acute cutaneous lupus usually develops after UVB exposure (*photosensitivity*). The malar rash usually lasts for several days and does not scar on resolution of the erythema.

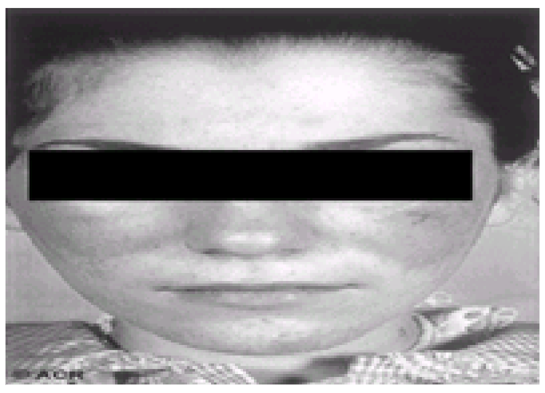

Figure 39.1. Malar rash. An erythematous facial eruption on the malar and chin areas. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (See Color Figure 39.1.)

On histologic examination, there is minimal degeneration of the basal cell layer of the epidermis. Immunofluorescence, however, is striking: IgG and complement are deposited at the dermal–epidermal junction in both affected (lesional) skin and typically in normal-appearing (*nonlesional*) skin as well. These changes are considered to be almost pathognomonic for lupus. These observations suggest that the basal cell layer of the epidermis is being injured through antibody-mediated activation of the complement system or by antibody itself.

The target(s) of these autoantibodies is unknown. Circumstantial evidence implicates the nuclear ribonucleoprotein Ro in the immunopathogenesis of cutaneous lupus. UVB exposure is known to cause the translocation of Ro to the cell surface in keratinocytes, as discussed previously (44). Anti-Ro autoantibodies bind *in vitro* to the surface of UV exposed keratinocytes (45) and presumably contribute to cell injury through a type II or III immunologic mechanism. Finally, photosensitivity in lupus patients is associated with the presence of anti-Ro antibodies. Whether anti-Ro antibody is directly contributing to tissue injury or is binding incidentally to damaged keratinocytes remains unknown, however.

Subacute cutaneous lupus erythematosus (SCLE) is a hyperkeratotic annular or psoriasiform erythematous rash with a predilection for the sun-exposed areas of skin. It is present in about 10% of lupus patients. Conversely, individuals may have this form of cutaneous lupus with minimal to no disease in other organ systems and have an excellent prognosis. The rash is more persistent than acute cutaneous lupus but rarely scars.

The epidermal changes are more prominent in SCLE than in acute cutaneous lupus: There is marked degeneration of the basal layer of the epidermis with a mononuclear cell infiltration of mostly CD4+ T cells about the dermal–epidermal junction and in the dermis around small blood vessels and appendages. Immunofluorescence again reveals IgG and complement deposition at the dermal–epidermal border in a granular pattern suggesting a type III mechanism of immunologic injury. There is an even stronger association with the anti-Ro response than in acute cutaneous lupus; in addition, most patients with SCLE also have the

MHC class II allele HLA-DR3 (61,62). The reasons for this genetic association between autoantibody production and MHC gene remain unknown. One can speculate that some peptide from the Ro antigen is being presented to CD4+ helper T cells by an APC bearing the DR3 molecule, but to date there is no strong evidence to support this hypothesis.

Chronic cutaneous lupus lesions are either circular (*discoid*) or irregular coalescent erythematous papules or plaques with adherent scale (Fig. 39.2). Often the borders of the lesion are erythematous, representing an advancing front of inflammation. The inflammatory cells are again mostly CD4+ T cells in the dermis. The centers of the lesions are often hypopigmented and atrophic. Like acute and subacute lesions, they usually appear on sun-exposed areas with a predilection for the ears, scalp, and face. Unlike acute and subacute cutaneous lupus, however, chronic cutaneous lupus leaves scars that can be quite disfiguring. Occasionally, these lesions may affect the extremities and trunk as well as the sun-exposed areas above the neck (*generalized discoid lupus*). Discoid lesions, like the malar rash of acute cutaneous lupus, are specific for lupus and are therefore included in the ACR diagnostic criteria.

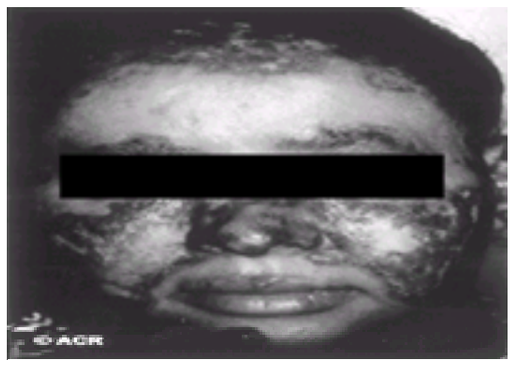

Figure 39.2. Discoid lupus. An erythematous and hyperpigmented rash on the face with central scarring and epidermal atrophy. Follicular plugging is also seen. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (See Color Figure 39.2.)

Chronic cutaneous lupus erythematosus (discoid lupus) lesions develop in up to 25% of SLE patients but may also occur in the absence of any systemic manifestations. These individuals with isolated discoid lupus typically lack any of the characteristic autoantibodies of SLE, including anti-Ro. About 5% to 10% of individuals presenting with discoid lupus without concurrent systemic involvement eventually develop other manifestations of SLE, such as immune-mediated thromobcytopenia.

The preceding discussion of acute, subacute, and chronic cutaneous lupus focused on the role of ubiquitous nuclear autoantigens, such as Ro in immunopathogenesis. If these forms of cutaneous lupus are due to complement activation by antibody (type III mechanism), then one must explain why an immune response against a ubiquitous nuclear antigen is focused at the dermal–epidermal junction in these conditions. Another type of cutaneous lupus (*bullous lupus*) is characterized by an immune response against an antigen normally expressed only in the skin and in the kidney: type VII collagen in the basement membranes of the epidermis and glomeruli, respectively. Direct immunofluorescence of bullous lupus reveals IgG, IgM, and IgA in the basement membrane of the skin, similar to dermatitis herpetiformis. Bullous lupus also is associated with renal disease in SLE, as one would predict based on the distribution of the autoantigen. This form of bullous disease is only seen in lupus.

Alopecia is a common finding in SLE and can be diffuse or patchy and scarring or nonscarring. Diffuse hair loss may accompany acute cutaneous lupus and is usually reversible. Short, fragile, broken hairs also can be seen in the front of the hairline (“lupus hairs”). Hair loss is permanent when it is due to the extensive scarring of discoid lesions.

Mucous membrane involvement with oral, genital, and nasal ulcerations is common in lupus and is seen in 25% to 40% of SLE patients. Ulcers may be seen on the buccal mucosa or the hard palate and are usually painless.

Other mucocutaneous findings in lupus include microvascular lesions such as periungual erythema, livedo reticularis, and Raynaud phenomenon. Small-vessel cutaneous vasculitis also may be seen with lupus. Urticaria and panniculitis (*lupus profundus*) have been described in lupus patients.

Musculoskeletal Manifestations

Joints, muscle, and their supporting structures, such as tendons and ligaments, are commonly involved in lupus, affecting more than 90% of patients (63), and joint pain (*arthralgia*) is often the symptom that brings patients to medical attention. Arthralgia and symmetric arthritis of the hands [wrists, proximal interphalangeal (PIP), and metacarpophalangeal (MCP) joints] and knees are the major joint manifestations, similar to rheumatoid arthritis (RA). In contrast to RA, however, joint involvement in lupus is more migratory and less sustained. Large articular effusions are uncommon, but periarticular swelling is more prominent. Inflammation of periarticular structures can lead to deformities resembling those seen in RA but characteristically leave the articular cartilage intact, that is, without joint erosions. In addition, arthritis in lupus rarely reaches the intensity seen in RA. Tendinitis and tendon rupture also are seen in patients with SLE. Subcutaneous nodules are found in up to 10% of SLE patients, usually in those with positive rheumatoid factor (64).

The immunologic mechanisms underlying arthritis in lupus remain unknown. Synovial biopsy reveals evidence of microvascular injury in the synovium with enlarged endothelial cells with perivascular infiltrates of mononuclear cells (65). These changes are not specific to lupus.

For patients with arthralgia and mild arthritis, analgesics or NSAIDs may provide adequate relief. Hydroxychloroquine is also effective. In patients with more severe arthritis not responding to hydroxychloroquine and NSAIDs, weekly methotrexate should be considered with careful monitoring of liver and renal function (66). Corticosteroid use should be avoided if possible for arthritis in lupus because of well-documented long-term cumulative toxicities (67).

Osteonecrosis (avascular necrosis) should be suspected in SLE patients with localized joint pain, especially in those who are or were on corticosteroids. It is most common in the femoral head, although other sites, such as the shoulders (humeral head), tibial plateau, and scaphoid can be involved (67). Conventional radiographs may be negative in early lesions. In these situations, a magnetic resonance imaging (MRI) scan should be done to detect early changes typical of osteonecrosis.

Muscle pain (*myalgia*) and muscle weakness, especially in the deltoid area and quadriceps, are common in lupus patients. Inflammatory myositis resembling polymyositis or dermatomyositis is rare in lupus, however, occurring in 5% to 10% of SLE patients. These patients often exhibit features of other systemic rheumatic diseases as well. Individuals with a high titer (often >1:10,000) of autoantibody against the small nuclear ribonucleoprotein U1 snRNP can develop an overlap syndrome of lupus, polymyositis, and systemic sclerosis that is known as *mixed connective tissue disease*. Medication-induced myopathy caused by corticosteroids (rarely by hydroxychloroquine) also should be considered in the differential diagnosis of proximal weakness in lupus.

Cardiac Manifestations

Any structure within the heart can be damaged by lupus, including the valves, pericardium, myocardium, conduction system, and coronary arteries. Valvular disease is common in lupus, with 50% to 60% of patients having abnormalities on transesophageal echocardiography (68). The mitral valve is most commonly affected, with thickening, vegetations, regurgitation, or stenosis. Initially, mononuclear cells infiltrate the valve leaflets, leading to thickening. Later, immune complexes, mononuclear cells, and fibrin and platelet thrombi accumulate, producing vegetations. The nature of the infiltrating leukocytes and immune complexes remains unknown. Resolution of the valvulitis usually leads to fibrosis and, in some cases, calcification, leading to varying degrees of valvular regurgitation. Similar observations have been made in inflamed pericardium and myocardium, suggesting a common mechanism of pathogenesis in cardiac disease. Although valvular abnormalities in lupus are usually clinically silent initially, they carry a significant potential (20%) for long-term morbidity and mortality, including stroke, embolic disease, heart failure, infectious endocarditis, and valve failure requiring replacement (68). Interestingly, the course and severity of the valvular disease usually do not mirror the activity of disease in other organs, suggesting that different pathogenic mechanisms are operative in cardiac disease compared with skin, joint, and renal disease.

The major cardiovascular morbidity in lupus is ischemic heart disease resulting from accelerated atherosclerotic coronary disease. Known risk factors include those common to atherosclerotic coronary disease (smoking, hypertension, hyperlipidemia, diabetes mellitus, plasma homocysteine levels) and those unique to lupus, namely, the dose and duration of corticosteroid use and the presence of antiphospholipid antibodies. Pericarditis is the most common cardiac complication attributable to lupus itself. It is usually asymptomatic, but pericardial effusions can be demonstrated in 50% to 60% of patients by echocardiography. Symptomatic pericarditis

occurs in about 25% of patients.

Pulmonary Manifestations

Any component of the pulmonary system, including the lung parenchyma, pleura, pulmonary vessels, and diaphragm, can be affected by SLE [reviewed in (69)]. Pleuritis with or without pleural effusion is the most common pulmonary manifestation of lupus, occurring in 60% to 70% of patients sometime during the course of their illness. Acute and chronic pneumonitis can be seen in lupus but are uncommon, occurring in fewer than 10% of patients. Acute pneumonitis often occurs with concurrent pulmonary hemorrhage resulting in hemoptysis and carries an extremely poor prognosis. Chronic pneumonitis leading to fibrosis is quite rare in lupus, in contrast to the interstitial lung disease of systemic sclerosis, Sjögren syndrome, and polymyositis. Pulmonary hypertension also can occur but is rare in the absence of parenchymal lung disease. Other pulmonary manifestations include pulmonary embolism, bronchiolitis obliterans with organizing pneumonia, and the shrinking lung syndrome. The shrinking (*vanishing*) lung syndrome is characterized by dyspnea, pleuritis, and a progressive decrease in lung volume in the absence of interstitial lung disease (70). It appears to be due to a myopathy or myositis selectively affecting the diaphragmatic muscles.

The immunopathogenesis of the pulmonary manifestations of lupus remains obscure. Similar to cardiac disease, immune complex deposition in the pulmonary microvasculature has been implicated. In acute pneumonitis, alveolar lining cell injury with immunoglobulin and complement deposition is seen with varying degrees of alveolar edema and hemorrhage. In patients who have acute pneumonitis with hemoptysis, the predominant finding on light microscopy has been inflammation and damage to alveolar capillaries with subsequent hemorrhage into the alveolar space. Vasculitis has been documented only rarely. Abnormal endothelial cell–leukocyte interaction may be responsible for the phenomenon of acute reversible hypoxemia in lupus, in which no parenchymal lung disease is demonstrable on computed tomography (CT) scanning. Leukocyte agglutination with subsequent occlusion of the microvasculature has been suggested as a mechanism of pulmonary and other organ dysfunction in lupus (71). This is presumably due to abnormal expression of the endothelial adhesion molecules E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) in lupus (72).

After infection has been excluded, high-dose corticosteroids with or without other immunosuppressive agents (such as cyclophosphamide) are given for severe pulmonary disease, such as acute hemorrhagic pneumonitis. In rapidly deteriorating patients who do not respond to this approach, intravenous pulse corticosteroids followed by plasma exchange with concomitant immunosuppressive therapy has been used (73,74). The treatment of interstitial lung disease in lupus depends on the degree of chronic (fibrotic) changes detected in the parenchyma. In patients who have interstitial lung disease, high-resolution chest CT (HRCT) can help to guide therapy. A ground-glass appearance on HRCT suggests active inflammation and warrants immunosuppressive therapy. In contrast, a reticular or honeycomb pattern on HRCT likely reflects fibrosis and therefore is unlikely to improve with immunosuppressive therapy. Patients with diaphragmatic myopathy (the shrinking lung syndrome) may benefit from high-dose corticosteroids as well (75,76).

Renal Manifestations

Renal disease is the major cause of morbidity in lupus [reviewed in (77)]; 50% of SLE patients develop clinically significant nephritis characterized by decreased glomerular filtration rate or abnormal urinalysis. The frequency of subclinical renal involvement, however, is greater than 90% because almost all patients with SLE have abnormal renal biopsies if immunofluorescence studies and electron microscopy are performed (56). Although the glomerulus suffers the brunt of the inflammatory process, the renal tubules and interstitium are not spared.

Lupus glomerulonephritis is characterized by the deposition of immune complexes of autoantibody within the glomerulus and is classified based on the site and severity of that deposition. The nature of these immune complexes and the subsequent activation of the complement system are discussed in the section on immunopathogenesis. The World Health Organization (WHO) Classification (Table 39.3) is the most commonly used framework. The clinical utility of this classification lies in its correlation with disease severity and prognosis for preservation of renal function. One must bear in mind that designations applied using this classification scheme are not fixed: Transition from one stage to another is common when sequential biopsies are compared in patients being treated for lupus nephritis, in particular the transition from focal to diffuse proliferative glomerular disease.

Class	Pathologic Finding
I	Normal
IIA	Mesangial deposits
IIB	Mesangial cellular proliferation
III	Focal proliferative glomerulonephritis
IV	Diffuse proliferative glomerulonephritis
V	Membranous glomerulonephritis

TABLE 39.3. World Health Organization Classification of Lupus Nephritis

Renal biopsies with no abnormalities on light microscopy, immunofluorescence studies (for deposition of immunoglobulin and complement), and electron microscopy are designated as WHO class I. The urinalysis and glomerular filtration rates are normal. In class II, or *mesangial nephritis*, IgG and complement deposition can be seen in the mesangium. This triggers recruitment and activation of phagocytic cells (neutrophils and macrophages) in the mesangium. The chemoattractants C3a and C5a are generated. In addition, proinflammatory cytokines are produced by activated T cells and macrophages, including tumor necrosis factor a (TNF-a), interferon-g (IFN-g), IL-6, and transforming growth factor-b (TGF-b). The mesangium is hypercellular on light microscopy. Continued deposition of immune complexes presumably overwhelms the capability of the mesangium to degrade the complexes, leading to “spillage” of immune reactants into adjacent structures, notably the subendothelial space.

Focal proliferative glomerulonephritis (class III nephritis) is characterized by the spread of the inflammatory process into the subendothelial space of the glomerular capillaries. This may be due to deposition of circulating immune complexes in the glomerular basement membrane (GBM) or through the binding of circulating autoantibody to “trapped” antigen in the GBM. These antibodies are usually IgG1 or IgG3, which are effective activators of the complement cascade. Light microscopy shows a proliferative glomerulonephritis that affects fewer than 50% of the glomeruli (“focal” disease) on renal biopsy, although one must consider the possibility of sampling error. Within affected glomeruli, some regions of the glomeruli are spared; that is, there is “segmental” involvement (Fig. 39.3). The changes within the glomerulus can be proliferative, necrotizing, sclerosing, or a combination of these. The clinical consequence is a small decrease in the glomerular filtration rate, proteinuria, and an active urinalysis with microscopic hematuria, proteinuria, and red blood cell or white blood cell casts. If the inflammatory process remains unchecked, some patients will progress to more extensive glomerular disease.

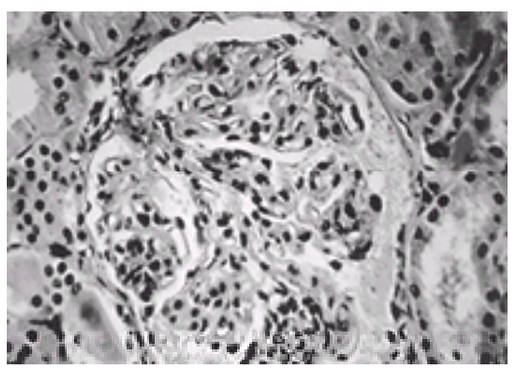

Figure 39.3. Focal proliferative glomerulonephritis. Marked hypercellularity with obliteration of capillary lumens are seen in two glomerular tufts on hematoxylin and eosin staining. The remainder of the glomerulus is normal or minimally affected. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.)

Class IV nephritis (*diffuse proliferative glomerulonephritis*) is the most ominous form of lupus nephritis because it carries the highest risk of progressing to renal failure. Most (>50%) of the glomeruli are affected with diffuse hypercellularity of mesangial and endothelial cells and capillary loop thickening (Fig. 39.4). Necrosis is often seen within the glomerulus. Immunofluorescence microscopy demonstrates extensive deposition of immunoglobulin and complement in the mesangium and capillary loops (Fig. 39.5). Electron microscopy shows extensive subendothelial and mesangial deposits. Patients with class IV nephritis typically present with severe renal insufficiency as well as significant proteinuria and microscopic hematuria.

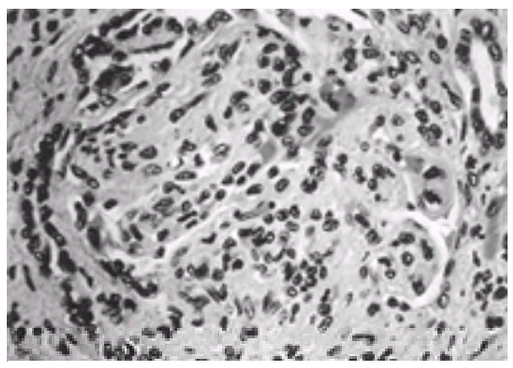

Figure 39.4. Diffuse proliferative glomerulonephritis. Marked hypercellularity with compression of capillary lumens affecting the entire glomerulus with obliteration of the normal glomerular architecture (hematoxylin and eosin staining, high power). (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.)

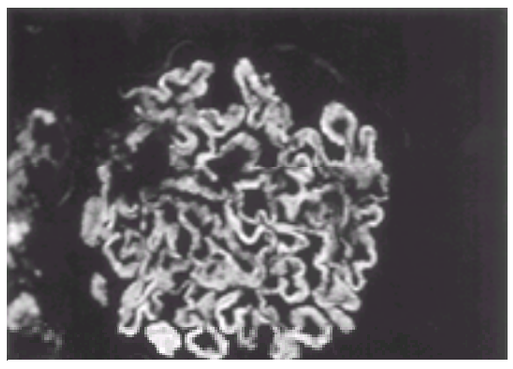

Figure 39.5. Immunofluorescence staining of lupus nephritis. The basement membrane of the glomerulus is partly outlined by irregular deposits of immunoglobulin G (IgG) detected with fluorescein-conjugated rabbit antihuman IgG. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.)

Class V (*membranous glomerulonephritis*) is characterized by the deposition of immune complexes in the urinary space below the epithelial cells (subepithelial space). These autoantibodies may be binding to antigens on epithelial cells or to cationic antigens that have traversed the GBM. Antibodies binding in the subepithelial space are usually IgG2 or IgG4 (78), which do not bind complement well and therefore would not trigger the same degree of inflammatory reaction as IgG1 or IgG3. Light microscopy shows a diffuse thickening of the peripheral capillary walls in the absence of cellular infiltrate (Fig. 39.6). Electron microscopy reveals subepithelial and intramembranous deposits. In general, affected patients have extensive proteinuria but only minimal elevations of the serum creatinine. Some patients, however, progress to chronic renal insufficiency.

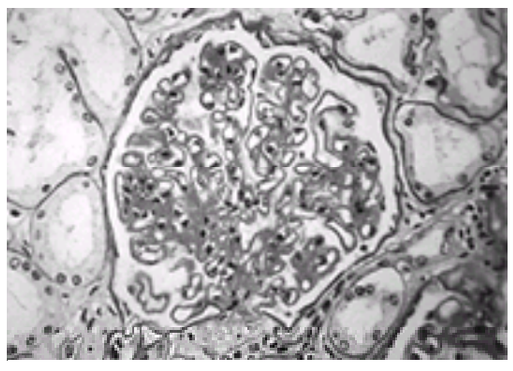

Figure 39.6. Membranous glomerulonephritis. Widespread, uniform thickening of capillary basement membranes of the glomerulus is seen on periodic acid-Schiff staining. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (See Color Figure 39.6.)

The treatment of lupus nephritis should be individualized based on the histopathologic type, activity and chronicity of nephritis, and rate of change in renal function. Patients with mesangial or mild focal proliferative nephritis can be treated with high-dose corticosteroids alone without cytotoxic drugs. Those with severe focal proliferative or diffuse proliferative lupus nephritis should be treated aggressively with a combination of immunosuppressive and cytotoxic drugs, such as cyclophosphamide and high-dose corticosteroids (79). This regimen is more efficacious than corticosteroids alone for severe proliferative nephritis but is associated with significant toxicity. The side effects of cyclophosphamide include increased risk of bacterial and viral infection, especially *Varicella* reactivation, hemorrhagic cystitis, and ovarian failure. There is also a clear increase in the risk of hematologic malignancy and bladder carcinoma. Although there is no well-accepted treatment for pure membranous lupus nephritis, patients with nephrotic range proteinuria or worsening renal function should be considered for high-dose corticosteroids with or without cytotoxic therapy such as intravenous cyclophosphamide (80).

Other therapeutic strategies have been used for severe lupus nephritis, CNS disease, or multisystem disease. Growing clinical evidence reports the successful use of the immunosuppressive/cytotoxic drug mycophenolate mofetil, either at presentation or in patients who have failed intravenous pulse cyclophosphamide therapy (81). In addition, plasma exchange with subsequent intravenous cyclophosphamide followed by prednisone and cyclophosphamide has achieved remission in more than half of the patients with severe lupus (most with lupus nephritis) at almost 4 years of follow-up (73,82). This regimen is associated with a substantially higher risk of potentially fatal bacterial and viral infection, however, than treatment with cyclophosphamide alone (83).

Several animal studies demonstrated the potential of immunobiological agents targeted at specific molecules in the treatment of SLE (84,85,86 and 87). These therapies are discussed fully in the section on immunopathogenesis. These agents interfere with the interaction of T cells with APCs such as B cells, thereby blocking B-cell activation and subsequent T-cell help for pathogenic autoantibody production. The infusion of anti-CD40 ligand antibody in lupus-prone mice not only prevented the development of nephritis but also improved survival in mice with established nephritis (85,86). Treatment of lupus-prone mice with CTLA4-Ig, a fusion protein of the T cell downregulatory molecule CTLA-4 to the Fc portion of a mouse IgG2a monoclonal antibody, decreases autoantibody production and increases survival (84,87). To date, however, these therapies have not been successful in small clinical trials in lupus patients.

Neuropsychiatric Manifestations

Lupus can affect any part of the nervous system, from the CNS, to the spinal cord, to the peripheral nervous system (PNS). Possible CNS manifestations include cognitive dysfunction, depression, psychosis, coma, seizure disorders, migraine, and other headache syndromes, aseptic meningitis, chorea, stroke (due to hemorrhage or thrombosis associated with the antiphospholipid syndrome), and cranial neuropathies (88). The most common neuropsychiatric manifestations of lupus are depression and psychosis. Attributing these changes to lupus itself is difficult and is a diagnosis of exclusion. It is paramount to evaluate patients for potentially reversible causes, such as infection, hypertension, metabolic abnormalities, and toxic effects of medications, particularly corticosteroids. No laboratory tests are available to distinguish reliably between neurologic disease secondary to lupus versus these other etiologies.

The pathogenesis of CNS lupus remains cryptic. Two mechanisms have been implicated: nonvasculitic small-vessel injury and direct cellular damage from autoantibodies. CNS vasculitis is rarely found on histopathologic examination; instead, vasculopathy with leukoagglutination without significant inflammatory infiltrate of vessel walls is usually seen (89). The mechanisms underlying vascular damage, including antiphospholipid antibodies, are unknown. The evidence implicating autoantibodies stems from the observation that IgG antibodies against neuronal antigens are seen in most individuals with diffuse CNS disease and lupus (90,91). These antibodies cross-react with lymphocyte antigens and are cytotoxic to lymphocytes *in vitro* (90). One candidate antigen on neurons is a 50-kd protein present in the membrane of synaptic terminals (92). Antineuronal antibodies are either absent or present in low titer in persons who have non-CNS lupus or other systemic rheumatic diseases. Persistent elevations of IgG anticardiolipin antibodies also have been associated with cognitive dysfunction in SLE (93). Other autoantibodies have been associated with different CNS manifestations of lupus: recent studies showed an association between psychiatric manifestations of SLE, especially depression, and elevated serum levels of antiribosomal P protein antibody (94). At present, however, this observation is of little clinical utility because of the lack of a commercially available test to determine the presence of this autoantibody.

Seizure disorders occur commonly in lupus, affecting 10% to 20% of patients. Seizures may be the only manifestation of lupus at presentation, with individuals years later developing the more common manifestations of disease. Conversely, many of the anticonvulsants used to treat seizure disorders can cause a positive ANA. These persons can therefore be mistakenly diagnosed as having SLE. Stroke in lupus can be due to many different factors, including hypertension secondary to renal insufficiency, thrombosis due to the presence of antiphospholipid antibodies, hemorrhage from severe thrombocytopenia, and embolic disease from infectious or noninfectious mitral valve endocarditis.

Peripheral nervous system disease in lupus may be motor, sensory, or mixed, and produce a relatively symmetric polyneuropathy or mononeuritis multiplex. Rarely, an acute ascending motor paralysis similar to Guillain-Barré syndrome and transverse myelitis occurs.

Unfortunately, no data from randomized controlled studies are available to guide the treatment of neuropsychiatric lupus. Once other causes have been excluded, therapy of neuropsychiatric lupus should be individualized based on the severity of illness and, if possible, on understanding the nature of the underlying process such as inflammation or thrombosis. High-dose intravenous corticosteroids (methylprednisolone) are usually given for severe disease such as cerebritis or acute transverse myelitis, typically followed by pulses of intravenous cyclophosphamide. Given the sparse inflammatory infiltrate seen on the brain or meningeal biopsies in most cases, this is of questionable value. Anticoagulation should be considered for patients who show evidence of cerebrovascular thrombosis.

Hematologic Manifestations

Leukopenia and thrombocytopenia are common hematologic findings in lupus [reviewed in (95)]. Because SLE patients often are taking immunosuppressive drugs, drug-induced hematologic problems always should be ruled out first. Lupus is unique among the systemic rheumatic diseases in that leukopenia rather than lymphocytosis is seen in active disease. For this reason, it is included in the ACR diagnostic criteria for lupus. Leukopenia ranges from 2,500/mm^3 to 4,000/mm^3 and rarely causes any clinical problem. It is usually due to lymphopenia, with a lymphocyte count of less than 1,500/mm^3.

Thrombocytopenia occurs in about 25% to 50% of SLE patients, and antiplatelet antibodies often are demonstrated in these patients. The antigen(s) is unknown. Usually, the thrombocytopenia is not severe enough to warrant specific therapy. Not all lupus patients with antiplatelet antibodies, however, have thrombocytopenia. A patient may have an apparently isolated case of idiopathic thrombocytopenia (ITP) and subsequently develop SLE. Rarely, thrombotic thrombocytopenic purpura (TTP) has been associated with lupus.

Various forms of anemia, including immune-mediated and nonimmune-mediated anemia, occur. The most common form of anemia in lupus is the anemia of chronic disease with low serum iron and total iron-binding capacity and a normal serum ferritin. Autoimmune hemolytic anemia with a positive Coombs test result occurs in 7% to 15% of SLE patients. Some lupus patients, however, have a positive Coombs test result without evidence of hemolytic anemia. Pure red cell aplasia resulting from antibodies against erythroblasts and pancytopenia has been reported rarely in SLE as well (96).

Coagulation abnormalities are also seen in lupus. Autoantibodies directed against protein–phospholipid complexes critical for the initiation of the clotting cascade by the intrinsic pathway (lupus anticoagulant antibodies) can prolong the partial thromboplastin time (PTT). Despite the prolongation of the PTT *in vitro*, patients with these antibodies are at risk for arterial or venous thrombosis (the antiphospholipid syndrome, or APS). Patients with thrombosis secondary to APS should be treated with chronic anticoagulation (97). Low-dose aspirin alone usually is prescribed prophylactically for patients with antiphospholipid antibodies or anticardiolipin antibodies without a history of a thrombosis, although data supporting this approach are lacking.

Splenomegaly and lymphadenopathy occur in lupus. The enlarged nodes are usually soft, nontender, and variable in size. Biopsy demonstrates reactive hyperplasia.

Patients with the anemia of chronic disease do not require specific therapy for the anemia. Coombs-positive autoimmune hemolytic anemia is treated with high-dose corticosteroids. Immunosuppressive drugs such as azathioprine and cyclophosphamide should be considered for refractory cases. Thrombocytopenia is usually not severe in lupus and does not require therapy. When the platelet count goes below 20,000/mm^3 or when there is active bleeding, high-dose corticosteroids are used. For patients who do not respond to high-dose corticosteroids or who require unacceptably high maintenance doses of prednisone, azathioprine, cyclophosphamide, the androgen danazol and splenectomy have been used (98). In emergency situations, intravenous immune globulin can raise platelet counts above dangerously low levels within 1 day. The effect, however, lasts only several weeks (99). Leukopenia is usually not severe enough in lupus to warrant specific immunosuppressive therapy.

Gastrointestinal Manifestations

Hepatomegaly and abnormal liver function tests are frequently detected in SLE patients. Medications such as NSAIDs, azathioprine, and methotrexate are most commonly responsible for abnormal liver function tests. In addition, autoimmune hepatitis should be considered in the differential diagnosis. About 25% to 50% of SLE patients have gastrointestinal symptoms, including dysphagia, abdominal pain, anorexia, nausea, and vomiting, that are most commonly due to side effects of medication. Rarely, these symptoms are due to mesenteric vasculitis, bowel perforation, sterile or spontaneous bacterial peritonitis, or pancreatitis.

Drug-induced Lupus

Certain medications, most commonly procainamide, can cause symptoms and immunologic abnormalities that are somewhat similar to (idiopathic) SLE (Table 39.4). Constitutional, musculoskeletal, and pleuropericardial symptoms dominate the clinical picture; cutaneous, renal, and CNS diseases are distinctly unusual [reviewed in (100)]. The syndrome of drug-induced lupus resolves with discontinuation of the offending agent. The immunologic hallmark of drug-induced lupus is the formation of autoantibodies against histones in all patients who have this syndrome. The panoply of autoantibodies that characterizes idiopathic lupus is not seen. The humoral response in drug-induced lupus usually is focused against the H2A–H2B component of histones complexed to DNA (101) and precedes the development of symptoms in both murine models of the disease and in humans (102). Most patients who develop antihistone antibodies while taking procainamide never develop the syndrome, however. One risk factor for the development of drug-induced lupus is decreased hepatic metabolism of these compounds, that is, the “slow acetylator phenotype,” which is due to decreased acetyltransferase activity in the p450 cytochrome system (103).

Drugs with strong evidence shown by prospective studies
Procainamide, hydralazine, chlorpromazine, isoniazid, methyldopa, minocycline
Drugs with circumstantial evidence
β-Adrenergic blocking agents, mephenytoin, phenytoin, quinidine, rifampin, sulfonamides

TABLE 39.4. Drugs Implicated in the Induction of Lupus-Like Disease

How drugs such as procainamide can trigger autoimmunity remains unclear. *In vitro*, they can be metabolized into cytotoxic compounds by myeloperoxidase in activated neutrophils (104). They may also directly alter lymphocyte function by decreasing DNA methylation, leading to increased expression of adhesion molecules such as LFA-1 (45). In addition, these compounds may augment the positive selection of autoreactive T cells in the thymus; injection of the thymus of nonautoimmune C57BL/6 mice with the hydroxylamine metabolite of procainamide leads to enhanced thymocyte proliferation against chromatin derived peptides. A T-cell–dependent antibody response against the H2A–H2B subunit of chromatin (105,106) follows, although an effect on peripheral tolerance cannot be excluded by these experiments.

Pregnancy and Lupus

There is an increased risk of spontaneous abortion, fetal loss, and prematurity in lupus [reviewed in (107)]. Whether pregnancy can induce a flare of disease remains controversial: Initial studies suggested an increased risk of a lupus flare during pregnancy, but later controlled studies reported that the risk of flare in pregnant women with lupus is the same as that in nonpregnant women, especially when disease activity is under control prior to pregnancy (108). Preeclampsia and eclampsia are other important considerations in the differential diagnosis of the pregnant lupus patient with hypertension and worsening renal function.

A mother with lupus may have a newborn with neonatal lupus characterized by a transient skin rash resembling that seen in acute cutaneous lupus, hepatitis, cytopenias, and heart block. Neonatal lupus is exclusively seen in a lupus mother with anti-Ro or La antibody; the risk for the syndrome is about 1% to 5% in children of mothers with these autoantibodies [reviewed in (109)]. The mechanism of neonatal lupus is believed to be secondary to maternal IgG anti-Ro or anti-La antibody that crosses the placenta and binds neonatal antigens in various organs, including the heart.

Laboratory Tests: Antinuclear Antibodies

The immunologic hallmark of lupus is the presence of ANAs in the serum; 95% to 99% of patients with SLE have ANAs when indirect immunofluorescence is used and human (Hep-2) cells are used as the source of antigen [reviewed in (110)]. For this reason, ANA testing has been incorporated into the evaluation for lupus. In some patients, the titer of certain autoantibodies is also a useful correlate of disease activity. The titer and pattern of staining of ANAs are important; four patterns of staining are recognized: (a) diffuse nuclear, (b) peripheral nuclear; (c) rim, speckled, and (d) nucleolar (Fig. 39.7, Fig. 39.8 and Fig. 39.9). Proper interpretation of the ANA test result by the physician is paramount: Many healthy individuals (5%–8% of the general population) have a positive ANA but never develop any symptoms or signs of a systemic rheumatic disease. In addition, persons who have other systemic rheumatic diseases, such as Sjögren syndrome, systemic sclerosis, and polymyositis/dermatomyositis and other organ-specific autoimmune disease (e.g., chronic autoimmune hepatitis) usually develop ANAs as well. Enzyme-linked immunosorbent assay (ELISA) testing using recombinant protein antigens or purified DNA is done following fluorescent ANA testing to determine the specificity of the ANA.

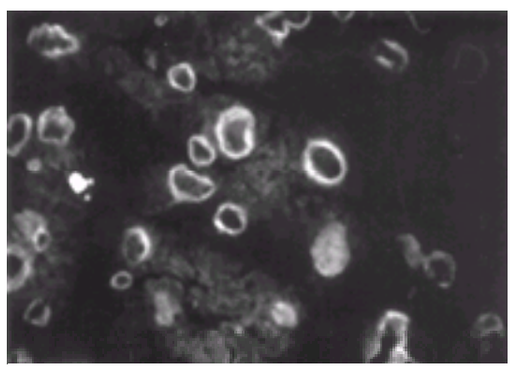

Figure 39.7. Antinuclear antibody: Rim (peripheral) pattern. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (See Color Figure 39.7.)

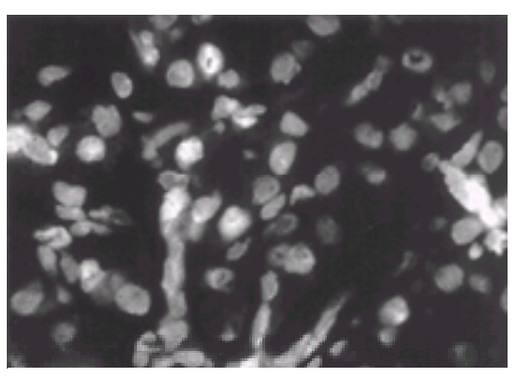

Figure 39.8. Antinuclear antibody: Homogeneous (diffuse) pattern. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (See Color Figure 39.8.)

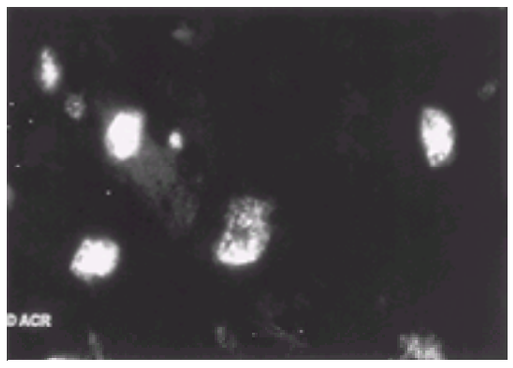

Figure 39.9. Antinuclear antibody: Speckled pattern. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.)

In SLE, ANAs can be divided into antibodies against chromatin-associated antigens [double-stranded (ds) DNA and histones] and antibodies against ribonucleoproteins (Ro, La, Sm, and U1 snRNP) (Table 39.5). Anti-dsDNA antibodies and anti-Sm antibodies are unique among the autoantibodies because they are seen only in lupus, with anti-dsDNA antibodies typically giving a diffuse or rim pattern and anti-Sm antibodies giving a speckled ANA test result. Antibodies to intact chromatin occur in about 90% of lupus patients, whereas anti-dsDNA antibodies are present in roughly 70%. In some lupus patients, the titer correlates with disease activity, especially renal disease. Anti-Sm antibody occurs in about 20% to 30% of SLE patients and is more common in African Americans with lupus (111).

Target of Antibody	Clinical Associations	Prevalence (%)
dsDNA	Highly specific for SLE Correlates with disease activity, especially with renal disease	70
Histone	SLE Drug-induced lupus	50–70 >90
Sm	Highly specific for SLE	20–30
U1-snRNP	SLE MCTD	30–40
Ro (SS-A)	SLE Neonatal lupus Subacute cutaneous lupus Photosensitivity Sjogren's syndrome	30–60
La (SS-B)	SLE Neonatal lupus Sjogren syndrome	10–15
Ribosomal P antigen	Psychiatric disease, especially depression	10–20

MCTD, mixed connective tissue disease; SLE, systemic lupus erythematosus; snRNP, small nuclear ribonucleoprotein.

TABLE 39.5. Autoantibodies and Their Clinical Association and Prevalence in Sle

Anti-U1RNP is detected in 30% to 40% of SLE patients, and it is also found in high titers in patients with mixed connective-tissue disease. Although commonly associated with Sjögren syndrome, anti-Ro and anti-La antibodies are found in about 30% to 40% and 10% to 15% of SLE patients, respectively. The presence of anti-Ro or anti-La antibodies in a pregnant woman is associated with a 1% to 5% chance of neonatal lupus developing in the infant. Antihistone antibodies are seen in patients with SLE and drug-induced lupus. Other less commonly found ANAs in SLE are anti-Ku antibody, antiproliferating cell nuclear antigen (anti-PCNA), anti-RNA polymerase antibody, and antiribosomal P protein antibody. ANA-negative SLE patients often have anti-Ro antibody by ELISA (112).

Laboratory Studies: Complement

One of the important pathogenic mechanisms of tissue injury in lupus is activation of the classical complement pathway by immune complexes formed between self-antigen and autoantibody. This consumes early components of the complement system. Therefore, patients with active lupus, particularly nephritis, usually have low C3, C4, and total hemolytic complement. Determination of low serum complement levels is useful in helping to distinguish lupus activity from mimics, such as infection. In addition, patients who have a hereditary deficiency of early complement proteins C4 and C2 develop lupus at a young age. Therefore, it is useful to screen for hereditary complement deficiency in children and young adults newly diagnosed with lupus.

Prognosis

Given the variability in the presentation and severity of illness, it is difficult to discern long-term outcomes in patients with lupus. The prognosis and course of disease are variable: Patients may follow a relapsing or relapsing and remitting course, with intermittent flares of lupus superimposed on a “baseline” of chronic disease activity. Alternatively, they may follow a course of chronic, active, progressive disease with cumulative organ damage. Some patients have long-term (>6 months) periods of disease quiescence with no discernible activity on standard measures of disease activity, such as the SLE disease activity index (SLEDAI). Unfortunately, at present, we have no predictors of which clinical course patients will follow and which complications will occur. Fortunately, the overall prognosis (survival) in SLE has improved over the last 3 decades since the introduction of corticosteroid and cyclophosphamide therapy and is now 80% to 90% at 10 years compared with 50% 30 years ago (113). The price for this advance has been steep: Increasing morbidity rates from cardiovascular disease, including stroke and myocardial infarction, have accompanied the use of high-dose corticosteroids for serious manifestations of lupus (114,115). The prevention of atherosclerosis in lupus patients will become paramount in the future.

In addition, for unknown reasons, prognosis is worse for African Americans with lupus, particularly those with renal disease (116). Significant effort should be made in the future to understand the genetic and environmental factors responsible for the poor outcome in some patients.

IMMUNOPATHOLOGY

Autoantibodies in Lupus

The production of high-titer IgG antibodies against ubiquitous intracellular components, including chromatin and ribonucleoproteins, is the hallmark of lupus in humans and in murine models of the disease (8,50). The deposition of immune complexes of these autoantibodies with their respective autoantigens in target organs, such as the kidney, leads to activation of complement and Fc receptor binding (117) with subsequent tissue injury. Presumably, similar mechanisms account for tissue inflammation in other organs, such as the skin and joints.

Proof of the pathogenicity of certain autoantibodies comes from studies of human polyclonal anti-dsDNA and murine monoclonal anti-dsDNA IgG antibodies, which induce glomerular pathology when passively transferred into nonautoimmune mice. In these animals, the glomerular filtration rate is reduced, pathologic proteinuria develops, and mesangioproliferative glomerulonephritis destroys the normal architecture of the glomerulus (118,119). These changes are quite similar to those seen in human lupus. Mice that transgenically express immunoglobulin genes coding for anti-dsDNA antibodies also develop immune complex–mediated proliferative glomerulonephritis (120,121).

Anti-dsDNA antibodies are preferentially deposited in the kidney and can be eluted from the kidneys of animals with spontaneous lupus glomerulonephritis in high concentration relative to their level in serum (122). In humans, increases in serum titer often precede, or are coincident with, worsening of renal disease (123,124). In addition, genetic disruption of the ability of B cells to produce antibody results in markedly diminished glomerular pathology in lupus-prone mice (125). Although following the titer of anti-dsDNA antibodies is clinically useful in many patients with nephritis, the presence of anti-dsDNA antibodies does not always positively correlate with development of renal disease (123,124), nor are they necessary for the development of glomerulonephritis in some patients. Autoantibodies other than those directed against chromatin also can incite immune complex renal disease, and end-organ injury may result from nonantibody-dependent mechanisms (125,126).

The precise site of interactions between autoantibody and autoantigen is unclear. Three hypotheses have been proposed to explain the process by which immune complexes containing antichromatin antibodies are formed [reviewed in (127)]. The first hypothesis proposes that the interaction between these autoantibodies and antigen occurs exclusively in the serum, with subsequent deposition in tissues. This explanation now appears unlikely as the sole reason for the pathogenicity of autoantibodies. Multiple attempts to detect immune complexes containing DNA in the serum of lupus patients have failed (128,129). Further, the passive transfer of preformed complexes containing anti-DNA antibodies in mice has not resulted in glomerular inflammation (130). A second hypothesis postulates that antichromatin antibodies may cross-react with protein and carbohydrate antigens, such as laminin, in the glomerulus to form immune complexes (131,132,133 and 134). Support for this hypothesis comes from the observation that a subset of antichromatin antibodies, generated by immunization with a foreign peptide, are cross-reactive with other autoantigens in lupus, such as the Sm ribonucleoprotein (135). An alternative possibility is that circulating antigen is trapped *in situ* within the kidney and subsequently binds anti-dsDNA antibodies. Evidence for this hypothesis comes from the observation that circulating nucleosomes, normally generated during the process of apoptosis (136,137 and 138), can bind to proteins within the GBM, particularly type IV collagen (139,140,141 and 142). Regardless of the precise mechanism(s) involved, immune complexes are located within the kidney. Phagocytic cells within the kidney bearing Fc and complement receptors then can recognize these complexes either by binding to the Fc portion of IgG or through recognition of opsonins generated by the classic complement pathway. The subsequent inflammatory response leads to destruction of the glomeruli. The importance of this mechanism of tissue injury in lupus is demonstrated in animals with an impaired ability to recognize immune complexes through the Fc portion of IgG. Lupus-prone NZB/NZW mice deficient in the g chain of the Fc receptor have significantly reduced glomerular pathology compared with wild-type controls (117).

It is therefore of great importance to identify the properties that determine the pathogenicity of these autoantibodies. No striking differences in V heavy or light immunoglobulin gene segment use has been found between lupus-prone and nonautoimmune mice or in studies of human lupus patients (143). This suggests that many different V genes are capable of generating an anti-DNA response and that the B-cell germ-line repertoire is not different in patients with systemic autoimmune disease. Therefore, properties of anti-dsDNA antibodies conferred after germ-line gene segment rearrangement strongly influence pathogenicity. These properties include the net charge of the antigen-binding region, the isotype, and the avidity of the antibody for DNA. Cationic antibodies are more likely to be pathogenic (144), presumably because of increased avidity for negatively charged antigens, such as DNA, and preferentially bind in the kidney (145). These cationic antibodies have a high frequency of arginine substitution in the V CDR3 region, and the position of these arginine substitutions is critical to glomerular binding (146). Isotypes that bind complement well (IgG2a) are more pathogenic in murine models than other isotypes (IgG1). Finally, antibodies directed against dsDNA in lupus-prone mice develop greater affinity for dsDNA over time. The net effect of these changes in antibody structure is to increase the affinity of the antibody for autoantigen over time.

ab T CELLS REQUIRED FOR AUTOANTIBODY GENESIS AND DISEASE IN LUPUS

The production of antibodies against chromatin and ribonucleoproteins is antigen driven in human and murine lupus. This conclusion is based on genetic analysis of high-affinity, pathogenic murine IgG antiDNA autoantibodies (147,148,149 and 150). Such work also was performed with human anti-DNA with similar results (151) showing clonal selection, somatic mutation, and selected distribution of replacement of silent mutations in the complementarity-determining regions (CDRs) of the antibody. Sequencing of antibodies directed against dsDNA suggested that these antibodies affinity matured in response to DNA, or perhaps an antigen that mimicked it in charge and conformation [reviewed in (152)]. Apparent antigen selection and somatic hypermutation of autoantibodies suggest that autoreactive B cells in lupus require autoantigen-specific cognate and contact-dependent ab T-cell help [reviewed in (153)], given the requirement of ab T cells for immunoglobulin somatic hypermutation and isotype switching in secondary lymphoid organs. Indeed, evidence supports the notion that autoantibody production in murine lupus is T cell dependent, originally coming from the demonstration that neonatal thymectomy of lupus-prone mice led to abrogation of IgG anti-dsDNA synthesis and glomerulonephritis and to improved mortality (154). Similar results were obtained after treatment of these animals with antibodies that depleted Thy-1-positive cells (155) or $CD4^+$ cells (156). Genetic studies have provided further evidence for the T-cell dependence of disease in murine lupus. Lupus-prone MRL/Fas^{lpr} mice with genetic disruption of $I\text{-}A^b$ lack $CD4^+$ T cells and have decreased penetrance of autoantibodies and glomerulonephritis (157). Similarly, autoantibodies were diminished in MRL/Fas^{lpr} and NZB mice with genetic disruption of CD4 ($CD4^+$ T-cell knockouts) (158) and in lupus-prone mice congenitally deficient in ab T cells. Either $TCRa^{-/-}$ or TCRb ($^{-/-}$ (159,160) Fas-deficient MRL/Fas^{lpr} and Fas-intact MRL/+ $^{Fas-lpr}$ mice have marked reduction in autoantibody production and end-organ disease with enhanced survival compared with abT-cell-intact controls (161).

Data from murine models of lupus support the hypothesis that $CD4^+$ T cells provide help to autoreactive B cells in the production of pathogenic autoantibodies. T cells have been isolated from (NZB x NZW)F_1 and from SNF1 [(SWR x NZB)F] lupus-prone mice that help anti-DNA production *in vitro* and accelerated disease when transferred to prenephritic mice *in vivo* (162,163,164 and 165). Such T cells are activated by peptides of DNA-binding histones from nucleosomes (163,164,166) or by peptides from anti-DNA Ig (165), consistent with the idea that they support anti-DNA autoantibody synthesis.

$CD4^+$ ab T cells derived from lupus nephritis patients have been shown to promote pathogenic anti-DNA production *in vitro* (167). These cells also proliferate when mixed with self-B cells in a class II MHC-restricted and CD154-CD40–dependent fashion (168,169,170,171 and 172). These results indicate that anti-DNA help in human lupus also is largely cognate and contact dependent. Moreover, CD4+ T cells from patients with lupus respond to peptides of chromatin proteins (167,172,179), supporting the hypothesis that they help anti-DNA B cells. The epitopes on chromatin recognized by $CD4^+$ T cells in human lupus are similar to those epitopes recognized by $CD4^+$ T cells in murine lupus that are pathogenic on adoptive transfer and that provide help to autoreactive B cells *in vitro* (167). In addition, these T-cell epitopes are physically close to the epitopes recognized by antichromatin antibodies. Therefore, autoreactive B cells may directly activate pathogenic T cells through recognition of a similar antigenic epitope, as depicted in Fig. 39.10 [reviewed in (173)]. These data suggest that the histone-DNA complex within the nucleus drives the pathogenic response against dsDNA in both human and murine lupus. In addition, diversification of the humoral response against chromatin occurs in a sequential and stereotypic pattern. Antibodies are initially directed against the H2A–H2B subunit of the nucleosome and then, through the process of epitope spreading, develop against the H3A–H3B subunit (174).

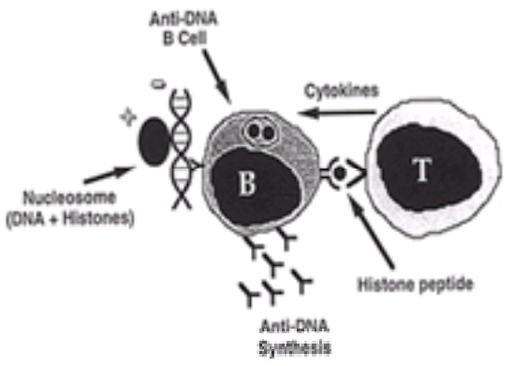

Figure 39.10. Generation of anti-dsDNA antibodies in lupus through help provided by histone-specific CD4+ T cells. In this model, surface immunoglobulin on an anti-DNA B cell can bind DNA as a component of a nucleosome, followed by internalization of the particle. Processing of histone components of the nucleosome results in presentation of histone peptides by major histocompatibility complex (MHC) class II molecules to activated, histone-specific CD4+ T cells. Cognate T-cell/B-cell help, in the setting of cytokines and costimulatory molecules (not shown), then results in the proliferation and differentiation of B cells with production of anti-dsDNA antibodies. Such T cells also could drive other clonally specific B cells capable of binding nucleosomal components to autoantibody synthesis, including those directed against chromatin, histones, or other nucleosomal components, such as high mobility group (HMG) proteins. (Adapted from Craft J, Fatenejad S. Self antigens and epitope spreading in systemic autoimmunity. *Arthritis Rheum* 1997;40:1374–1382, with permission.)

ab T-CELL HELP IN LUPUS IS AUTOANTIGEN SPECIFIC AND MHC RESTRICTED, AND MEDIATED BY CD154 (CD40 LIGAND)-CD40 INTERACTIONS

As in a normal immune response, ab T cell help in lupus is an MHC-restricted interaction between an ab T cell and B cell recognizing epitopes derived from the same antigen. This interaction is dependent on binding of CD40L (CD154) on the activated T cells to CD40 on the B-cell surface. Evidence for MHC restriction is provided by reconstitution studies of lethally irradiated lupus-prone mice in which autoantibody production depends on MHC class II matching between the donor and the recipient (175). The requirement for contact-dependent T-cell and B-cell interactions in murine lupus is further supported by the observation that disruption of ligand pairs necessary for mutual T-cell and B-cell co-stimulation results in diminished autoantibody synthesis and end-organ disease. Interruption of either CD154-CD40 or the CD28-B7.1/B7.2 ligand pair interactions in lupus-prone (NZB x NZW)F_1 and SNF_1 mice by antibodies or fusion protein (CTLA-4Ig) results in diminished autoantibody synthesis and end-organ disease (84,85,86 and 87,176). Similarly, mice with a disrupted CD154 locus on the lupus-prone MRL/Fas^{lpr} and MRL/+$^{Fas\_\ lpr}$ backgrounds have significant reductions in autoantibody levels and nephritis (177,178). Finally, soluble CD154, cleaved from the cell membrane by matrix metalloproteinases, may serve as an additional pathway to activation of both cognate and noncognate APCs (179,180). Soluble CD154 levels are elevated in lupus patients, particularly in those with active disease (182,183).

The acceleration of autoantibody production and clinical nephritis after transfer of autoantigen-specific T cells into pre-nephritic SNF or (NZB x NZW)F_1 mice indicated that these T cells are capable of promoting the lupus phenotype (163,164 and 165). To determine whether autoantigen-specific T cells are required for full disease penetrance, potentially autoreactive T cells in lupus-prone MRL/Fas^{lpr} mice were replaced with the AND TCR Va and Vb transgenes that recognize PCC in the context of $H\text{-}2^k$ (181,182). Despite transgenic T-cell activation, $TCR^{-/-}{}_a$ and TCR $b^{-/-}$ MRL/Fas^{lpr} mice lacked pathogenic autoantibody production, with absent renal, skin, and salivary gland disease (the latter two organs also are compromised in wild-type MRL mice). This study emphasized, once again, that the specificity of the responding T cell is critical for full penetrance of autoantibody production and disease in human and murine lupus.

Origin of Autoreactive T and B Cells in Lupus

The preceding studies provide a simple, concise model for lupus in which pathogenic autoantibody production by B cells requires cognate interaction between ab T cells and autoreactive B cells. The ontogeny of such cells in murine lupus has been addressed in studies of central tolerance, the process by which autoreactive T cells are deleted in the thymus. This work demonstrated that in lupus-prone animals, superantigen-induced deletion is intact (183,184 and 185) and that central deletion is comparable between Fas-deficient MRL/Fas^{lpr} and Fas-intact MRL/+ ^{Faslpr} congenic mice (186,187 and 188). A more recent study analyzed thymic deletion after administration of a conventional peptide antigen in MRL mice in comparison to a nonautoimmune strain, B10.BR, utilizing AND TCR transgenic mice (189) that recognize a pigeon cytochrome C (PCC) peptide (88,89,90,91,92,93,94,95,96,97,98,99,100,101,102,103 and 104) in the context of $H\text{-}2^k$. Identical reduction in the number of double-positive (DP) thymocytes, downregulation of the transgenic TCR, and an increase in the number of apoptotic thymocytes were observed in both the autoimmune and nonautoimmune strains (189). These experiments.suggest that negative selection to agonist antigens is intact in lupus and leads to the question of whether the initiation or regulation of T-cell activation (i.e., peripheral tolerance) is abnormal in lupus.

INTRINSIC ABNORMALITIES OF T-CELL ACTIVATION IN LUPUS

ab T cells are necessary for full penetrance of autoantibody production and disease in human and murine lupus; at present, it appears that activation of such cells in lupus is a consequence of peripheral tolerance abrogation. The events that lead to this are at present unknown. Accumulating evidence suggests that lupus T cells have intrinsic (genetic) defects that render them more susceptible to activation through their TCR–CD3 complex after contact with self-peptides. This hypothesis stems

from several observations. First, T cells from humans with SLE appear to have abnormalities in TCR signaling (190,191 and 192) and apoptosis (193,194) as well as in expression of effector molecules, including CD40 ligand (CD154) (168,171,179,180). Second, lupus-prone mice have increased numbers of activated T cells *in vivo* (195,196 and 197). Third, a recently identified genetic locus on chromosome 7 from lupus-prone New Zealand mice (NZM; New Zealand mixed) contributes to a heightened threshold of T-cell activation and a lower threshold for apoptotic death (40,198). Interpretation of studies of T cells isolated from humans and mice with lupus is complicated by the heterogeneity of such cells (for example, in the state of activation and consequences of repetitive stimulation *in vivo*). Moreover, these studies and the genetic studies of lupus mice have not shown conclusively that the identified defects are intrinsic to mature T cells rather than extrinsic (for example, in antigen presentation). Indeed, APCs, such as macrophages (199,200 and 201) and B cells (202,203), from lupus-prone mice are genetically abnormal compared with nonautoimmune controls, and such abnormalities could account for the previously identified T-cell defects. Indeed, the locus from chromosome 7 in the NZM background that contributes to enhanced T-cell activation (198) overlaps with the same locus from the Fas-deficient MRL background (MRL/Fas^{lpr}) that contributes to anti-DNA antibody production and lymphoaccumulation (204) and to renal disease (205). The latter locus is not yet known to be associated with other defects, such as in T-cell activation.

Understanding of the role of ab T cells in the etiology of lupus has been complicated by a paradox: Lupus is a disease apparently characterized by systemic T-cell activation; however, T cells isolated from lupus patients and mice have been reported to be hypoproliferative (206,207,208,209 and 210). Typically, demonstration of hypoproliferation has rested on studies in which T-cell proliferation to a nominal antigen was measured. In these studies, T cells isolated from lupus patients were hypoproliferative compared with those from healthy controls or from those with other rheumatic diseases. These conclusions are confounded, however, by the facts that (a) antigen-specific responses cannot be measured accurately without enumeration of antigen-specific T cells and equalization between groups; and (b) the proliferative response to mitogens is affected by the activation history of the T cell. It is most meaningful to compare the behavior of naive cells to naive cells and activated cells to their counterparts; however isolation of these cell populations has proved difficult. It may also be that the generalized activation of T- and B-lymphocytes seen in lupus leads to clonal expansion of self-reactive lymphocytes, skewing the lymphocyte repertoire toward a self-response and making responses to nominal antigens more difficult to detect. In any case, it will be difficult to separate the contributions of T cells and B cells to the disease process because clonal expansion of autoreactive T cells in lupus is dependent on B cells (148) and extensive T-cell and B-cell collaboration, mediated by CD154–CD40 interactions (176,178). This collaboration is enhanced by upregulated levels of CD40L on lupus T cells (168,171) and may explain the presence of chromatin- and ribonucleoprotein-specific ab T cells in the peripheral blood of human lupus patients (167,172,211,212 and 213).

The mechanism(s) underlying abnormal T-cell activation in lupus is unknown. Biochemical defects potentially affecting T-cell signaling have begun to be identified in lupus T cells, although the consequences of these abnormalities on T-cell function remain poorly understood. T cells isolated from the peripheral blood of patients with lupus show decreased activity of protein kinase A (PKA), a cyclic adenosine monophosphate (cAMP)-dependent serine/threonine kinase (214,215). Decreased PKA activity in lupus patients is due to inefficient binding of cAMP to PKA, leading to deficient phosphorylation of membrane associated proteins (214,215), among other effects. How this abnormality in such a ubiquitous and integral enzyme, which is present in most patients with lupus (216), might contribute to the development of generalized T-cell activation and systemic autoimmunity remains unknown. TCR-mediated signal transduction also has been found to be abnormal in lupus. T cells isolated from the peripheral blood of lupus patients have a greater flux of calcium from intracellular stores after TCR stimulation but, paradoxically, show deficient phosphorylation of the z chain of the CD3 complex (217). Finally, T cells from lupus patients have been reported to be deficient in the activation of the transcription factor NF-kB (218). These findings of diminished phosphorylation of the z chain of the CD3 complex and decreased induction of NFkB are surprising in light of the increasing evidence that lupus T cells are hyperresponsive to stimulation through the TCR (198).

Intrinsic Abnormalities of B-cell Activation in Lupus

Although the nature and extent of intrinsic T-cell abnormalities in lupus remain poorly defined, the case for intrinsic B-cell abnormalities is much clearer. Experimental evidence from murine and human lupus has consistently and strongly suggested that there is an intrinsic abnormality in lupus B cells that leads to dysregulated production of autoantibodies. This conclusion is supported by the demonstration that pre-B cells isolated from the fetal liver of lupus-prone (NZB x NZW) F_1 mice (NZB/W) transferred into severe combined immunodeficient (SCID) mice will lead to IgM and IgG hyperglobulinemia, production of ANAs, and, in some mice, lupuslike deposition of IgG in the kidney with significant proteinuria (219). The transfer of pre-B cells from nonautoimmune mice did not lead to these characteristic signs of autoimmunity. *In vitro* studies demonstrated that B cells in the peripheral lymphoid organs of NZB/W mice spontaneously secrete significantly more IgM from birth than control B cells (220). B cells from lupus-prone mice are also more sensitive to stimulation from T cells (221). These data are consistent with studies of human lupus that have demonstrated that B cells are hyperproliferative and produce more antibody spontaneously, including autoantibodies (222).

The molecular mechanisms responsible for this hypereffector phenotype of lupus B cells remain largely unknown. Abnormal cytokine production and expression of effector molecules on the cell surface have been investigated in both human and murine lupus. B cells and monocytes from lupus patients produce more IL-10 and are more sensitive to IL-10 stimulation (223). Further, B cells from lupus patients with active disease abnormally express CD40 ligand compared with those isolated from patients in remission or from healthy controls (168). Expression of CD40L could be induced on B cells from lupus patients in remission but not on control B cells, supporting the idea of an intrinsic difference in lupus B-cell phenotype. Taken together, these findings demonstrate that, in lupus, opportunities for T-cell and B-cell interactions exist that do not exist in unaffected persons. It seems likely that aberrant production of cytokines (such as IL-10) and expression of surface effector molecules (such as CD40L) set up a self-perpetuating cycle of lymphocyte activation that ultimately enhances disease progression.

Evidence suggests that signal transduction in B cells is abnormal in lupus. Abnormalities in both positive and negative regulation of B-cell receptor (BCR) signaling have been described. Human lupus B cells also differ in their biochemical imprint after BCR stimulation, having greater protein tyrosine phosphorylation and free calcium release than control B cells after BCR stimulation (224). The reason for this important difference is unknown. CD22 is a B-cell-specific transmembrane glycoprotein that is phosphorylated after BCR signaling and assists in the recruitment of SHP-1 phosphatase into the BCR signaling complex. It therefore serves as an important downregulator of B-cell activation (225). B cells from mice deficient in CD22 (through targeted gene knockout) are also hyperresponsive after BCR stimulation and develop high titers of IgG anti-dsDNA antibodies (225,226). The role of CD22 and other BCR-associated proteins, such as CD21, in regulating B-cell activity in lupus is unknown and is an important area for investigation.

The role of B cells as APCs also appears important to the development of lupus. MRL/Fas^{lpr} mice genetically deficient in B cells had a significantly reduced number of activated and memory T cells with a shift toward a naive phenotype compared with B-cell-intact animals (126). As a consequence, T-cell infiltration of the skin and kidney was markedly reduced. In summary, lupus B cells are intrinsically abnormal in terms of BCR signaling, with enhanced production of, and sensitivity to, cytokines trophic to B cells as well as aberrant expression of effector molecules. These abnormalities result in dysregulation of antibody production and may also lead to inappropriate activation of T cells and abnormal T-cell and B-cell collaboration.

Complement System and Fc Receptors

The complement system has a complex and paradoxical role in the pathogenesis of lupus. Activation of the classical complement pathway with local deposition of C3 is seen in the kidneys of lupus patients with proliferative glomerulonephritis. Deficiencies in the early components of the classical pathway, particularly C1q, however, are well known to predispose to lupus (227,228). This genetic correlation is particularly strong: Nearly 90% of patients completely deficient in C1q, C1r/C1s, or C4 develop SLE independent of race and ethnicity (228,229). Although still striking, the relation of C2 deficiency to disease is weaker because only approximately one third of patients with complete C2 deficiency develop lupus. This is likely due to the fact that C2 can be bypassed in the early activation of the complement system (228). Thus, complete absence of the early complement components, particularly C1q and C4, is sufficient to cause the development of autoimmunity independent of other genetic influences. Such individuals account for approximately 1% of all individuals with lupus (228). What is the link between early complement deficiency and the development of autoimmunity?

In lupus patients, particularly those with nephritis, phagocytic cells fail to clear immune complexes effectively from the peripheral lymphoid organs (230,231,232 and 233). Patients with active disease have the most pronounced impairment (232). Given the importance of immune complex deposition in the pathogenesis of nephritis and the role of C1q in binding immune complexes and facilitating their removal from the circulation, it is logical to hypothesize that patients with absent C1q will have impaired clearance of immune complexes. C1q may also bind the surface of apoptotic cells *in vitro* independent of the presence of antibody (234). Taken together, these data suggest that normal functioning of the complement system, through C1q, is necessary to remove immune complexes and apoptotic cells either with or without antibody binding. This hypothesis has not been directly tested in humans. An autoimmune syndrome characterized by the development of immune complex glomerulonephritis but with incomplete penetrance was seen in mice deficient in C1q (22). An increase in the number of apoptotic cells in the glomerulus was observed, suggesting that immune complexes of apoptotic cells with autoantibodies were being deposited in the kidney. The autoantibodies seen in these animals were directed against chromatin, the most common specificity seen in lupus. Development of autoimmunity depended on the strain studied. Glomerulonephritis was seen in 25% of the F intercross between 129 and C57BL/6 mice but not in the 129 parents. Therefore, one must be cautious in extrapolating these findings to the pathogenesis of human lupus. Given the low frequency of homozygous complement deficiency in the cohort of lupus patients, other mechanisms must account for tissue injury besides an impairment of clearance of immune complexes containing apoptotic cells.

Similarly, defects in the mechanisms for the removal or degradation of cellular debris may predispose to systemic autoimmunity. Self-antigens such as chromatin are present within the debris of apoptotic cells. An increase in the concentration of self-antigens above a certain immunologic threshold presumably breaks immunologic tolerance to these antigens. Mice engineered to be deficient in DNAse 1, the major nuclease present in the serum, develop antichromatin antibodies and immune complex–mediated glomerulonephritis (235). Lower levels of DNAse 1 were also seen in a small group of SLE patients, but how common this finding is in lupus and

whether it is primary or secondary to the disease process are unknown.

Other defects in the handling of circulating immune complexes have been described in lupus. In healthy individuals, circulating immune complexes containing complement fragment C3b bind to erythrocytes via complement receptor 1 (CR1). Red cells bearing immune complexes containing complement are then stripped of immune complexes in the spleen by phagocytic cells bearing receptors for the Fc portion of immunoglobulin. Red cells in lupus patients express lower levels of CR1 (236,237 and 238), but this is an acquired defect and not an inherited deficiency (239,240). Thus, it appears that this decrease in the ability of red cells in lupus to transport immune complexes to the spleen is not a primary event in the pathogenesis of the disease. Instead, it appears that abnormalities in expression of the Fc receptor for IgG2a may be more important as causative factors. In humans, two alleles with differing affinity for IgG2a confer either high or low affinity for the isotype. In one study, African-American lupus patients, particularly those with nephritis, appeared more likely to express only the low-affinity receptor (23), although another study reported no such association (26). In addition, a polymorphism in FcRIIIa (CD16) resulting in low binding of IgG has been associated with lupus, particularly nephritis (27). The significance of Fc receptors in the pathogenesis of lupus is illustrated in animals deficient in the ability to bind immune complexes through a targeted deletion in the common gamma chain of Fc receptors (117). Lupus-prone (NZB x NZW)F_1 mice with such a deletion were protected from the development of severe glomerulonephritis, despite the deposition of immune complexes in the kidney and the local activation of complement (117).

Vascular Injury in Lupus

The role of endothelium in the initiation and propagation of tissue injury in lupus is an increasingly important area of research (241). Endothelial cells are important mediators of inflammation and have been shown to regulate the migration of inflammatory cells into tissue via expression of adhesion molecules. They also can produce proinflammatory molecules such as nitrous oxide (NO) and the cytokines IL-6 and IL-8. Vascular endothelium is activated in lupus, as demonstrated by the increased expression of ICAM-1, VCAM-1, and E-selectin in clinically uninvolved skin in lupus patients (72). This suggests that the upregulation of adhesion molecules is an “upstream” event in lupus and occurs before the development of overt pathology. The stimuli triggering endothelial activation in lupus are largely unknown. The ligation of C1q receptors on endothelial cells leads to the upregulation of adhesion molecules *in vitro,* and immune complexes are an important ligand of C1q *in vivo*. Thus, the accumulation of immune complexes in patients with lupus nephritis could result in increased binding of C1q to endothelial cells, resulting in endothelial cell activation (242). Endothelial cells also express CD40 and are subsequently activated through interaction with CD40 ligand (CD154) (243). This raises the intriguing question of whether the aberrantly high and prolonged expression of CD40L on the surface of T and B cells in lupus (168,171,244) or elevated soluble levels in lupus (179,180) contribute to endothelial activation. The contribution of activated platelets expressing CD40L to endothelial activation via CD40L–CD40 interaction in lupus is also unknown (245). Of relevance here is the recent description of thromboembolic complications in monkeys treated with anti-CD40 ligand antibodies in an attempt to block the rejection of renal allografts (246).

Activation of vascular endothelium may be due in part to increased production of NO in lupus. Elevated production of NO has been found in both patients with active lupus (247) and lupus-prone MRL/*Fas*^{lpr} mice (248). In persons with lupus, NO levels correlate with disease activity (247). The importance of the production of NO in the pathogenesis of murine lupus was demonstrated in MRL *lpr* mice deficient in the inducible form of NO synthetase (iNOS). These mice have markedly diminished vascular disease but unchanged glomerulonephritis (249). Significant sources of NO production in lupus patients are the vascular endothelium and also keratinocytes (72), where it is produced even in clinically uninvolved skin. The clinical sequelae of vascular disease in lupus extend beyond inflammatory lesions in the skin and kidneys. Even as the prognosis for patients with lupus has improved, morbidity and mortality rates from cardiovascular disease and stroke have increased (250). The cause of the accelerated atherosclerosis remains unknown but may involve the perturbations of the vascular endothelium discussed herein.

GENETICS OF LUPUS

That SLE is a disease with a substantial genetic component is well documented by studies of monozygotic twins with lupus, with a concordance rate of 25% to 50% (16). Some of the most exciting and dramatic developments in understanding the pathogenesis of SLE have come from studies aimed at unraveling the genetics of murine lupus. Concurrent progress in understanding the genetics of human lupus has been made as well.

Advances in understanding the genetics of murine lupus come from studies of the (NZB x NZW)F_1 and New Zealand mixed (NZM) models of lupus, and, to a lesser degree, from the MRL model. The advantages of studying genetics in murine models are that (a) causal relationships between proposed susceptibility loci and disease can be identified through the introduction of candidate loci in nonautoimmune hosts (generation of congenic mice); (b) the relationships between susceptibility loci can be assessed through the use of mice congenic for multiple such loci; and (c) disease-related genes identified in lupus-prone mice can suggest mechanisms of disease and pathogenesis and can be used to screen for similar disease-associated genes in humans. Lessons from murine studies will be discussed first, followed by a review of human studies.

Disease-associated loci on chromosomes 1, 4, and 7 in both (NZB x NZW)F mice (including the NZM strain) and MRL mice have been identified by analysis of F_1 progeny backcrossed to parental strains [reviewed in (251)]. In addition, there is a weaker association with the MHC locus on chromosome 17. Using differences in short tandem repeats of DNA flanking loci of interest, mice can be bred to express selectively these loci on the host's normal genetic background (congenic mice). These studies revealed that a locus on chromosome 1 (Sle1), about 100 cM long, is sufficient to abrogate B-cell tolerance when bred into otherwise normal C57BL/6 mice (252). These mice develop ANAs directed against chromatin, specifically against the H2A/H2B component of the nucleosome. This is analogous to the process by which antibodies specific for each part of the nucleosomal complex arise sequentially in human patients. T-cell tolerance to chromatin is also lost in these animals, suggesting that expression of this locus leads to the presentation of chromatin in an immunogenic manner. Despite these gross defects in B-cell and T-cell functions, lymphocyte responses to mitogenic stimuli are normal and Sle 1 congenic mice do not develop immune complex–mediated glomerulonephritis.

A locus on chromosome 4 (Sle2) confers an intrinsic B-cell defect (204). B cells from mice congenic for this locus are hyperresponsive to stimulation through the BCR or with soluble CD40L, duplicating the phenotype of B cells in both lupus patients and in autoimmune mice. They secrete more IgM when immunized with nominal antigens but do not develop an elevated IgG response. These mice have no defects in T-cell function, nor do they develop autoimmune pathology. This strongly suggests that a T-cell defect is necessary to turn B-cell hyperresponsiveness into overt autoimmunity.

A locus on chromosome 7 (Sle3) confers an intrinsic T-cell abnormality (198). In these animals, T-cell tolerance to chromatin is broken such that T-cell reactivity against all nucleosomal components can be demonstrated. CD4+ T cells are hyperresponsive to stimulation with anti-CD3 and show a decrease in activation-induced cell death. Perhaps as a consequence of promiscuous T-cell activation and help, these mice develop low-titer ANAs but usually do not develop overt autoimmune disease. Bicongenic nonautoimmune mice bearing both susceptibility loci from chromosome 1 and chromosome 7 develop expanded pools of activated B and T cells, high-titer anti-dsDNA antibodies, and highly penetrant, lethal glomerulonephritis in female patients (40). This suggests synergistic interaction between the genes conferring intrinsic abnormalities in B- and T-cell function, leading to fulminant autoimmune disease.

Two linkage studies in Fas-deficient MRL-*Fas*^{lpr} mice also identified a centromeric region of chromosome 7 associated with disease: an (MRL/*Fas*^{lpr} x *Mus. castaneous*) x MRL/Fas^{lpr} backcross 1 analysis-linked *Lrdm2* on chromosome 7 to renal disease (205) and a more recent (MRL/*Fas*^{lpr} x C57BL/6-Fas^{lpr})F_1 cross-found linkage between *Lmb3* (from the MRL background) and lymphadenopathy and splenomegaly, with a weaker association with anti-DNA autoantibody production (254). *Lrdm2* and *Lmb3* overlap with the lupus susceptibility locus *Lbw5*, mapped using an (NZB x NZW)F_1 cross (255), and with Sle3, the susceptibility locus from the NZM background (198). Finding these overlapping susceptibility loci in different animal models of lupus suggests that a limited number of genes can interact to predispose to systemic autoimmunity.

ROLE OF APOPTOSIS IN THE INITIATION OF SYSTEMIC AUTOIMMUNITY

A rapidly growing body of work investigated the role of programmed cell death (apoptosis) in the initiation of systemic autoimmunity. The impetus of this work is the observation that nuclear and cytoplasmic (auto)antigens targeted by the adaptive immune system in lupus are generated on the surface of cells during apoptosis, exposing them to the immune system (42). The hypothesis that antigens generated during apoptosis initiate systemic autoimmunity is predicated on two ideas: (a) that tolerance to self-antigens may be broken because these antigens are generated at a high enough concentration to cross an “immunologic threshold” and (b) that self-antigens are altered during apoptosis to create new epitopes that the immune system has not seen before and therefore cannot have developed tolerance for these epitopes. Presumably, differences in the responsiveness of the adaptive immune system to these stiumli would lead to a differential adaptive immune response in individuals with lupus compared with healthy individuals (i.e., autoimmunity versus maintenance of peripheral tolerance).

Antigenic targets of lupus autoantibodies have been found on the surface of keratinocytes induced to undergo apoptosis by UVB irradiation. These include both cytoplasmic (e.g., the Ro protein, ribosomal antigens, and calreticulin) and nuclear antigens [other Ro proteins, La, snRNPs, Ku, DNA-dependent protein kinase and poly-ADP ribose polymerase (PARP)] (42). This finding is of particular interest because of the known role of UVB in initiating and exacerbating skin disease and systemic flares in those predisposed to develop lupus. In addition, many of these protein antigens appear to have been modified from their original structure by caspase-mediated cleavage, generating novel protein fragments that are now potentially immunogenic (256,257 and 258). Not all antigenic targets in lupus are cleaved during apoptosis, however.

Apoptosis is distinguished from necrosis by its ability to induce cell death without causing inflammation. This blockage of inflammation depends on the rapid clearance of apoptotic cells by phagocytic cells before the debris of apoptotic cells is recognized by circulating leukocytes. Defects in macrophage clearance of apoptotic cells *in vitro* have been reported in lupus patients (259), suggesting that the concentration of potential autoantigens may be higher in lupus. This defect alone, however, should not induce autoimmunity because B and T cells should be tolerant to autoantigens, even if they are presented more frequently. Thus, we must ask whether there are additional defects in the process of antigen processing and presentation that contribute to the abrogation of tolerance. Growing evidence supports the notion that dendritic cells capable of phagocytosing apoptotic cells can tolerize peripheral T cells (260,261). It will remain to be seen whether this pathway is fully functional in persons who have lupus.

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40 SJÖGREN'S SYNDROME

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HISTORICAL FEATURES

Sjögren's syndrome is named after the Swedish ophthalmologist Henrik Sjögren (1). However, the disease was addressed during the 19th century in a number of case reports, between the years of 1882 and 1924, describing various combinations of dry mouth, dry eyes, and chronic arthritis (reviewed in 2). Mikulicz (3) reported in 1892 a man with bilateral parotid and lacrimal gland enlargement associated with massive round cell infiltration. Somewhat later Gourgerot (4), in 1925, described three patients with salivary and mucous gland atrophy and insufficiency. In 1927, Mulock Houwer (5) reported the association of filamentary keratitis, the major ocular manifestation of the syndrome, with chronic arthritis. Subsequently in 1933, Henrik Sjögren (1) reported in his classic doctoral dissertation detailed clinical and histologic findings in 19 women with xerostomia and keratoconjunctivitis sicca, of whom 13 diplayed chronic arthritis. Later on, Morgan and Castleman (6) in 1953 established that Sjögren's syndrome and Mikulicz's disease were the same entity. The link between Sjögren's syndrome and malignant lymphoma was described in a classic paper in 1964 (7). The distinction between primary and secondary Sjögren's syndrome was suggested in 1965 (8). From the diagnostic point of view, the first histologic grading assessing the infiltration of labial glands was described in 1968 (9). More recently, a set of preliminary classification criteria was identified by a European Concerted Action in 1993, and they have been widely accepted (10).

IMMUNOLOGIC HIGHLIGHTS

The salivary and lacrimal glands are the principal targets of a T-cell–mediated chronic inflammation, giving rise to deficient function and leading to dry eyes (keratoconjounctivitis sicca) and mouth (xerostomia). However, other exocrine glands including those of the pancreas, sweat glands, and mucus-secreting glands of the bowel, bronchial tree, and vagina also may be affected. The Sjögren's syndrome–associated (Ro/SSA) autoantibodies in the sera were described in 1969 (11) and were later found to be present also in secretions (12). A number of other autoantibodies have been described, but none has received significant clinical recognition (2). Gene interaction and complementation of the immune response was shown for human leukocyte antigen (HLA) and the RNA proteins Ro/SSA and La/SSB (13).

EPIDEMIOLOGY

General

Sjögren's syndrome is a worldwide disease and may occur at all ages. However, the peak incidence is in the fourth and fifth decades of life, with a female-to-male ratio of 9:1. A number of studies have shown a great variation in the frequency of Sjögren's syndrome (reviewed in 2). The prevalence studies demonstrate that sicca symptoms and primary Sjögren's syndrome affect a considerable portion of the population; precise numbers are dependent on the age group studied and on the criteria used (14). Subjects identified as having primary Sjögren's syndrome in population studies often have mild to moderate complaints, and many of them have been found not to be aware of the disease. A cautious but realistic estimate from the studies presented so far is that primary Sjögren's syndrome is a disease with a prevalence not exceeding 0.6% of the general population (six per 1,000).

For the diagnosis of Sjögren's syndrome, the most practical criterion to use is the recently modified European criteria, which include a list of exclusions (15). In addition to the subjective symptoms of dry eyes and dry mouth, the following objective items should be fulfilled: (a) ocular signs by Schirmer's I test and/or rose bengal score; (b) focal sialadenitis by histopathology; (c) salivary gland involvement by either salivary scintigraphy, parotid sialography, or unstimulated salivary flow; and (d) autoantibodies of Ro/SSA and/or La/SSB specificity.

Genetics

A prominent feature of Sjögren's syndrome is its genetic predisposition (reviewed in 16). Several families involving two or more cases of Sjögren's syndrome have been described (17), and a family history with relatives having other autoimmune diseases is common (30% to 35%) for Sjögren's syndrome patients.

The polymorphic major histocompatibility complex (MHC) genes are well-documented genetic risk factors for the development of autoimmune diseases overall (18). With regard to Sjögren's syndrome, the most relevant MHC complex genes are the class II genes, more specifically the HLA-DR and DQ alleles (19). Patients of different ethnic origin show different HLA gene associations (20). In whites of northern and western European background, including North American whites, Sjögren's syndrome is one of several autoimmune diseases associated with the haplotypes HLA-B8, DRw52, and DR3. An association with DR2 has been reported in Scandinavians (21) and DR5 in Greeks (22). All of the haplotypes are in strong linkage disequilibrium, causing difficulties in establishing which of the genes contains the locus conferring the risk.

HLA class II allele association has been reported to differ among anti-Ro/SSA positive subjects according to the presence or absence of coexisting anti-La/SSB (23). Distinct HLA haplotypes have been associated with a certain degree of autoantibody diversification in Sjögren's syndrome patients (24). Of particular importance is that a stronger correlation has been found between anti-Ro/SSA autoantibodies and DR3/DR2 than that with the disease itself (25,26). Autoantibodies to Ro/SSA and La/SSB have been found to be associated with DR3 and DQA alleles (27). Sjögren's syndrome patients with DQ1/DQ2 alleles have a much more severe autoimmune disease than do patients with any other allelic combination at HLA-DQ (13). Recently the DR3-DQ2 haplotype has been indicated as a possible marker for a more active immune response in Finnish Sjögren's patients (28).

Environment

Among the possible etiologic and triggering factors involved in Sjögren's syndrome, the discussion about a relationship between viral infections causing development of autoimmune reactions began some decades ago. The putative role of different viruses in Sjögren's syndrome can be viewed in the light that salivary glands are a site of latent viral infections. Potential viral triggers include a number of viruses including Epstein-Barr virus (EBV), widely studied in relation to Sjögren's syndrome (29). A higher prevalence of serum human herpesvirus-6 (HHV-6) specific antibodies also has been detected in patients with Sjögren's syndrome than in normal individuals (36% vs. 10%) (30). However, other studies could not confirm this finding (31,32). Difficulties in analyzing the possible role of viral infection in relation to development of Sjögren's syndrome is hampered by the high prevalence of both herpesviruses (EBV and HHV-6) in the healthy population.

Retroviruses are known to infect cells of the immune system and cause abnormalities in immune regulation. High serum titers of anti–human T lymphotropic virus type I (HTLV-I) antibodies and a high prevalence of salivary immunoglobulin A (IgA) class anti–HTLV-I antibodies in patients with Sjögren's syndrome were reported endemically in Japan (33).

Hepatitis C virus (HCV) infection has in some populations been frequently (14%) detected in patients with primary Sjögren's syndrome (34). Analysis of the association between chronic lymphocytic sialadenitis and chronic HCV liver disease showed that histologic features of Sjögren's syndrome were significantly more common in HCV-infected patients (57%) compared with controls (5%) (35).

Lymphotropic viruses have the potential to trigger the autoimmune process. Some of the immunoreactive regions within the La/SS-B protein have been found to have sequence similarities with proteins of EBV, HHV-6, and human immunodeficiency virus (HIV)-I (36). It seems reasonable that these viruses can promote autoantibody (particularly anti-La/SS-B) production through molecular mimicry or exposure of La/SS-B homolog sequences on cellular surfaces after translocation of cryptic self-determinants.

CLINICAL PRESENTATION

Signs and Symptoms

Sjögren's syndrome has a wide variety of clinical features (reviewed in 2). Onset of the disease is insidious, and patients have difficulty in determining when the disease actually started. Keratoconjunctivitis sicca and xerostomia (so-called sicca complex) are the main clinical presentations in adults, whereas bilateral parotid swelling (Fig. 40.1) can be an obvious sign at juvenile disease onset. In more than half of the patients, an extraglandular manifestation may develop during the evolution of the disease. Occasionally systemic features may lead to diagnosis.

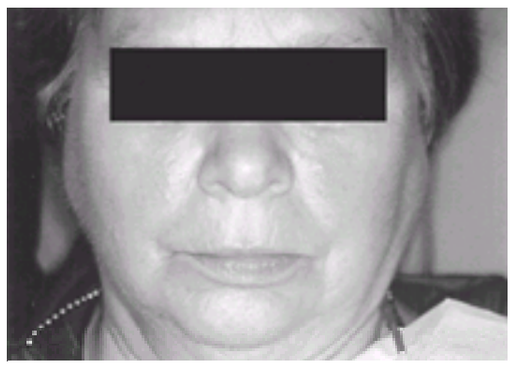

Figure 40.1. Bilateral parotid gland enlargement in Sjögren's syndrome.

The spectrum of the disease extends from an organ-specific autoimmune disorder to a range of systemic manifestations (musculoskeletal, pulmonary, gastric, hematologic, dermatologic, renal, and nervous system involvement). Sjögren's syndrome may develop alone (primary) or in association with almost any of the autoimmune rheumatic diseases (secondary), the most frequent being rheumatoid arthritis and systemic lupus erythematosus.

Arthritis, Raynaud phenomenon, and purpuric vasculitis on the lower extremities are the most common extraglandular manifestations of primary Sjögren's syndrome. A high incidence of pulmonary involvement with functional abnormalities has been described, although they are usually subclinical and rarely progressive. A wide range of neurologic disorders has been reported, the peripheral nervous system being most frequently affected. Interstitial nephritis in a subclinical form and as a cause of renal tubular acidosis or nephritogenic diabetes insipidus occurs in about 30% of the patients.

Lymphomas, almost exclusively of B-cell lineage, are a characteristic but unusual feature of Sjögren's syndrome, occurring in about 5% of the patients (37). This complication of Sjögren's syndrome is found particularly in patients with high levels of immunoglobulins, autoantibodies, and cryoglobulins. When the lymphoma develops, the immunoglobulin levels often decrease, and the autoantibodies might disappear.

Concerning secondary Sjögren's syndrome, rheumatoid arthritis patients with sicca complex tend to have more severe disease, with frequent extraarticular manifestations including vasculitis, presenting as digital infarcts and/or cutaneous ulcers. In systemic lupus erythematosus, patients with concomitant Sjögren's syndrome have a lower frequency of glomerulonephritis and a relatively good prognosis. Primary biliary cirrhosis and scleroderma, although rare in general, are frequently complicated by Sjögren's syndrome. Other autoimmune diseases that have been described in association with Sjögren's syndrome include polymyositis, mixed connective tissue disease, chronic active hepatitis, and Hashimoto thyroiditis (38).

Laboratory Tests

The most commonly used tests for the detection of dry eyes are the Schirmer I and the rose bengal (alternatively Lissamine green dye) and subsequent scoring according to van Bijsterveld. Schirmer I is performed using standardized tear test strips. In the European classification criteria (10), Schirmer I test is positive when the wetting is less than 5 mm in 5 minutes. The scoring according to van Bijsterveld detects destroyed conjunctival epithelium induced by dryness.

Saliva-production tests are simple screening tests for salivary gland involvement in Sjögren's syndrome. Saliva, which is produced by the three major and numerous minor submucosal salivary glands, exhibits great flow variations among healthy individuals and in the same individual under diverse conditions (39,40). The test should therefore be standardized; the unstimulated whole saliva collection test is performed for 15 minutes, and the test is considered positive when 1.5 mL or less whole saliva is collected, being well below the normal mean range.

Other tests used to evaluate salivary gland involvement include parotid sialography and salivary gland scintigraphy. The sialography typically shows sialectasias in contrast to the fine arborization seen in normal parotid ductules. In the scintigraphic test, ^{99m}technetium-pertechnate is given intravenously, and in Sjögren's syndrome patients, the typical finding is decreased uptake in response to stimulation of the parotid and submandibular salivary glands. This test is a sensitive and valid method to measure abnormalities in salivary gland function in the hands of skilled personnel (41).

Anti-La/SSB antibodies were first defined by the immunodiffusion technique in association with anti-Ro60 precipitins (11). Recent studies have shown that up to 40% of anti-La–positive sera are negative on immunodiffusion and detectable only by immunoblot or enzyme-linked immunosorbent assay (ELISA). These are termed nonprecipitating anti-La antibodies (42,43). Anti-La is invariably accompanied by anti-Ro, reflecting the physical association of these molecules in Ro/La ribonucleoprotein particles (Fig. 40.2). In contrast, anti-Ro antibodies frequently occur in the absence of anti-La reactivity.

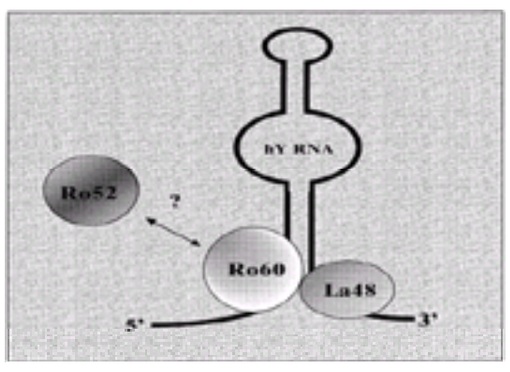

Figure 40.2. Structure of important autoantigens (Ro/SSA and La/SSB) in Sjögren's syndrome. hY RNA, human small cytoplasmic Y RNA.

The reported frequencies of anti-Ro and anti-La depend on the methods of detection and referral bias of the center performing the study. Overall, anti-Ro precipitins

occur in approximately 60% to 75% of primary Sjögren's syndrome and also are observed in cases of secondary Sjögren's syndrome, irrespective of the association with systemic lupus erythematosus, progressive systemic sclerosis, rheumatoid arthritis, or primary biliary cirrhosis (44). Anti-La antibodies were initially reported to occur in up to 40% of patients with primary Sjögren's syndrome. Even higher frequencies were reported when anti-La was analyzed by ELISA or immunoblotting (45). Further studies have shown that combined detection of anti-La and anti-Ro antibodies have a higher diagnostic specificity for primary Sjögren's syndrome than does anti-Ro alone (46).

Although the pathogenetic role of anti-Ro and anti-La in Sjögren's syndrome is not established, positive serology is associated with a high frequency of palpable purpura, leukopenia, lymphopenia, and hypergammaglobulinemia, and with more severe glandular disease (47,48 and 49). Recent studies also have found salivary enrichment of anti-Ro and anti-La in patients with Sjögren's syndrome, suggesting local autoantibody production in salivary glands (12,50) as well as presence of Ro52, Ro60, and La autoantibody-producing cells in salivary gland biopsy samples from patients with Sjögren's syndrome (51,52).

Pathology

The labial salivary gland biopsy has an important role in establishing the diagnosis of Sjögren's syndrome. It is performed preferentially according to the procedure described by Daniels (53,54). After local anesthesia, a 1.5- to 2-cm incision is made parallel to the vermilion border in the middle of the lower lip, between the midline and the corner of the mouth. At least five lobes of labial glands are then obtained by blunt dissection. After routine histologic fixation and preparation, the biopsy is evaluated according to a procedure in which an inflammatory focus is defined as an accumulation of at least 50 mononuclear leukocytes per 4 mm^2 (Fig. 40.3) (54). According to the European criteria (10), a biopsy is positive at focus score 1 or more per 4 mm^2, whereas the California criteria (55) define positive biopsy as more than one focus. Occasionally epimyoepithelial islands are seen in labial gland biopsies, but these are more common in the major glands. One differential diagnostic feature is the granulomatous inflammation as seen in connection with, for example, sarcoidosis.

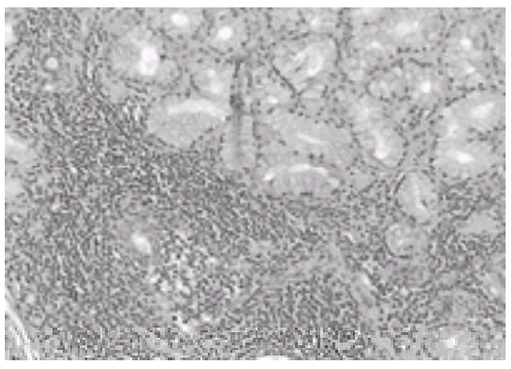

Figure 40.3. Focal sialadenitis in salivary gland with surrounding normal-appearing parenchyma in a patient with Sjögren's syndrome.

The specificity of a positive labial salivary gland biopsy is 86.2%, and the sensitivity is 82.4% in patients with primary Sjögren's syndrome diagnosed according to the European criteria (56). The focal infiltration of lymphoid cells in the salivary glands is a progressive process, as demonstrated by increase of focus score over time (57). The focus score is connected to the presence of keratoconjunctivitis sicca and autoantibodies (48,58), whereas the correlation with xerostomia is less evident (57).

Another pattern of inflammation in labial salivary gland biopsy is chronic sialadenitis, characterized by scattered mononuclear cell infiltration without focal aggregates and accompanied by degenerative changes (acinar atrophy, ductal hyperplasia, fibrosis, and/or fatty infiltration). This pattern is not considered to be typical for primary Sjögren's syndrome and often leads to glandular atrophy and xerostomia.

Treatment and Prognosis

At present, treatment for most patients is essentially symptomatic. The patient should be seen regularly by a rheumatologist as well as an ophthalmologist and dentist to prevent and treat the consequences of mucosal dryness, in addition to extraglandular manifestations and other associated complications.

Artificial tears often alleviate the patient's ocular complaints, and are of importance in preventing corneal damage and conjunctivitis (59). The use of topical steroids is not recommended because of a high risk of secondary bacterial and viral infections in the eye.

Another treatment option for dry eye is “punctal occlusion” by using a variety of “plugs” to occlude the punctal openings at the inner aspects of the eyelids (60). With this procedure, the instilled artificial tears will remain in the eye for a longer time.

The management of dry mouth aims to prevent and treat infections, gum disease, and dental caries. To reduce the risk of caries, it is necessary to keep good oral hygiene and use sugarless sweets and chewing gums to stimulate residual salivary flow. Artificial saliva products and special toothpaste may also be of benefit for certain patients, and fluoride supplementation is advocated. Eradication of oral candidiasis usually provides significant improvement of oral symptoms.

Oral pilocarpine has recently been shown to be a safe treatment and to provide significant subjective and objective benefits for patients with Sjögren's syndrome with symptoms associated with xerostomia (61). Another potential therapy includes systemic use of interferon-a (IFN-a), which may be of benefit for the symptoms associated with xerostomia (62,63). Cemiveline, a novel quinuclidine derivative of acetylcholine exhibiting high affinity for the muscarinic M_3 receptor, has long-lasting sialogogic action and few side effects (64).

Hydroxychloroquine may be useful as an immunomodulating agent reducing immune activation and lymphoproliferation and is sometimes used in patients with Sjögren's syndrome (65,66). Administration of systemic steroids also has been suggested to improve the signs and symptoms of Sjögren's syndrome, but they are mainly used for treatment of severe extraglandular complications such as pulmonary and renal involvement (67).

There are few studies on the natural course of primary Sjögren's syndrome, but it has been described as a step-wise, gradual progression from a disorder mainly in exocrine glands, to systemic extraglandular features, and finally to lymphoid neoplasia development (68). However, in general, primary Sjögren's syndrome is characterized by a stable and rather mild course of glandular and extraglandular manifestations, in contrast to the increased risk for development of malignant lymphoma (37).

Serology can be useful in predicting the subsequent outcome and complications in patients with primary Sjögren's syndrome. The presence of anti-Ro/SSA antibodies may identify patients with systemic disease (69), and in anti-Ro/SSA/anti-La/SSB–positive patients, the relative risk of developing non-Hodgkin lymphoma has been reported as high as 49.7 after 10 years' follow-up (70). The development of extraglandular manifestations seems to be influenced by a number of factors including the MHC HLA B8 and DR3 expression (71).

Spontaneous symptomatic improvement has been described in 12% of patients with primary Sjögren's syndrome, especially in elderly patients with some clinical overlap with systemic lupus erythematosus (69). In a recent study on survivorship in a population-based cohort followed up from 1976 to 1992, the authors did not demonstrate increased mortality of patients with primary Sjögren's syndrome (72).

IMMUNOPATHOLOGY

Humoral Responses

A large number of autoantibodies have been reported in both primary and secondary Sjögren's syndrome, reflecting both B-cell activation and a loss of immune tolerance in the B-cell compartment (Table 40.1) (73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91,92,93,94,95,96 and 97). Over the past few years, there has been significant progress in defining the fine specificity of these antibodies (Fig. 40.4) (98,99,100,101,102,103,104,105,106,107 and 108) and characterizing their target autoantigens. In some cases, the presence of these antibodies is related to the extent and severity of disease in Sjögren's syndrome.

Autoantibodies	Frequency in Primary Sjogren's Syndrome (%)	References
Non-organ-specific		
Anti-nuclear antibodies (ANA)	50–80	(73,76)
Anti-Ro/SSA	50–70	(2,73,74,75,76,77,78)
Anti-La/SSB	30–60	(2,73,74,76)
Anti-Sm	2	(74)
Anti-RNP	7	(74)
Anti-DNA	0	(74)
Rheumatoid factor	50–80	(2,73,79,80,81)
Anti-mitochondrial	13	(82)
Anti-neutrophil cytoplasmic	10–25	(83,84)
Anti–alfa-fodrin	55–95	(85,86)
Anti-muscarinic M3 receptor	100	(87,88,89,90,91)
Other antigens		(92,93,94)
Organ-specific		
Anti-smooth muscle	30	(74)
Anti-salivary duct	20–40	(74,95,96)
Anti-thyroid	10–50	(73,74)
Anti-gastric mucosa	5–30	(73,74)
Other tissues		(82,97)

TABLE 40.1. The Association of Autoantibodies and Sjögren's Syndrome

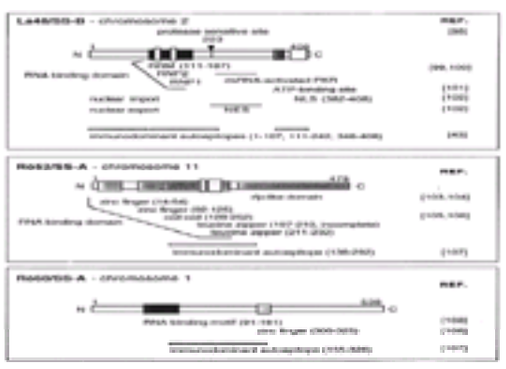

Figure 40.4. Structure, function, and immunobiology of La, Ro52, and Ro60.

The B cells make up roughly 20% of the infiltrating cell population in affected glands. The B cells produce immunoglobulins with autoantibody activity for IgG (rheumatoid factor), Ro/SSA, and La/SSB (52). A substantial number of the B cells are of $CD5^+$ phenotype (B-1 cells) (109). Production of IgG predominates in Sjögren's syndrome, whereas synthesis of IgA is more abundant in normal salivary glands.

Non–organ-specific autoantibodies anti-Ro/SSA and anti-La/SSB are the diagnostically most important and the best-characterized autoantibodies in primary Sjögren's syndrome (2). The majority of anti-Ro–positive sera also react with the denatured form of a 52-kd protein termed Ro52, which is structurally distinct from Ro60 and probably does not directly associate with the Ro ribonucleoprotein particle (Fig. 40.2) (77,78). However, the two Ro proteins colocalize to surface membrane blebs on apoptotic cells, where they may become targets of an autoimmune response (110). Human monoclonal antibodies reactive with continuous and conformation-dependent epitopes on Ro52 have recently been cloned from a patient with primary Sjögren's syndrome (111).

Anti-thyroid microsomal and anti-gastric parietal cell antibodies occur in about one third of patients with both primary and secondary Sjögren's syndrome, but other organ-specific antibodies are infrequent (38). Antibodies to salivary duct antigens were described more than 30 years ago, but they have remained poorly characterized, and their clinical significance is uncertain (95,96).

Several other autoantibodies have been reported to be frequently present in the sera of patients with primary Sjögren's syndrome, including antibodies directed against carbonic anhydrase (92,93), proteasomal subunits (94), and a-fodrin (85). These findings are intriguing but await independent confirmation in larger cohorts of Sjögren's syndrome patients. The finding of serum autoantibodies directed against the muscarinic M_3 receptor (expressed in salivary and lacrimal glands) in the majority of patients is an important advance in understanding the pathogenesis of impaired glandular function in Sjögren's syndrome (87,88). Recent studies in the nonobese diabetic (NOD) mouse have indicated that muscarinic receptor autoantibodies are directed against the agonist binding site of the molecule on the cell surface and interfere with secretory function of exocrine tissues in Sjögren's syndrome (89). Inhibitory effects of these autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome has recently been experimentally shown (90). However, the clinical significance of these antibodies in Sjögren's syndrome remains to be elucidated (91).

Rheumatoid factor is detected in the serum and saliva of 60% to 80% of primary Sjögren's syndrome patients (79,80). There appears to be little role for somatic hypermutation in their generation in contrast to that of rheumatoid factor in rheumatoid arthritis (81). A significant number of patients with primary Sjögren's syndrome have mixed oligoclonal cryoglobulins, many of them having IgM rheumatoid factor activity (112). The latter frequently possess cross-reactive idiotypes, notably the 17.109 (V kappa III b related) and G-6 (VH1 related) idiotypes, which may serve as markers for lymphoma development in primary Sjögren's syndrome (113,114).

Oligoclonal or monoclonal B-cell expansion, arising mainly from salivary glands but also from visceral organs and lymph nodes, has been reported to occur in 14% to 100% of Sjögren's syndrome patients (115). In this respect, Sjögren's syndrome appears to be an intermediate between autoimmunity and malignancy, and it is suggested that patients with evidence of clonal expansions of B cells in their salivary glands are at high risk of developing malignant lymphoma (116,117 and 118). Various studies have reported that between 25% and 80% of salivary lymphoid infiltrates have morphologic and/or immunophenotypic evidence of low-grade lymphomas (119). However, there is no absolute correlation between clonality and the development of lymphoma. Although a high proportion of lymphoid cells may show evidence of immunoglobulin gene rearrangements, clonality does not necessarily predict progression to clinically overt lymphoma. The clinical benefit of immunogenotypic analysis in the clinical diagnosis of salivary gland lymphoma in Sjögren's syndrome remains to be defined (120,121). A recent study reported that a history of swollen salivary glands, lymphadenopathy, and leg ulcers predicted lymphoma development in patients with primary Sjögren's syndrome (122).

Cellular Responses

Immunohistologic analysis of lymphoid cell infiltration in exocrine glands in Sjögren's syndrome shows a predominance of T cells (Fig. 40.5A) with fewer B cells and macrophages (2) (Table 40.2). Adhesion molecules and activated lymphocyte function–associated antigen type 1 (LFA-1) promote homing and occasionally characteristic cell clustering similar to that of follicular structures of lymph nodes. Expression of the mucosal lymphocyte integrin $a^E b_7$ and its ligand E-cadherin suggest a mucosal origin of a subpopulation of the infiltrating cells (123). There is an increased expression of HLA-DR/DP/DQ molecules on acinar and ductal epithelial cells (Fig. 40.5B) (124), presumably due to local production of IFN-g by activated T cells. The majority of T cells in the lymphocytic infiltrates are $CD4^+$ T-helper cells with a CD4/CD8 ratio well over 2. Most of these T cells bear the memory phenotype $CD45RO^+$ and express the a/b T-cell receptor and LFA-1, and may contribute significantly to B-cell hyperactivity. There is indication of oligoclonal expansion of certain TCR V_b family–expressing lymphocytes (125). The peripheral blood in Sjögren's syndrome has yielded findings similar to those in salivary glands, although a difference in magnitude is occasionally evident (Table 40.3).

Infiltration of $CD3^+$ $CD4^+$ $CD45RO^+$ T cells
Mucosal lymphocyte integrin $a^E b_7^+$ cells
Activated B cells (IgG > IgM > IgA)
Production of autoantibodies and monoclonal immunoglobulins
Absence of natural killer cells
Increased expression of adhesion molecules (VCAM-1, LFA-1)
Cytokine production (IL-6, IL-10, TNF-α, IFN-γ)
Dysregulated lymphocytic apoptosis
Aberrant HLA-DR expression on glandular epithelial cells

Ig, immunoglobulin; VCAM, vascular cell adhesion molecule; LFA, leucocyte function–associated antigen; TNF, tumor necrosis factor; IFN, interferon; HLA, human leucocyte antigen.

TABLE 40.2. Immunologic Findings in Salivary Glands in Sjögren's Syndrome

Hypergammaglobuminemia
Autoantibodies (serum and actively secreting cells of ANA, RF, anti-Ro/SSA and anti-La/SSB)
Monoclonal immunoglobulins
Reduced T-cell function
Decreased natural killer cell function
Increased B-1 cells
Increased lymphocytic apoptosis

ANA, antinuclear antibody; RF, rheumatoid factor.

TABLE 40.3. Immunologic Findings in the Peripheral Blood in Sjögren's Syndrome

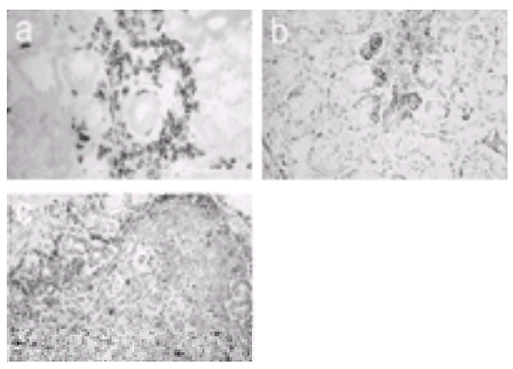

Figure 40.5. Immunohistologic features in minor salivary glands with focal sialadenitis from patients with Sjögren's syndrome disclose **(A)** a large proportion of CD3-positive T cells, **(B)** induced expression of human leukocyte antigen (HLA)-DR on glandular epithelium, and **(C)** few CD25-positive cells.

Immune-mediated Tissue Destruction

Highly upregulated expression of HLA molecules, and the more recently demonstrated B-7 costimulatory molecules (126), by salivary gland epithelium in Sjögren's syndrome (Fig. 40.5B) is a potentially effective local antigen-presenting mechanism whereby HLA antigens could be involved in exocrine glandular destruction mediated directly or indirectly by $CD4^+$ T cells. Such interaction may lead to further production of cytokines and stimulation of B-cell proliferation and differentiation. Indeed, high levels of interleukin (IL)-1b, IL-6, and tumor necrosis factor-a (TNF-a), three tissue-destructive cytokines, are produced by epithelial cells. IL-10 and IFN-g are produced mainly by infiltrating T cells, whereas IL-6 and IL-10 also are produced in increased amounts in peripheral blood (127). A low capacity to produce IL-2 in Sjögren's syndrome might be due to absence of T-cell costimulatory signals, resulting in the induction of anergy in the responding T-cell population, but other explanations also are possible.

Even though the mechanism(s) behind the characteristic glandular destruction of Sjögren's syndrome salivary glands remains obscure, immunopathologic findings demonstrate that infiltrating cytotoxic T cells (CTLs) could play a role in this event. On recognition of a proper MHC/antigen complex presented by a target cell, CTLs induce cell death through one of two main and independent pathways, the perforin-mediated or the Fas-mediated pathway. Interestingly, expression of Fas also has been detected among infiltrating mononuclear cells in salivary glands of MRL/*lpr* mice, a murine model displaying features similar to those of human systemic lupus erythematosus and Sjögren's syndrome (128).

Expression of Fas, Fas-L, Bcl-2, and other apoptosis-associated genes/proteins has been detected by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical staining of minor salivary glands in patients with Sjögren's syndrome (129,130). In particular, ductal and acinar epithelial cells, but to some degree also infiltrating mononuclear cells express abnormal levels of Fas and FasL, especially in cases with heavy mononuclear cell infiltration. Ductal epithelial cells expressing Fas were usually situated inside or close to a dense focus (131). Most *in situ* studies have clearly shown a low-grade or even absent apoptosis among infiltrating mononuclear cells (129,130 and 131). The presence of granzyme A in Sjögren glands (132) suggests that rather than apoptosis, the perforin pathway of CTL-mediated killing may be involved in destruction of salivary glands.

Among the salivary gland–infiltrating T cells, some express activation markers such as CD25 (Fig. 40.5C), protooncogene products, and HLA-DR, but few T cells proliferate as determined by cell-cycle studies. It seems difficult also to stimulate the T lymphocytes in Sjögren's syndrome with the autoantigens Ro/SSA and La/SSB (133). These findings suggest that many cells are of memory T-cell phenotype; either few of them are autoantigen specific, or alternatively, many of them are in a state of anergy. In both cases, lack of stimulation of T cells also will hamper the apoptotic signals.

Immunopathogenesis (Summary)

The etiology and pathogenesis of Sjögren's syndrome is still a matter of speculation, although several hypotheses prevail. Nevertheless, there is considerable evidence that some as-yet-unknown initiating factor(s) set against the appropriate genetic background may evoke immunologically mediated inflammatory mechanisms, which result in the chronic exocrine gland lesions (Fig. 40.6). T-cell–mediated autoimmune responses in the glandular tissue and dysregulated apoptosis are currently considered to be of central importance in the pathogenesis. A plethora of autoantibodies has been linked to this autoimmune exocrinopathy, although their role is not always well-defined. Accordingly, B-cell activation is a very consistent immunoregulatory abnormality in Sjögren's syndrome.

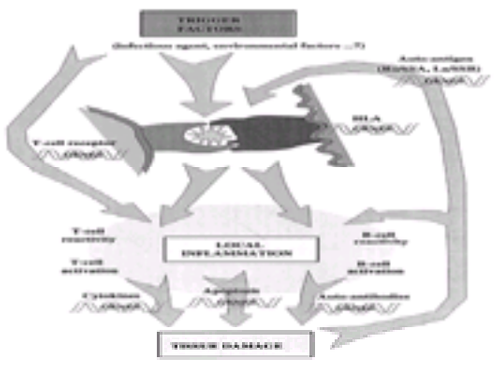

Figure 40.6. A schematic presentation of potential immune components in the pathogenesis of Sjögren's syndrome.

ANIMAL MODELS

As already alluded to, some genetic associations may predispose to Sjögren's syndrome, in particular, the genes encoding products of the MHC and immune receptors, but also other genes. It is thus natural to seek more knowledge using genetically well characterized, inbred animal models that are available for study (134). The current challenge is to find links between a particular genetic background and phenotypic expression(s) of disease.

Any proposed animal model should fulfill certain criteria and features found in the human disease. Moreover, the clinical symptoms of Sjögren's syndrome in humans usually appear relatively late in life, thus making examination of early events difficult. An animal model of the disease would make it possible to study earlier events and to identify potentially important immune reactions in the pathogenesis. Finally, both immune manipulation and the effects of drug therapy can be studied in animals (134).

The earlier attempts to induce Sjögren's syndrome in animals by injection with salivary gland extracts with or without adjuvants and/or other supplements would give rise to a transient inflammation, which was self-limiting and did not mirror the human disease in either the temporal course of events or the serologic profile. The better models of Sjögren's syndrome are the mice with spontaneous autoimmune disease with long-lasting and progressive exocrinopathy, but even in these cases, the disorder has at best represented only secondary Sjögren's syndrome (134). Both anti-Ro (135) and anti-La (136) have been recently detected in murine models of spontaneous Sjögren's syndrome. Because these autoantibodies are the dominant serologic marker in patients with primary Sjögren's syndrome, this finding is an important starting point for future work.

Interesting observations were made in the MRL/lpr mouse in which the *lpr* genotype has been identified as a mutation in the gene encoding Fas, which is a cell-surface receptor that mediates apoptosis. Apoptotic cells were absent or appeared at very low frequency among the infiltrating mononuclear cells in salivary glands. Based on the analysis of the apoptotic activity, the T cells seemed to be rescued from apoptosis because of a failure in signaling (128).

To analyze Fas and TNF receptor I apoptosis pathways in inflammatory salivary gland disease induced by murine cytomegalovirus infection, different strains of mice were infected (137). Both Fas- and TNF receptor I–mediated apoptosis were found to contribute to the clearance of murine cytomegalovirus-infected cells in salivary glands. However, because Fas-mediated apoptosis is necessary for the downmodulation of the immune response, a defect in this process can lead to a postinfectious, chronic inflammatory response that resembles Sjögren's syndrome.

To understand the role of IL-10 in Sjögren's syndrome, transgenic mice were constructed (138). The continuous overexpression of IL-10, induced apoptosis in glandular tissue and infiltration of lymphocytes consists of primarily $FasL^+$, $CD4^+$ T cells.

NOD.B10.H2b mice have been found to exhibit exocrine gland lymphocytic infiltration typical of Sjögren's syndrome–like disease and dysfunction observed in NOD mice, but without the insulitis and diabetes (139). These findings indicate that murine sicca syndrome occurs independent of autoimmune diabetes and that the congenic NOD.B10.H2b mouse represents a novel murine model of primary Sjögren's syndrome.

FUTURE DIRECTIONS

The search for susceptibility genes in families with Sjögren's syndrome is ongoing with the same approach as in the other chronic autoimmune diseases and with utilization of two major strategies: the position-independent candidate gene approach with mutation screening of suspected disease-related genes and full genome scanning (microsatellite analysis) in humans as well as in animal models to determine susceptibility chromosomal regions, which later will be used in a positional candidate gene strategy.

Another challenge in Sjögren's syndrome will be to stratify the disease process including genetic and environmental triggers (Fig. 40.6). Identification of new genetic markers and better characterization of novel autoantibodies (e.g., those directed against muscarinic receptors in exocrine glands) may lead to the development of better diagnostic and prognostic tests in Sjögren's syndrome including its systemic complications.

Sjögren's syndrome is considered to represent an ideal disease to study the mechanisms underlying autoimmunity because its manifestations are both organ specific and systemic. The significance of such studies is underlined by the high prevalence of Sjögren's syndrome as a common but often neglected systemic autoimmune disease often found in the female and aging population.

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41 SYSTEMIC SCLEROSIS (SCLERODERMA)

Richard M. Silver, M.D., and Marcy B. Bolster, M.D.

Systemic sclerosis (SSc; scleroderma) is a disease of unknown etiology, the hallmark of which is induration of the skin. Although it was long regarded as a bland fibrotic process, there is now ample evidence of an active inflammatory and immunologic process underlying its pathogenesis. Characteristic microvascular and immunologic abnormalities are present in the majority of patients. It remains to be determined precisely how the immunologic and microvascular changes relate to the overproduction of connective tissue by the fibroblast, but data suggest that cytokines and growth factors derived from a prolonged immune response may influence the behavior of fibroblasts and endothelial cells, leading to overproduction of extracellular matrix proteins and to microvascular injury. In this chapter, the clinical features of scleroderma are reviewed, and the possible role of the immune response in the pathogenesis of scleroderma and related disorders is discussed.

CLASSIFICATION

Scleroderma may occur as a localized or a systemic disease. In its localized form, scleroderma is confined to the skin and adjacent tissues, where it is classified as linear scleroderma (including scleroderma *en coup de sabre*) or morphea (plaquelike, guttate, or generalized). In its systemic form (SSc), scleroderma may affect a number of visceral organs. Here the classification is somewhat controversial and is based on the extent of cutaneous involvement. One widely accepted classification is shown in Table 41.1, in which diffuse (particularly truncal) skin involvement is distinguished from limited skin involvement; the latter category encompasses the CREST [calcinosis, Raynaud phenomenon (RP), esophageal dysmotility, sclerodactyly, and telangiectasia] variant of scleroderma. Limited cutaneous SSc also includes what has been variously designated acrosclerosis, types I and II scleroderma of Barnett, types I and II of the German classification, intermediate cutaneous SSc of Giordano, and SSc *sine* scleroderma (99).

Diffuse cutaneous SSc (dSSc)[a]
Onset of Raynaud phenomenon within 1 yr of onset of skin changes (puffy or tiddebound)
Truncal and acral skin involvement
Presence of tendon friction rubs
Early and significant incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease, and myocardial involvement
Absence of anticentromere antibodies (ACAs)
Nailfold capillary dilatation and capillary dropout[b]
Antitopoisomerase (anti-Scl-70) antibodies (30% of patients) or anti-RNA polymerase antibodies (30% of patients)
Limited cutaneous SSc (lSSc)
Raynaud phenomenon for years (occasionally decades)
Skin involvement limited to hands, face, feet, and forearms (acral) or absent (no truncal involvement)
A significant late incidence of pulmonary hypertension, with or without interstitial lung disease, trigeminal neuralgia, skin calcifications, and telangiectasia
A high incidence of ACAs (70%–80%)
Dilated nailfold capillary loops, usually without capillary dropout

[a] Experienced observers note some patients with dSSc who do not develop organ insufficiency and suggest the term chronic dSSc for these patients.
[b] Nailfold capillary dilatation and destruction also may be seen in patients with dermatomyositis, overlap syndromes, and undifferentiated connective tissue disease. These syndromes may be considered part of the spectrum of scleroderma-associated disorders.
Modified from LeRoy EC, et al. Scleroderma (systemic sclerosis): Classification, subsets and pathogenesis. J Rheumatol 1988;15:202 with permission.

TABLE 41.1. Subsets of Systemic Sclerosis (ssc)

Although some have presented a conceptual framework of three subtypes of SSc [digital, proximal extremity, and truncal (110)], serologic and microvascular data now support the simpler classification shown in Table 41.1 (99). By classifying patients into subsets early in the course of the illness, one may identify patients at greater or lesser risk to develop certain visceral complications and provide more homogeneous populations of patients for studies of pathogenesis, clinical manifestations, and treatment. For example, limited cutaneous SSc tends to be a more protracted illness with a better prognosis, but late in the course of the disease, these patients may develop distinctive complications (e.g., pulmonary arterial hypertension). Diffuse cutaneous SSc is characterized by more extensive visceral organ involvement early in the course of the disease and a poorer prognosis compared with limited cutaneous SSc.

EPIDEMIOLOGY

Epidemiologic studies of scleroderma have been impeded by the lack of a specific diagnostic test and the relative rarity of the disease. Widely accepted criteria for the classification of SSc were not developed until 1980. Standardized criteria were developed to provide a uniform basis of classification and to facilitate comparison of patient groups. The American College of Rheumatology (ACR) clinical criteria for SSc are listed in Table 41.2.

Major criterion
Proximal scleroderma: typical sclerodermatous skin changes (tightness, thickening, and nonpitting induration, excluding localized forms of scleroderma) involving areas proximal to the metacarpophalangeal or metatarsophalangeal joints, affecting other parts of the extremities, face, neck, or trunk (thorax or abdomen); usually bilateral, symmetric, and almost always including similar changes in the digits (sclerodactyly)
Minor criteria
1. *Sclerodactyly*: sclerodermatous skin changes (as described above) limited to digits (fingers and/or toes)
2. *Digital pitting scars or loss of substance from the finger pad*: depressed areas at tips of digits or loss of digital-pad tissue as a result of digital ischemia rather than trauma or exogenous causes
3. *Bibasilar pulmonary fibrosis*: bilateral reticular pattern of linear or linear-nodular densities that are most pronounced in basilar portions of the lungs on standard chest roentgenogram; may assume appearance of diffuse mottling or "honeycomb lung," and should not be attributable to primary lung disease

Classification as definite systemic sclerosis requires the presence of (a) the major criterion or (b) two of three minor criteria. See (98).

TABLE 41.2. Preliminary Criteria for the Classification of Systemic Sclerosis (scleroderma)

A review of epidemiologic studies of SSc found the incidence to vary between 2 and 10 cases per million (153). Female individuals are affected more commonly than are males. The female-to-male ratio varies with age, peaking at 15:1 during the childbearing years. SSc is rare in childhood, when localized forms of scleroderma (e.g.,

morphea) are more common. The peak incidence of SSc occurs in the fifth and sixth decades (153). SSc appears to be more frequent among African-American female subjects, and African Americans have a poorer prognosis.

Few studies have been designed to investigate cases occurring in a random sample of the general population. Instead, most epidemiologic studies have relied on information obtained from hospital records and death certificates, which likely underestimate the true incidence and prevalence of SSc. In such studies, prevalence has ranged from four to 126 cases per million (153). However, a random community-based survey conducted in South Carolina estimated a prevalence of between 19 and 75 cases per 100,000 (107,108). The prevalence may be nearly 20-fold higher if one includes patients not fulfilling the ACR criteria but having substantive features of SSc [for example, RP with SSc-type capillary abnormalities (see later)].

CLINICAL FEATURES

Raynaud Phenomenon and Microvascular Disease

RP refers to episodic digital ischemia provoked by cold or emotion. Although classically described as triphasic (i.e., pallor, followed by cyanosis, and then hyperemia), a three-color response does not occur universally. Pallor appears to be the most reliable sign and hyperemia the least reliable sign in individuals lacking the classic triphasic response (106).

The establishment of the presence or absence of RP is important when evaluating a patient with thickened skin. The absence of RP should raise the possibility that one might be dealing with a disease other than SSc (Table 41.3), because 95% of SSc patients have RP.

Eosinophilic fasciitis
Chemical-induced disorders
Bleomycin fibrosis
Vinyl chloride disease
Trichloroethylene fibrosis
Toxic oil syndrome
Tryptophan-associated eosinophilia-myalgia syndrome (EMS)
Graft-versus-host disease
Digital sclerosis of diabetes mellitus (diabetic cheiroarthy)
Infiltrating carcinomas
Scleredema
Scleromyxedema (papular mucinosis)
Werner syndrome
Progeria
Rothmund syndrome
Acrodermatitis chronica atrophicans
Lichen sclerosis et atrophicus
Carcinoid syndrome
Phenylketonuria
Porphyria cutanea tarda
Congenital porphyria
Primary amyloidosis
Acromegaly

TABLE 41.3. Diseases with Cutaneous Features Resembling Scleroderma

The duration of RP before skin involvement is an important clinical point. Patients with diffuse cutaneous SSc and a tendency to early visceral organ damage usually have a brief duration of RP before the development of skin changes, whereas patients with limited cutaneous SSc usually have many years (often decades) of RP before overt skin and visceral involvement. Another important issue for the clinician is the evaluation of the individual with RP for the presence of an underlying connective tissue disease (CTD). RP per se is quite common in the general population, yet only a fraction of such individuals will develop a CTD (132). One recent survey found the prevalence of RP to be 4.6% among adults living in South Carolina (107). The prevalence may be higher in colder climates. Because RP may herald the development of serious disease, especially CTD, studies have been undertaken to test whether certain ancillary tests may be predictive of the evolution to an overt CTD. Two markers appear to have predictive value for the subsequent development of CTD (usually SSc): (a) abnormal nailfold capillaries, and (b) antinuclear antibodies (ANAs).

The nearly universal occurrence of RP and the presence of vascular lesions in all organs affected by SSc underscore the importance of the vasculature in the pathogenesis of SSc (125). Changes in the microvasculature can be observed *in vivo* in the capillary bed of nailfold skin (Fig. 41.1) (106). Two major categories of abnormalities have been identified: one is an *active* pattern, characterized by loss of capillaries and accompanied by disorganization of the capillary bed; the other is a *slow* pattern, characterized by enlargement of nailfold capillaries with minimal or no capillary loss (28). The active pattern has been shown to relate to the more rapidly progressing (diffuse cutaneous SSc) form of the disease, whereas the slow pattern is related to limited cutaneous SSc.

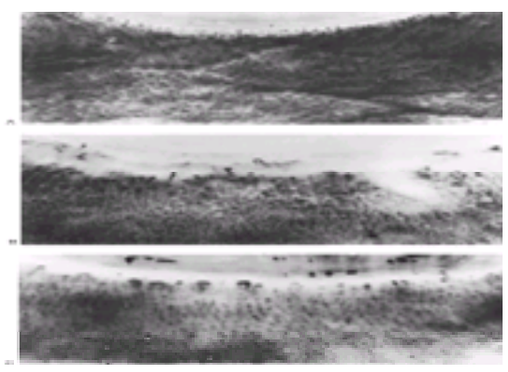

Figure 41.1. A: Normal capillaries visualized by *in vivo* nailfold capillary microscopy. **B:** "Active" pattern characterized by loss of capillaries and disorganization of the capillary bed, often seen in diffuse systemic sclerosis. **C:** "Slow" pattern characterized by enlargement of capillaries with minimal or no capillary loss, often seen in limited cutaneous systemic sclerosis. (**A:** Courtesy of Dr. H. R. Maricq. **B**, **C:** From Maricq HR. The microcirculation in scleroderma and allied diseases. (*Adv Microcirc* 1982;10:17, with permission).

An abnormal capillary pattern detected by nailfold capillary microscopy in a patient with RP indicates an increased risk for the presence of or eventual transition to a CTD (57). A prospective study of patients with RP confirmed this finding and concluded that the presence of abnormal capillaries was the most significant predictor of the subsequent development of SSc (49). In addition, the degree of capillary loss assessed by nailfold capillary microscopy may be correlated with clinical and serologic features and may assist in the subclassification of SSc, that is, diffuse cutaneous versus limited cutaneous (Table 41.1) (28).

Another marker that may be useful in predicting the presence or eventual development of CTD in the individual with RP is the presence of ANAs. Antibody specificity of ANA has been shown to be predictive of the development of specific subtypes of SSc (84,193). In a prospective study of patients with RP and undifferentiated CTD, the presence of ANAs was associated with the evolution of symptoms of CTD, usually SSc (84). Anticentromere antibody (ACA) was associated with limited cutaneous SSc (sensitivity of 60%, specificity of 98%) and anti–Scl-70 antibody was associated with diffuse cutaneous SSc (sensitivity of 38%, specificity of 100%). It is recommended that all patients with RP have a complete clinical evaluation, including nailfold capillary microscopy and ANA testing capable of detecting specific antigens associated with subsets of SSc.

Skin Involvement

Sclerosis of the skin is the hallmark of SSc, although rare patients may have typical visceral involvement in the absence of apparent skin disease (SSc *sine* scleroderma) (101). Skin thickening is the definitive diagnostic criterion of SSc in the majority of patients, and the distribution of involved skin serves as the means of classifying patients into one or another subset of SSc (Table 41.1).

Three phases of dermal involvement have been described. First, there is an edematous phase, often seen as stiff, puffy fingers and hands. In this phase, the condition is often difficult to distinguish from other CTDs or carpal tunnel syndrome; nailfold capillary microscopy and determination of the presence and type of ANAs are of greatest diagnostic utility during this phase of SSc. An indurative phase, characterized by tightness of the skin, usually follows the edematous phase. Here, sclerodactyly and the classic expressionless face with a pinched nose and puckered mouth (*mauskopf*) make the diagnosis unmistakable. Hypo- and

hyperpigmentation of the skin may occur. Ultimately an atrophic phase occurs, when the skin may actually soften.

Pitting scars over the fingertips, often accompanied by loss of substance of the finger pad, are characteristic findings in both limited and diffuse cutaneous SSc. Digital-pitting scars are one of the minor criteria for SSc and are not seen in primary RP (previously termed Raynaud disease). Telangiectasias are found on the hands and face, especially in the limited cutaneous form of SSc (Fig. 41.2). Calcinosis occurs on the volar aspect of the fingertips and over the joints, where ulceration may result in the extrusion of gritty material containing hydroxyapatite.

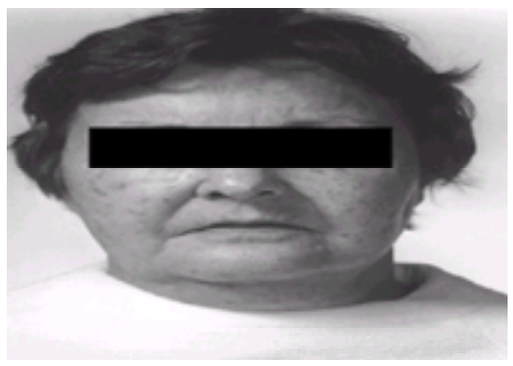

Figure 41.2. Face of woman with the CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) variant of systemic sclerosis.

Scleroderma skin has a thinned epidermis with loss of rete pegs and increased collagen within the dermis. In the inflammatory phase, a mononuclear cell infiltrate (lymphocytes, plasma cells, and histiocytic-type cells) is present in the dermis. During the indurative phase, there is a marked increase in thickness of the skin, accompanied by an increase in hydroxyproline content (142). The presence of fibroblasts containing well-developed rough endoplasmic reticulum and extracellular fine collagen fibrils supports the concept of increased collagen synthesis by scleroderma fibroblasts (50). The 10-nm-thick microfibrils previously thought to be early, immature collagen fibrils contain the glycoprotein fibrillin and are deposited with collagen in excessive amounts in the lower dermis of patients with localized or systemic scleroderma (51). Antifibrillin antibodies are present in both a murine model (tsk-1) and in some patients with SSc (see later).

Musculoskeletal Involvement

Musculoskeletal disease occurs in a significant percentage of patients with SSc. Autopsy studies have demonstrated muscle disease in up to 40% of patients (38), a figure that is greater than that for clinically recognized muscle disease. This discrepancy may be attributable to the occurrence of an indolent, often subclinical, myopathy that occurs with greater frequency than the more acute, inflammatory myopathy resembling polymyositis-dermatomyositis. The former is characterized by mild proximal muscle weakness and atrophy (usually without muscle tenderness), slight elevation of muscle enzyme levels (aldolase more often than creatine phosphokinase), and subtle or absent abnormalities on electromyography (35). The latter occurs in approximately 5% of SSc patients and is indistinguishable from polymyositis. Some patients with features of SSc and polymyositis have anti-PM/Scl antibodies. The PM/Scl autoantigen has been cloned and shown to encode a 75-kd acidic protein of the nucleolar complex (5). Examination of muscle biopsy specimens has revealed increased collagen deposition in interstitial and perivascular areas. Muscle fiber atrophy, degeneration, and necrosis have been observed adjacent to or far removed from areas of interstitial inflammation.

Articular complaints may be a prominent feature of SSc, particularly early in the course of the disease when arthralgia, swelling, and stiffness often occur. Later, the range of motion of joints may be limited because of overlying hidebound skin and fibrosis within the joint or tendons. Crepitus and grating sensations (tendon friction rubs) may arise from the joint or tendon sheaths. The combination of skin, joint, and tendon involvement may result in rapid development of contractures, particularly affecting the hands.

Synovial fluid analysis reveals noninflammatory joint fluid with cell counts of less than 2,000 per microliter, consisting predominantly of mononuclear cells. Radiographs usually do not reveal erosive joint disease, but in some patients, an erosive arthropathy may affect the distal and proximal interphalangeal joints and the first carpometacarpal joint. Resorption of the soft tissue at the fingertips is characteristic. Subcutaneous calcification and osseous resorption also may occur.

Gastrointestinal Tract Involvement

Gastrointestinal disease is the most commonly recognized visceral manifestation of SSc. Dysphagia for solid foods and heartburn, the most common symptoms, are due to esophageal dysmotility (distal two thirds) and esophageal reflux. Nearly 85% of SSc patients have esophageal reflux due to decreased lower esophageal sphincter pressure (198). Clinical evidence of esophageal involvement may precede cutaneous disease, particularly in patients with limited cutaneous SSc. Smooth muscle atrophy and fibrosis are the most common pathologic lesions (38). The striated muscle of the upper esophagus is spared. Inflammation, erosions, or ulcerations of the mucosa may result from reflux of gastric contents. Stricture or Barrett esophagus may occur as a complication of prolonged peptic esophagitis (Fig. 41.3).

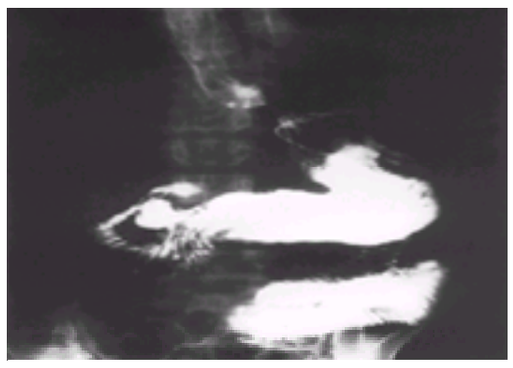

Figure 41.3. Barium esophagram showing stricture formation in the distal esophagus, secondary to chronic reflux esophagitis. (Courtesy of Dr. J. Stanley.)

Peristaltic abnormalities may delay gastric emptying and may reduce motility of the small and large intestines, giving rise to pseudoobstruction or malabsorption secondary to bacterial overgrowth. Wide-neck diverticula may be seen on barium studies, occurring especially on the antimesenteric border of the transverse and descending colon.

Liver disease is uncommon, but biliary cirrhosis may occur, usually in association with limited cutaneous SSc. Such patients usually have jaundice, pruritus, and antimitochondrial antibodies. A significant percentage of patients with primary biliary cirrhosis have features of scleroderma and ACAs (105).

Pulmonary Involvement

Lung disease ranks second to gastrointestinal tract disease in frequency of visceral involvement. Dyspnea and hypoxia may result from interstitial inflammation and fibrosis, or they may be the result of pulmonary hypertension occurring in the absence of parenchymal lung disease (23). With recent advances in the treatment of SSc-related renal disease, lung disease has become the most frequent cause of death from SSc.

Dyspnea on exertion is the most common pulmonary symptom and is often accompanied by a nonproductive cough. Examination usually reveals inspiratory “Velcro” crackles that are most prominent at the lung bases. Chest radiographs frequently show diffuse linear and nodular fibrosis in the lower two thirds of the lung fields (Fig. 41.4), but the chest radiograph is a relatively insensitive test of lung involvement in SSc. High-resolution computed tomography (HRCT) may demonstrate abnormalities before the onset of dyspnea or the detection of abnormalities by routine chest radiography (58). Tests of pulmonary function (PFTs) often reveal restrictive lung disease

with reduced lung volumes and decreased diffusing capacity. Moderate [forced vital capacity (FVC) 50% to 75% predicted] or severe (FVC less than 50% predicted) restrictive lung disease can be detected in 40% of SSc patients, and there is an increased incidence of anti–Scl-70 antibody and diffuse cutaneous skin disease in SSc patients with this degree of restrictive lung disease (170,171). HRCT findings include hazy opacification of the alveolar spaces (ground-glass opacification), irregular pleural margins, septal and subpleural fibrosis, honeycombing, subpleural cysts, and lymphadenopathy (187).

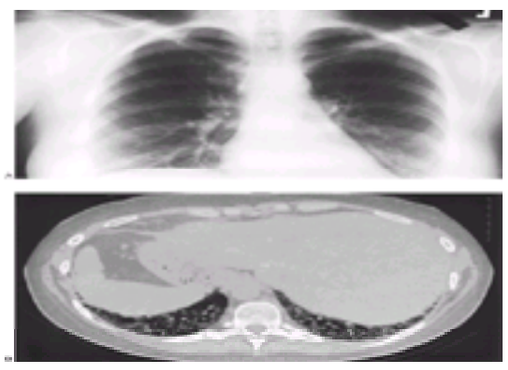

Figure 41.4. Chest radiograph **(A)** and high-resolution computed tomographic scan **(B)** of a 34-year-old woman with diffuse cutaneous systemic sclerosis of 3 years' duration.

An inflammatory component to the lung disease can be demonstrated in a significant percentage of SSc patients. Bronchoalveolar lavage (BAL) fluid reveals evidence of alveolitis, sometimes before the occurrence of dyspnea, abnormal results on PFTs, or abnormal-appearing chest radiographs (154). Most studies have shown an increased total number of cells (mostly activated alveolar macrophages) and an increased percentage and absolute number of neutrophils and eosinophils, similar to that described for idiopathic pulmonary fibrosis (129,130). A neutrophilia on BAL has been associated with symptoms of dyspnea, PFT abnormalities, and ground-glass opacification on HRCT scans (137,187,189).

The course of interstitial lung disease in patients with SSc is variable and not predicted by traditional tests, such as initial chest radiograph or PFTs, or by demographic data (149). Normal BAL findings have been associated with a stable pulmonary course, but alveolitis appears to be associated with worsening dyspnea, worsening appearance on chest radiographs, and significantly greater declines in FVC and diffusing capacity for carbon monoxide (D_{LCO}) (156).

Products of activated alveolar macrophages such as fibronectin, a glycoprotein that may serve as a chemoattractant and growth factor for fibroblasts, may play a role in the pathogenesis of SSc-related lung disease. Alveolar macrophages from patients with scleroderma release significantly more fibronectin than do those from controls, and the level of fibronectin is positively correlated with the degree of alveolitis and negatively correlated with the D_{LCO} (91). Other cytokines and growth factors of SSc patients may be important mediators of lung fibrosis (see later).

Pulmonary hypertension (in the absence of significant interstitial fibrosis) occurs in 5% to 10% of patients with limited cutaneous SSc, and only rarely in patients with diffuse cutaneous SSc. It is usually a late manifestation in patients with limited cutaneous SSc, typically occurring more than 10 years from disease onset. Patients with pulmonary hypertension experience dyspnea and fatigue, though up to one third of patients may be asymptomatic (175). Some patients with limited cutaneous SSc have an isolated decrease in the D_{LCO} on PFTs, and this has been associated with the development of severe pulmonary hypertension (169). Doppler echocardiography is a useful noninvasive means of estimating pulmonary artery pressures (117) and has a 90% sensitivity and 75% specificity for pulmonary hypertension as compared with right heart catheterization (41).

A case/control autopsy study showed that intimal thickening and luminal narrowing occur in both major subsets of SSc, but that such pulmonary vascular changes are most pronounced in patients with limited cutaneous SSc (3). Pulmonary vasospasm (visceral RP) may play a role in the pathogenesis of pulmonary arterial hypertension, but by the time that such patients are seen with dyspnea and hypoxia, significant pulmonary vascular resistance may have resulted from structural luminal narrowing.

There are other less frequent pulmonary manifestations of SSc. Pleural fibrosis is noted on HRCT scans and at autopsy more frequently than is clinically suspected (38,186). Pleural effusions also rarely occur in SSc. There is an increased frequency of lung cancer (adenocarcinoma, squamous cell carcinoma, and alveolar cell carcinoma) in scleroderma patients (178). Hemoptysis, or an enlarging infiltrate or mass lesion, suggests the presence of a malignancy and warrants appropriate evaluation. Spontaneous pneumothorax may occur because of rupture of a subpleural cystic airspace (pneumatocele). Aspiration pneumonia occurs as a result of esophageal dysmotility and the markedly reduced lower esophageal sphincter pressure. An uncommon pulmonary complication of SSc is diffuse alveolar hemorrhage. This may occur in the setting of acute renal failure or as a rare complication of lung fibrosis. Endobronchial telangiectasia also may be seen as hemoptysis and can be diagnosed by bronchoscopy.

Renal Involvement

Renal involvement is common in SSc, occurring predominantly in patients with diffuse cutaneous disease. Proteinuria (usually less than 500 mg/24 hours), azotemia, or hypertension occurs in 45% of SSc patients (26). Of all the visceral organs affected by SSc, disease of the kidney has been associated with the highest mortality. Until the introduction of dialysis and potent antihypertensive drugs, renovascular hypertension was uniformly fatal. Significant risk factors for the onset of scleroderma renal crisis include anemia, pericardial effusion, congestive heart failure, and rapid progression of skin thickening (166). Factors portending a poor prognosis in scleroderma renal crisis include (a) male gender, (b) advanced age, (c) poorly controlled blood pressure within 3 days of symptom onset, (d) congestive heart failure, and/or (e) severe renal insufficiency (creatinine greater than 3.0 mg/dL) at the time of initial therapeutic intervention (166).

The characteristic renal histopathology, similar to that seen in malignant hypertension, is concentric, subendothelial intimal proliferation affecting small arcuate and interlobular arteries (Fig. 41.5). The functional correlate of this structural abnormality is reduced renal cortical blood flow, which may be compromised further by vasospasm (visceral RP) (18). The renin-angiotensin system plays a major role in the pathogenesis of malignant hypertension secondary to SSc. Many patients have elevated plasma renin activity, which may precede or coincide with rapid deterioration in renal function, and many will respond to angiotensin-converting enzyme (ACE) inhibitor therapy. The use of captopril and other converting enzyme inhibitors has been associated with a marked reduction in mortality from SSc-related renal crisis (168), but not all patients recover renal function despite normalization of blood pressure.

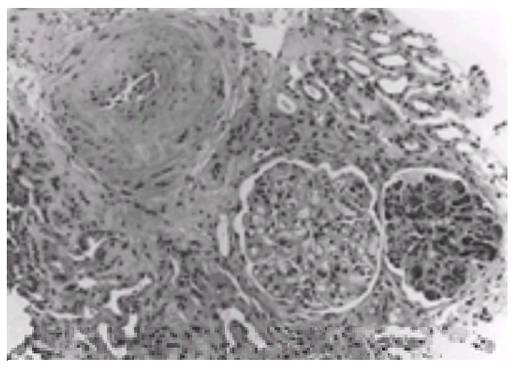

Figure 41.5. Intimal proliferation and reduplication of basement membrane and narrowing of vessel lumen in renal arteriole.

Some patients may develop acute renal failure without hypertension (65). When compared with SSc patients with hypertensive renal crisis, such patients are more likely to have microangiopathic hemolytic anemia and thrombocytopenia, and are more likely to have been treated with high doses of corticosteroids before onset of the

renal crisis. The risk of precipitating renal failure is a relative contraindication to the use of high-dose corticosteroids in SSc.

Involvement of the lower urinary tract occurs rarely, seen with microscopic hematuria with or without urinary urgency. Biopsy of the urinary bladder may reveal interstitial fibrosis (96).

Cardiac Involvement

The landmark article by Weiss et al. (188) established the entity of scleroderma heart disease. The authors set the stage for future investigation, suggesting that the pathologic changes were due to vascular disease in the setting of normal large and medium-sized coronary arteries. Heart disease may be seen as heart failure, arrhythmias, conduction disturbances, or chest pain, all of which may be the result of vascular disease and fibrosis. Pericardial effusions also occur, yet cardiac tamponade is rare. Symptoms tend to be slowly progressive and may be refractory to therapy.

Myocardial scarring in the presence of normal extramural coronary arteries is probably antedated by contraction band necrosis. This classic feature of scleroderma-associated heart disease can be produced experimentally by a transient interruption of blood flow. Evidence exists for cold-induced left ventricular dysfunction (44) and cold-induced regional perfusion defects (6). Structural lesions of the coronary microcirculation also may be a major determinant of cardiac involvement in SSc. Coronary angiograms usually appear normal, supporting the notion that myocardial dysfunction is secondary to microcirculatory disturbances. Some patients with primary scleroderma heart disease have reduced coronary blood flow and coronary reserve after maximal coronary vasodilation (124). Coronary vasospasm appears to be reversible with calcium channel blockers such as nifedipine (77).

Sjögren Syndrome

Symptoms of the sicca syndrome, particularly xerostomia and xerophthalmia, frequently occur in patients with SSc (128). Mononuclear cell infiltrates were found in biopsy tissue from labial salivary glands from nearly one third of unselected patients. An additional one third showed periglandular and interglandular fibrosis without significant inflammation. The presence of antibodies to SS-A (Ro) and SS-B (La) is correlated with histologic evidence of Sjögren syndrome, but not with salivary gland fibrosis.

Neurologic Involvement

Primary central nervous system involvement is rare in SSc. Trigeminal sensory neuralgia may occur, rarely as the presenting manifestation (182), and is more likely to occur in patients with SSc having an overlap syndrome (myositis and antiribonucleoprotein antibodies) than in those with pure SSc (47). Peripheral neuropathy occurs occasionally. Carpal tunnel syndrome may be present, particularly during the early, edematous phase of the disease.

Endocrine Involvement

Thyroid dysfunction may exist in a significant percentage of SSc patients (54). When present, hypothyroidism may be associated with high levels of antithyroid antibodies. Fibrosis of the thyroid gland may occur with or without autoimmune thyroiditis.

Erectile impotence occurs in male patients with SSc (95). It may be an early manifestation of the disease, usually with normal libido and normal testosterone and gonadotropin levels. Autonomic nervous system dysfunction and vascular abnormalities are postulated to play a role in its pathogenesis.

Fertility may be decreased in women with SSc. Scleroderma renal crisis may occur during pregnancy, accounting for maternal and fetal death. It is recommended that patients with early, diffuse cutaneous SSc (who are intrinsically at higher risk for renal crisis) delay pregnancy until their disease stabilizes (167,172). There does not appear to be an increased frequency of miscarriage, but the risk of premature and small full-term babies is increased (167,172).

ETIOLOGY AND PATHOGENESIS

Genetic Aspects

Only a few cases of familial scleroderma have been reported (25,112), and the twin concordance rate is low (56). It is interesting that asymptomatic relatives of SSc patients have a higher incidence of nonspecific ANAs than do controls (176). Because spouses also have an increased rate of ANA positivity (102), perhaps a shared environment is the dominant predisposing factor for autoantibody formation within families. A very high prevalence of SSc has been reported in Choctaw Native Americans residing in Oklahoma (10), believed to be due in part to a common ancestral founder ten generations ago (180). A candidate gene, fibrillin 1, which is defective in a mouse model of SSc (tight skin1,*tsk1*), and located on chromosome 15 in humans, was screened in Choctaw SSc cases and controls, and a 2-cM haplotype containing the fibrillin 1 gene was shown to be associated with SSc in this population (180). Choctaw SSc cases do not have the duplication of the fibrillin 1 gene seen in the *tsk1*mouse.

The resemblance of some forms of graft-versus-host disease (GVHD) to SSc, together with the observation that bidirectional cell traffic at the maternal/fetal interface can result in persistent microchimerism, has led some to speculate that microchimerism may play a role in the pathogenesis of some cases of SSc (121). Low levels of microchimerism can be detected in healthy women, but class II human leukocyte antigen (HLA) compatibility of a child appears to be more common for SSc patients, suggesting a possible role for microchimerism in the pathogenesis of SSc (13,14,120). In addition, cells containing Y-chromosome sequences were detected by *fluorescence in situ* hybridization in affected skin from some female SSc patients, but not in normal women (14). It remains to be determined whether there is a causal link between microchimerism and SSc, or whether this observation is an epiphenomenon (121).

Several population studies showed an increase in the frequency of certain class II HLA types, especially DR1, DR3, and DR5 (19), which may correlate with specific autoantibody profiles of SSc. The DR1 allele may be associated with ACAs, and hence the limited cutaneous subset of SSc, whereas DR5 may be associated with ACA or Scl-70 antibodies (4,53). The increased frequencies of HLA-DR5 and HLA-DR1 in ACA-positive patients may reflect linkage disequilibrium with DQ7 and DQ5, respectively (138). DNA typing with oligonucleotide probes suggests an important role for the presence of a DQ allele having a polar glycine or tyrosine rather than leucine at position 26 of the DQB1 domain; 100% of ACA-positive white patients with SSc, compared with 69% of ACA-negative patients with SSc, 71% of white controls, and 100% of ACA-positive nonwhite patients with SSc, had such a polar amino acid at this position (138). The second hypervariable region of the HLA-DQB1 chain may form the candidate epitope associated with the ACA response (138). Anti–Scl-70 (topoisomerase) antibodies have been found to be associated with DRw11 and a particular sequence (aa 71-77) of the DQB1 chain in white SSc patients (115), and either the DQBB1*0601 or*0301 allele in Japanese SSc patients (94).

Environmental Factors

Exposure to a variety of environmental agents has been implicated as a cause of scleroderma and scleroderma-like conditions (Table 41.4). The female preponderance normally seen in SSc is not seen in many with an environmental exposure implicated as a cause of disease, and it has been suggested that environmental exposure may be the major cause of SSc among male subjects (61).

Silica dust exposure
Silicone breast augmentation
Paraffin breast augmentation
Epoxy resins
Aromatic hydrocarbons: toluene, benzene, xylene
Aliphatic hydrocarbons: vinyl chloride, trichloroethylene, perchloroethylene
Drugs
Bleomycin
5-Hydroxy-L-tryptophan
L-Tryptophan (eosinophilia-myalgia syndrome)
Adulterated rapeseed oil (toxic oil syndrome)
Biogenic amines: appetite suppressants
Cocaine

TABLE 41.4. Chemicals and Toxins Implicated in Scleroderma and Scleroderma-Like Disorders

Exposure to silica has been implicated in some cases of SSc. This was first noted among Scottish stone masons exposed to silica dust, and later among gold miners and underground coal miners (45,141). It has been estimated that the likelihood of developing SSc is 25-fold higher in persons exposed to silica and is 110-fold higher in persons with silicosis (61). Silica-induced and idiopathic SSc appear to share similar pathophysiology, and silica *in vitro* can activate macrophages, leading to release of tumor necrosis factor (TNF)-a, interleukin (IL)-1 and IL-6, and can activate endothelial cells to express intercellular adhesion molecule (ICAM)-1 (62). Although augmentation mammoplasty with silicone or paraffin injections, or with silicone gel–filled implants, has been implicated in a number of patients with SSc (92,164), case/control and cohort studies failed to show a significant association between silicone breast implants and CTDs (144).

Exposure to vinyl chloride has been associated with the development of scleroderma-like cutaneous changes and RP, as well as acroosteolysis, pulmonary fibrosis, portal fibrosis, and hepatic hemangiosarcoma. Organic solvent exposure, either occupational or avocational, appears to be related to an increased risk of SSc (122,123).

Another illness having scleroderma-like features is the toxic oil syndrome (TOS), which affected nearly 20,000 people in Spain in 1981. It was associated with the ingestion of contaminated rapeseed oil (7). The early phase of the disease was characterized by noncardiogenic pulmonary edema, fever, skin eruptions, and eosinophilia. An intermediate phase was characterized by fasciitis and scleroderma-like skin changes, myalgia and muscle cramps, and severe peripheral neuropathy. The chronic phase was marked by softening of the affected skin, but neuropathy and muscle cramps persisted, and some patients died of pulmonary hypertension.

A very similar epidemic known as the *eosinophilia-myalgia syndrome* (EMS) occurred in the United States in 1989 (18,66,157), in association with the ingestion of L-tryptophan. One impurity in implicated batches of L-tryptophan, identified as 1,1'-ethylidenebis-(L-tryptophan) (EBT), was related to changes made in the manufacturing process (111). A similar illness was described 10 years earlier, in association with the ingestion of 5-hydroxy-L-tryptophan and carbidopa (174).

EMS patients often had acute dyspnea and cough with skin eruptions, myalgia, and eosinophilia. In many patients, fasciitis and scleroderma-like skin lesions developed, similar to what was seen in the TOS epidemic. Peripheral neuropathy was seen in both syndromes. It is unclear whether these similar epidemics share a common etiologic agent, but in each there was significant eosinophilia and evidence of altered metabolism of L-tryptophan (158). In the early phase of each syndrome, the metabolism of L-tryptophan via the L-kynurenine pathway was accentuated, perhaps by the induction of the rate-limiting enzyme, indoleamine-2,3-dioxygenase, by cytokines such as interferon-g (IFN-g) (158). This resulted in high levels of L-kynurenine and quinolinic acid, which may have played a role in the fibrosis and neuropathy seen in each syndrome.

Vascular Mechanisms

Clinical and morphologic abnormalities of the vascular system are well recognized in SSc, leading some to hypothesize that the endothelium is the major target organ (98). Morphologic abnormalities have been noted in vessels of almost every organ studied (125). Small arteries and arterioles demonstrate thickening of the intima with narrowing of the vascular lumen. The vascular media is normal or thinned, and the adventitia may be surrounded by a cuff of fibrous tissue, frequently obliterating periarterial lymphatics and capillaries. Ultrastructural studies reveal evidence of endothelial injury, with swelling and destruction of endothelial cells and reduplication of the capillary basement membrane.

Endothelin, a potent vasocontrictor, is present in increased concentrations in the plasma of patients with primary RP and SSc (195). It is tempting to speculate that in SSc, there exists a relative imbalance between endogenous vasodilators, such as nitric oxide and prostacyclin, and endogenous vasoconstrictors, such as endothelin and platelet-release products. Other vasoactive substances also may be important. For example, the presence of increased numbers of activated mast cells in skin affected by SSc suggests that histamine and other mast cell constituents may play a direct or secondary role in the vascular abnormalities (31).

Endothelial injury appears to be an early and important event in the pathogenesis of SSc, but the mechanisms leading to microvascular damage are poorly understood. Latent or persistent human cytomegalovirus (HCMV) infection has been postulated to be an accelerating factor in various autoimmune vasculopathies, including SSc (129). Elevated prevalence and levels of immunoglobulin G (IgG) HCMV antibodies have been observed in SSc patients (119).

A number of studies suggested that endothelial injury in SSc may be the result of an immune response directed against the endothelium. Antibody-dependent cell-mediated cytotoxicity (ADCC) toward endothelial cell targets has been demonstrated with some SSc sera (130). Antiendothelial cell antibodies (AECAs) present in sera of SSc patients may be pathogenic by inducing endothelial cell apoptosis (24). Studies in both an avian model of SSc and skin from patients with SSc indicated that endothelial cells undergo apoptosis early in the disease course, and suggest a role for AECAs (151). Non–antibody-mediated injury also may occur, because it has been shown that IL-2–activated lymphocytes [lymphokine-activated killer (LAK) cells] are cytotoxic to endothelial cells *in vitro*. One study found no evidence of LAK cell activity in the peripheral blood of SSc patients, but did not preclude the presence of such in the skin and other organs affected by SSc (155). In fact, the progenitor of the LAK cell [natural killer (NK) cell] adheres to endothelium and rapidly disappears from the circulation on exposure to IL-2 (12). This mechanism of endothelial cytotoxicity may be relevant, as evidence exists for enhanced production of IL-2 in patients with SSc (75). A factor resembling granzyme has been identified in SSc patients, both in sera and in skin lesions (76). Upregulation of class II major histocompatibility complex (MHC) molecule expression and other markers of endothelial cell activation, such as adhesion molecules, further supports the notion of endothelial cell perturbation in SSc (33,64,68,69,103,126,162).

Connective Tissue Aspects

The characteristic feature of SSc is excessive deposition of collagen and other extracellular matrix components (fibrillin, fibronectin, and glycosaminoglycans) in the skin and other organs. LeRoy (97) observed that SSc dermal fibroblasts synthesize excessive collagen *in vitro* and that this increased capacity to synthesize collagen persists *in vitro* for a number of passages, before declining toward normal levels. Increased synthesis of glycosaminoglycans and fibronectin also has been demonstrated. Messenger RNAs (mRNAs) of matrix proteins are increased in SSc dermal fibroblasts, largely the result of increased rates of transcription (79); increased half-lives of the mRNAs also may contribute to elevated matrix synthesis (80). Furthermore, scleroderma fibroblasts express more tissue inhibitors of metalloproteinases 1 (TIMP-1), which may contribute to excess matrix accumulation (90,93).

Studies using *in situ* hybridization showed that only a proportion of dermal fibroblasts are responsible for the high rate of connective tissue matrix synthesis (82). Another phenotypic feature of the scleroderma fibroblast is high expression of the surface molecule ICAM-1 (118). Such cells can bind to normal T lymphocytes *in vitro*, and this binding is inhibited by antibodies to ICAM-1 or to lymphocyte function-associated antigen-1 (LFA-1) (1). Circulating ICAM-1 levels are elevated in SSc, and proinflammatory cytokines enhance the release of ICAM-1 from SSc peripheral blood mononuclear cells (PBMCs) *in vitro* (68). Thus evidence is accumulating to support a physical relationship between T lymphocytes and fibroblasts, as well as cytokine-mediated effects (see later).

Transforming growth factor-b (TGF-b) enhances transcription of collagen mRNA through the activation of a promoter region of the collagen gene (70). When injected into experimental animals, TGF-b causes a mononuclear cell inflammatory response and fibrosis (140). Also of potential relevance to SSc, TGF-b induces the autocrine production of two potent fibroblast mitogens, platelet-derived growth factor (PDGF) (163), and connective tissue growth factor (CTGF) (146) and inhibits endothelial cell proliferation *in vitro* (177). Exposure to TGF-b *in vitro* leads to an increased expression of the PDGF-a receptor on dermal fibroblasts from SSc patients (194). Furthermore, exposure to TGF-b *in vitro* increases adherence of normal fibroblasts to extracellular matrix components to levels seen in scleroderma. Such an effect of TGF-b may be mediated by the cell-surface integrin b_1, which is expressed in scleroderma skin (162). TGF-b, PDGF, and CTGF have all been shown to be present in the skin of SSc patients (67), and serum CTGF levels were increased in patients with SSc and correlated with extent of skin sclerosis and pulmonary fibrosis (148). These cytokines may, therefore, play a role in the proliferation and activation of SSc fibroblasts. Such a role is bolstered by the observation that dermal fibroblasts from scleroderma patients exhibit overexpression of receptors for TGF-b as compared with normal dermal fibroblasts (87). Elevated TGF-b receptor levels correlated with elevated a 2 (I) collagen mRNA levels in scleroderma fibroblasts (87).

Immunologic Aspects

The frequent occurrence of mononuclear cell infiltrates in the dermis of early scleroderma lesions, as well as the presence of ANA in the majority of patients, suggests that cellular and humoral immunity may play a role in the pathogenesis of SSc. Additional support for such a role is the occurrence of scleroderma-like lesions in patients with chronic GVHD. Evidence suggests that SSc may be immunologically triggered and associated with expansion of fibrogenic clones of tissue fibroblasts (9). The major aspects of immune dysfunction described in patients with SSc, as well as the possible effect of the immune response on vascular and connective tissue metabolism, are discussed later.

HUMORAL IMMUNITY

Hypergammaglobulinemia occurs in a significant proportion of patients, with the largest elevation usually involving the IgG fraction (34). Immunoglobulin, C3, and fibrinogen are deposited in tissues in a variable percentage of patients. When a biopsy was performed on more than one site, and particularly if the nailfold was selected as a site for biopsy, the majority of SSc patients were noted to have deposits of immunoglobulin, C3, and fibrinogen (29). Granular deposits of IgM are present at the dermal/epidermal junction of patients with SSc. Autoantibodies from the epidermis of patients with SSc have been shown to activate complement *in vitro* (134),

and ongoing complement activation *in vivo* is suggested by elevated plasma levels of fragments C3d, C4d, and factor Ba (150), as well as the detection of activated complement C5b-9 and C5aR in SSc microvasculature (165).

ANAs occur frequently in SSc patients and their first-degree relatives, up to 95% of the former and 57% of the latter when HEp-2 is used as substrate (Fig. 41.6) (133,176). There are three major intracellular targets of ANAs in SSc. The first is ACA, which recognizes three human chromosomal antigens and is very specific for RP and limited cutaneous SSc (43). The association of ACA with certain nailfold capillary abnormalities has been noted (28). ACAs have been detected in approximately 40% of all patients with SSc, but are relatively specific for limited cutaneous SSc (50% to 96% positive) and, thus, identify a subgroup of SSc patients with less severe internal organ involvement and a more favorable prognosis.

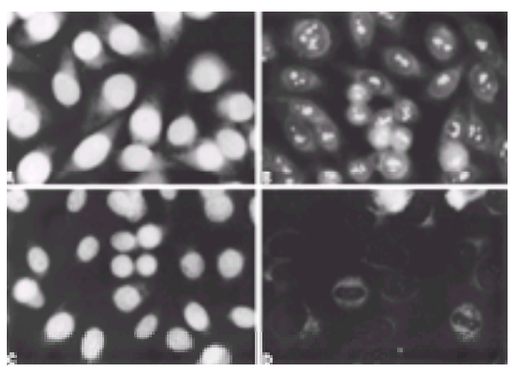

Figure 41.6. Patterns of nuclear staining seen in systemic sclerosis, using HEp-2 cells as a substrate. **A:** Homogeneous, which occurs less frequently and may be confused with diffuse, fine speckles. **B:** Nucleolar. **C:** Discrete large speckles. **D:** Centromere. (×40). (Photomicrographs courtesy of Drs. Z. Chen and S. K. Ainsworth.)

Anti–Scl-70, found in 30% to 70% of patients with diffuse cutaneous SSc, is an autoantibody directed against the nuclear enzyme DNA topoisomerase I (152). The prevalence of anti–Scl-70 antibodies varies among patients with different genetic or racial backgrounds and occurs with a relatively low frequency in scleroderma overall; nevertheless, it appears to be a highly specific marker of diffuse cutaneous SSc. Autoantibodies to RNA polymerases (RNAPs) are found in approximately half of patients with diffuse cutaneous SSc and are responsible for the speckled staining on the nucleolus in the immunofluorescence ANA test (136). Other antinucleolar antibody systems include PM-Scl, Th/To, and fibrillarin (small nucleolar U3 ribonucleoprotein) (135). Autoantibodies to the latter have been associated with high urinary mercury levels in SSc patients (11).

Although the distributions of each of the antigenic targets of SSc autoantibodies differ, they all are enriched within the nucleolus at some time during the cell cycle (179). Metals also are enriched in the nucleolus, and it has been observed that several SSc autoantigens are uniquely susceptible to cleavage by reactive oxygen species in a metal-dependent manner (27), thus potentially revealing cryptic antigenic epitopes and eliciting an MHC class II–driven autoimmune response.

Antibodies to fibrillin-1, the major structural glycoprotein of connective tissue microfibrils, are present in a significant proportion of white, Japanese, and Choctaw American Indian patients with SSc (181). This is of interest because the tsk1 mouse, a murine model of fibrosis and perhaps SSc, has a gene duplication that results in an abnormally large fibrillin-1 protein (89). These mice spontaneously produce anti–fibrillin-1 antibodies, as well as other SSc-specific autoantibodies (116). It remains to be determined whether the immune response to fibrillin or other matrix or intracellular components plays a primary role in disease pathogenesis or is a secondary phenomenon.

CELLULAR IMMUNITY

One of the earliest clinical observations suggesting a role for cell-mediated immunity in the pathogenesis of SSc was a scleroderma-like lesion in patients with GVHD (52). Scleroderma-like lesions also have been observed in a murine model of GVHD across minor histocompatibility barriers (73). The histopathology of this model is cutaneous fibrosis, loss of adipose tissue, atrophy of dermal appendages, and a T-lymphocyte and macrophage cellular infiltrate. Of interest is the observation that stainable mast cells seemingly disappear from the skin of mice with GVHD (32). Subsequent studies showed that abundant mast cells are present but not apparent with traditional staining techniques because they had lost their granules (30). By electron microscopy, these “phantom” mast cells have been shown to be highly activated.

Mast cells also are present in the skin and internal organs of patients with SSc. Granulated mast cells are increased in the skin, particularly the reticular dermis, of patients with early SSc (63). Tryptase-positive, chymase-positive mast cells have been detected in scleroderma skin specimens (72). One major product of mast cells, histamine, is elevated in the plasma of SSc patients, particularly those with diffuse cutaneous disease (46). Mast cell degranulation also has been noted in the tsk1 murine model of scleroderma (185). Mast cells are present in many fibrosing conditions besides SSc, and their exact role in fibrosis remains unclear. The mast cell may interact with both the endothelial cell and the fibroblast, each of which appears to be a critical element in the pathogenesis of SSc, but the exact role of the mast cell remains unknown (31).

The presence of perivascular lymphocytes and macrophages in the dermis of patients with SSc, particularly those with early, active skin disease, has long been appreciated (50). Studies indicate that such cells are activated, expressing class II MHC antigens (55). Macrophages from lungs of scleroderma patients exhibit a number of features indicative of *in vivo* activation, including spontaneous production and release of fibronectin (91). T lymphocytes are the predominant lymphocytic cell, with the helper/inducer phenotype ($CD4^+$) being more abundant than $CD3^+/CD8^+$ lymphocytes. The majority of the lymphocytes express HLA-DR antigen. Activated lymphocytes, predominantly $CD8^+$, are also present in lungs of SSc patients with alveolitis (196). Activated NK cells expressing CD56 and HLA-DR also are present in acute scleroderma skin lesions (55). Class II HLA antigens are expressed by dermal endothelial cells and perivascular fibroblasts in the deep dermis of acute scleroderma lesions (55), which suggests that these cells have been activated and are capable of serving as antigen-presenting cells.

Early studies reported a decreased number of circulating T lymphocytes (71,191). Flow cytometry demonstrated significantly reduced numbers and percentages of $CD8^+$ and $CD4^+$ T lymphocytes in patients with acute disease compared with those with chronic disease and normal controls (55). The $CD4^+/CD8^+$ ratio may be increased because of a diminished suppressor T-lymphocyte subset. Nonspecific suppressor cell function is diminished in some patients (71,85), but antigen-specific suppressor cell activity is usually normal and $CD4^+$ $CD45RA^+$ (suppressor-inducer) cells are not significantly different from normal (78,88). Evidence of T-cell activation includes increased expression of HLA-DR molecules and IL-2 receptors, and increased serum levels of soluble IL-2R (190).

Peripheral blood NK cell numbers and activity are reduced in patients with SSc (85,155). IL-2 stimulation of NK cells gives rise to LAK cells, which also are reduced in some SSc patients (85,155). This is enigmatic, as significantly elevated levels of IL-2 are present in sera of patients with SSc (75), and SSc serum does not contain inhibitors of LAK cells (85). One plausible explanation for the decreased LAK activity seen in SSc is that the precursors of LAK cells may have left the circulation to sites of disease activity. IL-2 induces NK-cell adhesion to endothelial cells, and NK cells promptly disappear from the circulation of cancer patients treated with high-dose IL-2 (12). Indeed, $CD16^+$ cells are increased in the skin of some SSc patients (109). Such cells not only are capable of binding to endothelial cells but also may mediate endothelial cell cytotoxicity by release of serine proteases (granzymes) (40).

The presence of mononuclear cells in the dermis of SSc patients has generated interest in the products of such cells and their potential effects on fibroblasts and endothelial cells. IFN-g is capable of downregulating fibroblast collagen synthesis *in vitro*. IFN-g exerts its action at the level of transcription, by decreasing mRNA for procollagen (42, 81). A 72-hour exposure to IFN-g reduced procollagen mRNA levels in scleroderma fibroblast cell lines to levels exhibited by control fibroblasts (81). Other cytokines also may be important in the pathogenesis of SSc. IL-1 is mitogenic to fibroblasts, as is an inhibitor to IL-1 produced by mononuclear cells from SSc patients (145). Elevated levels of IL-1 in serum, PBMC supernatant, and dermal fibroblast culture medium have been reported, and SSc fibroblasts express more IL-1R, perhaps making them more sensitive to IL-1 stimulation (86). Lymphotoxin and tumor necrosis factor-a (TNF-a), present in some SSc sera, also may be important mediators (59,74). TNF-a can injure endothelial cells *in vitro* and stimulate fibroblast proliferation (74,147). Depending on culture conditions *in vitro*, TNF-a can modulate the expression of type I and type III collagen gene expression, as well as the expression of MMP-1 and TIMP-1 by dermal fibroblasts (146). Increased levels of sTNF-a–receptor type I are detected in sera of many scleroderma patients, and seem to correlate with disease severity (104). IL-2, IL-4, IL-6, and IL-8 also are detectable in the sera and bronchoalveolar lavage fluid (BALF) of some SSc patients (22,60,190). Increased IL-4 levels may reflect activation of either T lymphocytes or mast cells. This may be relevant to the pathogenesis of SSc, because IL-4 induces proliferation of normal dermal fibroblasts in a dose-dependent fashion (48). A T-helper 2 pattern of cytokine production by T cells in blood and BALF is suggested (190), with increased IL-4 and reduced production of IFN-g.

TREATMENT

Effective treatment of SSc has been hampered by lack of knowledge regarding its etiology and pathogenesis and by difficulties in classifying patients into subsets and staging the degree of disease activity. In a study of 264 patients with SSc, overall survival was reduced, with a cumulative survival rate of less than 80% at 2 years, 50% at 8.5 years, and 30% at 12 years after entry (Fig. 41.7) (8). Factors associated with reduced survival included older age (older than 64 years), reduced renal function (blood urea nitrogen greater than 16 mg/dL), anemia (hemoglobin 11 g/dL or greater), reduced D_{LCO} (50% or less predicted), reduced total serum protein (6 g/dL or less), and reduced FVC (less than 80% predicted) (8). Nevertheless, significant improvement in outcome has been achieved, particularly with advances in supportive care. The latter is best illustrated by the improvement in survival associated with the introduction of ACE inhibitors for treating renovascular hypertension (168). Preventive and supportive treatments of this and other complications of SSc are outlined in Table 41.5.

TABLE 41.5. Supportive Measures for Treating Systemic Sclerosis

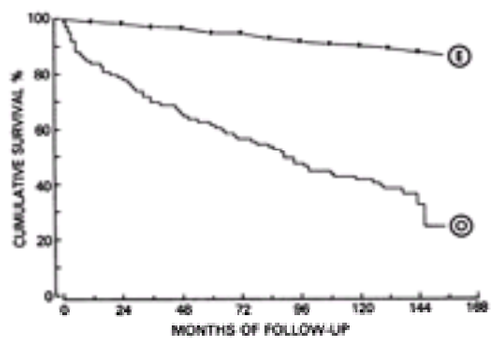

Figure 41.7. Observed survival curve for 264 patients with systemic sclerosis (*O*) and expected survival for a general population matched to the systemic sclerosis group for date of entry into the study, duration of follow-up, age, sex, and race (*E*). (From Altman RD, Medsger TA Jr, Bloch DA, et al. Predictors of survival in systemic sclerosis (scleroderma). *Arthritis Rheum* 1991;34:403, with permission)

Studies in the treatment of scleroderma lung disease have provided promising results (21). In SSc patients with alveolitis, several open studies have demonstrated the efficacy of daily oral cyclophosphamide in improving both the symptoms of dyspnea and FVC (2,17,159,171). A placebo-controlled trial, which is currently under way, is needed to assess efficacy and toxicity of oral cyclophosphamide. Single-lung transplantation has been performed in few SSc patients (100).

Isolated pulmonary hypertension, occurring predominantly in limited cutaneous SSc patients, has been historically very difficult to treat (21). Recent advances in the therapy of primary pulmonary hypertension have included the use of high-dose calcium channel blockers (139) and the prostacyclin analog epoprostenol (15,143). In SSc patients, trials have been performed using Iloprost, a prostacyclin analog (39,16,127) and inhaled nitric oxide (192), each of which demonstrated promising results. Recently a trial of epoprostenol was performed in patients with CTD and pulmonary hypertension (113), demonstrating significant improvements in pulmonary hemodynamics and exercise tolerance.

No drug has been shown in a prospective, double-blind controlled trial to be an effective treatment of SSc (160). A number of drugs and treatments have been reported to be effective, but few have withstood vigorous scrutiny (114,149). Although D-penicillamine and colchicine have been used extensively, many have found their effects to be marginal in those patients who can tolerate long-term treatment. A study comparing high-dose (750 to 1,000 mg per day) with low-dose D-penicillamine (125 mg every other day) did not demonstrate improved efficacy in terms of skin score, incidence of renal crisis, or mortality in SSc patients; however, the high-dose group had 80% of adverse reactions requiring withdrawal from the study (37). The delayed and partial response to a drug such as D-penicillamine may relate to its proposed site of action being rather distal in the pathogenetic sequence of the disease, that is, inhibition of collagen cross-links (Fig. 41.8).

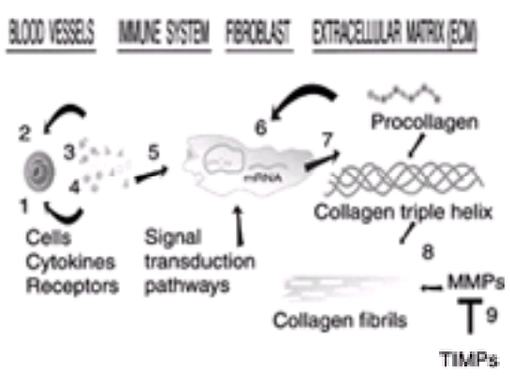

Figure 41.8. Pathogenetic basis of therapy for systemic sclerosis. Potential levels of action include (*1*) inhibit platelet aggregation; (*2*) inhibit endothelial injury; (*3*) inhibit immune cell function and cytokine production; (*4*) inhibit mast cell degranulation; (*5*) block cytokine or block cytokine receptor; (*6*) inhibit matrix protein transcription or translation; (*7*) inhibit collagen secretion assembly; (*8*) stimulate collagenase and other matrix metalloproteinase (*MMP*) activity; and (*9*) block tissue inhibitors of matrix metalloproteinases (*TIMP*).

Drugs targeted at the immune system also have been used, but results have thus far been disappointing. In a randomized double-blind placebo-controlled study of IFN-a, which inhibits collagen mRNA transcription *in vitro*, no improvement in skin score was demonstrated, and there was a significant decline in lung function (both of FVC and of D_{LCO}) (20). Short-term clinical trials of recombinant human IFNg have yielded mixed results (83,131). Open trials of cyclosporine (Cyclosporin A) suggest efficacy in some patients, but renal toxicity often precludes its use. A clinical trial using recombinant human relaxin, a hormone that inhibits TGF-b–induced fibroblast collagen synthesis and that also induces collagenase (184), was halted due to inefficacy. Autologous hematopoietic stem cell transplantation trials also are under way (36,161).

Although SSc remains an incurable disease, significant advances in supportive therapies have been made. Future therapies targeted to the immune system and to downstream steps in the process of extracellular matrix formation will likely prove to be more effective.

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of the manuscript.

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42 IDIOPATHIC INFLAMMATORY MYOPATHIES: DERMATOMYOSITIS, POLYMYOSITIS, AND RELATED DISORDERS

Lauren M. Pachman, M.D. and Frederick W. Miller, M.D., Ph.D.

The idiopathic inflammatory myopathies (IIMs) comprise a very diverse group of syndromes that have in common chronic muscle inflammation of unknown cause, resulting in muscular weakness. Inflammatory muscle disease was apparently not recognized until the mid-1800s. The more easily discerned disease, dermatomyositis, was first described by Unverricht (89). It was considered the major variant of inflammatory myopathy for almost 50 years until the mid-1900s, when it became clear that most myositis lacks a dermal component. In the mid-1960s Banker and Victor (2) found that dermatomyositis in the child was characterized by a greater degree of vascular inflammation and thrombosis than that seen in most other inflammatory myopathies. In the past, the use of muscle biopsies to aid in diagnosis was less frequent than it is now; therefore the extent and diversity of alterations in muscle physiology in rheumatic disease are just beginning to be appreciated. In the past 15 years there has been a concerted effort to focus on a more accurate definition of variants of IIMs. Recent histochemical, genetic, and immunologic tools have allowed investigators to divide these syndromes into more homogeneous groups.

DIAGNOSIS AND CRITERIA

The rarity and heterogeneity of the IIMs have inhibited our understanding of the range of clinical constellations and laboratory attributes that distinguish each entity from the other closely related neuromuscular and rheumatic disorders. The underlying pathology of the IIMs is chronic inflammation in muscle of unknown cause that results in the clinical features on which classification criteria have been based. Although not perfect, and in need of reassessment, five criteria (Table 42.1), proposed more than 25 years ago by Bohan and Peter (5), are still useful in sorting out the many disorders that can be confused with the IIM. Analysis of the utility of these criteria (5) suggests that their sensitivity ranges from 45% to 92%, whereas their specificity, compared with that for systemic lupus or systemic sclerosis, is 93% (48).

1. Symmetric, often progressive, proximal muscle weakness
2. Characteristic electromyographic (EMG) triad seen in myositis
   Short-duration, small, low-amplitude polyphasic potentials
   Fibrillation potentials, seen even at rest
   Bizarre high-frequency repetitive discharges
3. Elevations of serum levels of muscle-associated enzymes
   Creatine kinase (CK)
   Aldolase
   Lactate dehydrogenase (LDH)
   Transaminases (AST/SGPT and AST/SGOT)
4. Evidence of chronic inflammation in muscle biopsy
   Necrosis of type I and type II muscle fibers
   Degeneration and regeneration of myofibers with variation in myofiber size
   Interstitial or perivascular mononuclear cells
5. Characteristic rashes of dermatomyositis
   Scaly erythematous eruptions over the metacarpophalangeal or interphalangeal joints (Gottron papules)
   Periorbital purplish discoloration (heliotrope rash)
   Erythematous scaly rashes over the face, neck (V-sign), upper back and arms (shawl sign), and extensor tendons (linear extensor erythema) and other extensor surfaces

Definite disease: all criteria 1–4, except for dermatomyositis, in which any three of the first four criteria plus the rash are adequate.
Probable disease: any three of the first four criteria, except for dermatomyositis, in which only two of the first four criteria plus the rash are adequate.
Possible disease: only two of the first four criteria, except for dermatomyositis, in which only one of the first four criteria plus the rash are adequate.

TABLE 42.1. Criteria for Diagnosis of Idiopathic Inflammatory Myopathy

The first criterion is symmetric proximal muscle weakness, which is present in nearly all patients at some time during their illness. The second is electromyographic (EMG) abnormalities characteristic of inflammatory myopathy. Although most patients show one or more of these EMG abnormalities, they may be focal and present only in paraspinal muscles. Elevated serum levels of muscle-associated enzymes is the third criterion. These include creatine kinase (CK), aldolase, lactate dehydrogenase (LDH), and the transaminases. All these enzyme levels generally correlate with one another, but, despite the presence of intense focal muscle inflammation, they may be normal in some patients at all times in the disease course, close to the onset of symptoms, after the initiation of therapy, during remission, or after chronic severe myositis associated with significant muscle atrophy. The fourth criterion is histopathologic evidence of chronic muscle inflammation: a muscle biopsy specimen showing degeneration, necrosis, and regeneration of myocytes; phagocytosis; and chronic inflammatory infiltrates composed of interstitial or perivascular mononuclear cells. If not guided by magnetic resonance imaging (MRI) or ultrasound, the biopsy specimen may appear normal or be nondiagnostic (67), because of the focal distribution of the pathologic changes in the untreated patient, or because of the effects of therapy. Patients with dermatomyositis demonstrate the fifth criterion, the characteristic rashes of the disease. The classic pathognomonic signs include scaly erythematous papules overlying the metacarpophalangeal and interphalangeal joints (Gottron papules), or overlying the elbows, knees, and malleoli, and a purplish, often edematous, discoloration around the eyes (heliotrope rash) (Fig. 42.1). Other rashes that are characteristic of (but not limited to) dermatomyositis include erythematous scaly involvement of the face, neck, and anterior part of the chest (V-sign rash), of the shawl area (shawl sign rash), and over extensor tendons, particularly of the hands (linear extensor erythema). Evidence of dilated capillaries can be seen on the eyelid margins, the palate, gingiva, and edge of the nail-folds in many children and a smaller proportion of adults with dermatomyositis.

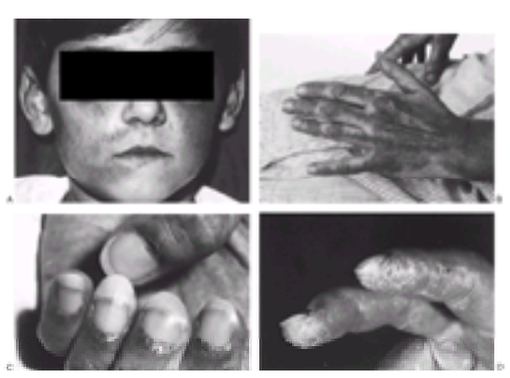

Figure 42.1. Clinical manifestations of the idiopathic inflammatory myopathies. **A:** The rash of a child with juvenile dermatomyositis, displaying periorbital erythema crossing the nasal bridge with edema and microinfarcts of the upper eyelids and the skin under the eyebrow. **B:** Linear extensor erythema and Gottron papules seen in an adult with dermatomyositis. **C:** Cuticular overgrowth and vasculitic changes at the nailbeds in an adult with dermatomyositis. **D:** Mechanic's hands: scaly, cracking skin over the lateral and palmar aspects of the fingers are commonly seen in adult patients with myositis associated with antisynthetase autoantibodies.

Inasmuch as a number of disorders may satisfy one or more of these criteria, the history and physical examination must be carefully performed to exclude the many entities that can closely mimic IIM (Table 42.2). Some of these diseases can be differentiated based on the clinical setting in which they occur, and others, by laboratory and muscle biopsy findings. Many of these disorders have an inflammatory myopathic component, but one of the pathologic features distinguishing IIM and its mimics is that in the latter, the inflammation is secondary to another cause of myopathy. In general, this secondary inflammation is indicated by the presence of polymorphonuclear cells or macrophages surrounding previously damaged myocytes in muscle biopsy specimens. In contrast, inflammation in IIMs appears as the primary event, with mononuclear cells surrounding and invading otherwise normal-appearing muscle cells.

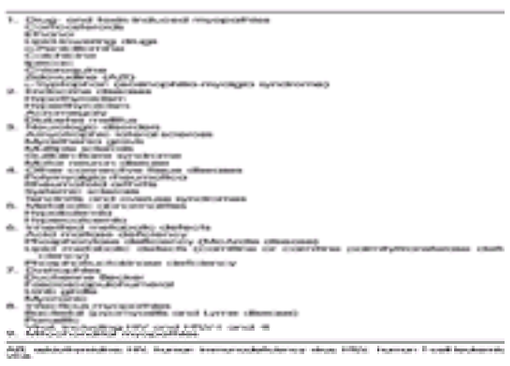

TABLE 42.2. Differential Diagnosis of Idiopathic Inflammatory Myopathy

The recent discovery of a group of autoantibodies only seen in myositis patients (the *myositis-specific autoantibodies*) (MSAs) (42) and the information that MRI provides in assessment of muscle inflammation and damage (75) have improved our capacity to diagnose and treat certain individuals with IIM. These findings, as well as other aspects of the IIM, including genetic testing, markers of immune activation and tissue damage, should undergo assessment of their usefulness in the development of new, more sensitive and specific classification criteria for the IIM (87). Recent advances in understanding the genetic basis for many of the metabolic and dystrophic myopathies should allow development of more accurate genetic diagnostic tools to help distinguish the IIMs from these other often difficult to diagnose disorders.

EPIDEMIOLOGY

In a global sense, the IIM syndromes are some of the most rare causes of myopathy and are far less common than the inflammatory myopathies that occur as a result of bacterial or parasitic infection and are prevalent worldwide. Although many epidemiologic studies of IIM combine polymyositis and dermatomyositis, recognition of some of the rarer variants, particularly inclusion body myositis (IBM), has occurred only recently. Nonetheless, IBM is likely the most common form of idiopathic myositis in persons older than 50 years (19). There appears to be a bimodal age distribution of onset in individuals with IIM. One peak occurs at ages 5 to 14 years, and the other at 45 to 64 years (48). In 1970, the incidence of these diseases was estimated to be 1.0 to 3.2 cases per year per 1 million white Americans and 7.7 for black Americans (48), but more recent data suggest that the incidence and prevalence have been increasing over the last three decades (56). Mortality appears to be influenced by age and gender; nonwhite females seem to have the highest mortality, and children with IIM seem to have a low mortality rate (30,31).

The incidence of juvenile dermatomyositis has just been established as 3.1 per million children per year for white, Hispanic, and African-Americans (50); it is 10 to 20 times more common than polymyositis. The child with juvenile dermatomyositis tends to have a more acute and severe onset (24), after an antecedent infection, which is often upper respiratory (66). The rash precedes identification of weakness in 50% (67). Seasonal factors may be related to the onset of symptoms in adults with polymyositis, as suggested by one study from Greece (45). In both the pediatric and adult myositis populations overall, there is a female predominance of about 2:1, although this may vary with specific ethnic background.

CLASSIFICATION

Once the heterogeneity of these patients was recognized, attempts were made to categorize them into more understandable groups. Although many classifications have been proposed, we have found that the most useful one incorporates both clinical and serologic information (Table 42.3). As helpful as the clinical classification of IIM has been, recent data suggest that dividing patients into serologic groups based on the presence or absence of certain autoantibodies found only in myositis patients, the MSAs, results in even more homogeneous patient groups (41).

Clinical classification
Primary idiopathic polymyositis
Primary idiopathic dermatomyositis
Myositis associated with another connective tissue disease (overlap myositis)
Myositis associated with cancer
Juvenile myositis
Inclusion body myositis
Miscellaneous myositis (eosinophilic, granulomatous, focal nodular, ocular, and others)
Serologic classification[a]
Myositis associated with antisynthetase autoantibodies
Myositis associated with anti-M-2 autoantibodies
Myositis associated with anti-signal recognition particle autoantibodies
Myositis associated with other myositis-specific autoantibodies[b]
Myositis without any of the above-mentioned autoantibodies

[a] See Table 42.6.
[b] Including anti-MAS, anti-PL12, and other autoantibodies (see Table 42.6)

TABLE 42.3. Classifications of the Idiopathic Inflammatory Myopathies

The MSAs are directed against and inhibit the function of translational components, proteins, and ribonucleic acids involved in the process of protein synthesis (51). Members of this family of autoantibodies include autoantibodies directed against one of five aminoacyl-transfer RNA (tRNA) synthetases, alanyl-tRNA, elongation factors, proteins of the signal-recognition particle (SRP), nuclear helicases, and several RNAs and proteins not yet identified. In addition to their interesting immunologic aspects, the myositis-specific autoantibodies have clinical utility in helping to assess the diverse signs and symptoms of patients with myositis and by assisting in their therapy. This is because each of these autoantibodies is associated with particular epidemiologic, clinical, immunogenetic, therapeutic, and prognostic features. Thus patients with each of these autoantibodies can be considered to have different syndromes that share the common feature of chronic inflammation in muscle.

CLINICAL FEATURES

Primary Idiopathic Polymyositis

Patients with myositis have many nonspecific symptoms that may complicate and delay diagnosis and treatment. Symmetric, progressive, proximal muscle weakness, however, is the primary clinical finding in polymyositis. It most often develops subacutely over weeks to months, but can develop acutely over days or insidiously over years. Patients often complain of an inability to climb stairs, arise from a seated position, reach for heavy objects, or comb their hair. Although proximal muscles of the neck, shoulders, and hips are more commonly and more severely affected, distal muscles of the limbs also can be involved. Some patients have fatigability of muscle function, and others have myalgias that limit their activity and that make assessment of true weakness difficult. As a group, patients with polymyositis have the most severe myositis and have the poorest response to therapy, compared with all other clinical subsets (41).

Myositis patients may have involvement of other organ systems that can alter the presentation and prognosis (Table 42.4). The systemic nature of these diseases is seen in general symptoms such as fatigue, fever, and malaise, or by pathology in specific organ systems. Cardiopulmonary involvement, with arrhythmias, dyspnea, or interstitial pulmonary infiltrates, is an especially worrisome, poor prognostic sign. Dysphagia or vasculitis affecting the gastrointestinal tract may be so severe as to require nasogastric feeding or parenteral nutrition, but is more commonly reflected in nasal speech, reflux symptoms (which may associated with change in the quality

of the voice), or gastrointestinal bleeding.

TABLE 42.4. Systemic Manifestations of Idiopathic Inflammatory Myopathy

Primary Idiopathic Dermatomyositis

Patients with dermatomyositis are distinguished from those with polymyositis by the skin changes described previously. The rash can precede, develop concurrent with, or follow the development of muscle weakness. Furthermore, the rashes can change over time. Occasional patients have classic dermatomyositis rashes and some other organ manifestations of connective tissue disease such as arthritis or interstitial lung disease, but clinical muscle weakness does not develop. This condition has been referred to as *amyopathic dermatomyositis* or *dermatomyositis sine myositis*. A number of these patients do have evidence of mild myositis, as demonstrated by minor enzyme elevations, by mild EMG or histologic changes (17), or by the appearance of the muscles on MRI scans (21). They may develop other symptoms of chronic disease (e.g., loss of range of motion) over time. The disease severity and responsiveness of dermatomyositis patients as a group tend to be intermediate between those of polymyositis and myositis-overlap patients.

Myositis Associated with Another Connective Tissue Disease

Patients may meet all the criteria for IIM and those for another connective tissue disease. These patients, sometimes referred to as having *overlap myositis*, most frequently have myositis in association with systemic sclerosis, systemic lupus erythematosus, or rheumatoid arthritis (adult patients who are rheumatoid factor positive or negative as well as seronegative patients with systemic-onset juvenile rheumatoid arthritis can be seen in this category). In addition, virtually any of the many rheumatic and autoimmune disease syndromes may be present (70). Because the myositis in these patients is generally the mildest of all the clinical myositis groups and most easily treated, these patients have a relatively good prognosis (41).

Myositis Associated with Cancer

Patients with both myositis and cancer have been recognized for more than 70 years, and the incidence of malignancy in these patients has been estimated to be up to 5 times that of the general population. Patients older than 40 years who have dermatomyositis appear to be at the greatest risk of cancer. A recent large population-based study estimated the risk of cancer to be 1.8 times higher in people with polymyositis and 2.4 times higher in those with dermatomyositis, although all forms of malignancy were reported to be roughly in the same proportion as in the general population (84). Although cancer can precede, develop concurrently, or follow the myositis, most cases occur within 2 years of the onset of myositis. Further evidence that the cancer is associated with the pathogenesis of myositis is provided by the finding that in some patients, surgical removal of the cancer can result in complete resolution of all signs and symptoms of myositis, and conversely, the return of the cancer is often heralded by the recurrence of myositis. The prognosis of these patients depends greatly on the prognosis of their cancer, as this is the primary cause of their death.

Although the association between cancer and myositis is well established, the more important question is how to approach the new patient with myositis in this regard. Our experience is that a thorough history and physical examination, with careful attention to any abnormalities suggestive of malignancy, will detect most cancers. The most useful tests include chest radiograph films; stool guaiac studies; mammography; careful lymph node, breast, and pelvic examinations; and in women with risk factors for ovarian cancer, abdominal ultrasound with serum levels of CA-125 antigen. Multiple x-ray studies or invasive procedures screening for cancer without a clinical reason are unlikely to be beneficial.

Juvenile Myositis

In children, acute infectious myositis can be distinguished from a chronic inflammatory process by the propensity of the former to be characterized by severe pain in calf muscles, which rapidly resolves in 1 to 4 weeks (49). This acute myositis, often accompanied by myoglobinuria, EMG changes, and CK elevations, is frequently associated with identification of influenza A and B on viral culture (14) and muscle biopsy specimens (22). In contrast, the onset of myositis in juvenile dermatomyositis is often insidious and may be preceded by a recurrent photosensitive dermatitis (92), low-grade fever, or both. Prominent physical findings include soft palatal weakness, resulting in a nasal tonality in speech that often has a raspy quality, and a positive Gower sign in addition to symmetric proximal muscle weakness. Vasculitis is the hallmark of this illness and is reflected by abnormal nail-fold capillaries (85), capillary thrombosis (2), and dropout (7), with occasional subsequent infarction of affected tissue, including skin, muscle, and gastrointestinal tract (11). The focal distribution of disease is revealed by MRI (Fig. 42.2) (34), which may assist in identification of a biopsy site and may explain cases with obvious symptoms but normal histology. Studies of lung function may show only a restrictive ventilatory defect (64), without impairment of perfusion or evidence of pulmonary fibrosis. One of the most useful associations with a global assessment of disease activity is the increased proportion of peripheral blood B cells (percentage CD19) (60), which are not activated (61). The increased %$CD19^+$ is frequently associated with a decrease in circulating $CD8^+$ cells, often resulting in an increased CD4/CD8 ratio. The extent of vasculitis may be associated with elevated plasma levels of von Willebrand factor antigen (82); the inflammatory component may be reflected by elevated neopterin, a macrophage-derived cytokine (91). Levels of one or all the muscle-associated enzymes may be elevated. An increased erythrocyte sedimentation rate and leukocytosis occasionally are seen, and immunoglobulin M (IgM) may be increased early in the disease (62). The most common autoimmune serologic marker is an antinuclear antigen (ANA), which is seen in a speckled pattern in more than 60% of patients with juvenile dermatomyositis (65). The specific antigens to which these antibodies bind have been identified in only 10% of children (20); the most common identified autoantibody in these patients, known as anti–Mi-2, is directed against a 220-kd nuclear helicase that is a component of a complex of nuclear proteins. This serologic subgroup appears to have a milder disease course (88). In addition to anti–Mi-2 autoantibodies, all the other MSAs, including anti-SRP and the anti-synthetases that were originally identified in adults, also occur in children (80).

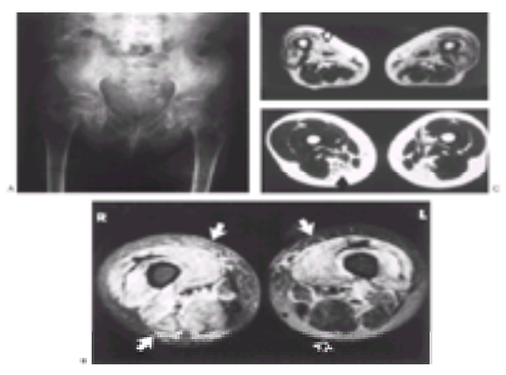

Figure 42.2. Radiographic findings in the idiopathic inflammatory myopathies. **A:** Radiographic evidence of extensive soft tissue calcification in a child with active juvenile dermatomyositis (JDM), which continued over several years. There is marked osteopenia. **B:** Magnetic resonance image (MRI) (T_2-weighted with fat suppression) through midthigh of a child with JDM. There is diffuse abnormal high signal within the quadriceps muscle group and the hamstring muscle group on the right (*arrows*). There is normal signal and relative sparing of the hamstring muscles on the left (*open arrow*). **C:** MRI (T_1-weighted) of long-standing inclusion body myositis. **Top:** Significant symmetric atrophy of the anterior thigh muscles and fatty replacement of many of the other muscle groups. **Bottom:** Clinically active adult dermatomyositis showing involvement of the posterior thigh muscles. (From Fraser DD, Frank JA, Dalakas M, et al. MRI in inflammatory myopathies. *J Rheumatol*

1991;8:1693, with permission.)

Delay in diagnosis and therapy with the consequent increase in disease duration and severity is associated with an increased frequency of soft tissue calcifications, usually at pressure points (6), which occur in 20% to 30% of affected children (67). The calcifications in patients with juvenile dermatomyositis have the same crystalline structure as those seen in patients with scleroderma. g-Carboxyglutamic acid, a component of the vitamin K–dependent pathway, is present in these calcifications; children with dermatomyositis have an increased urinary excretion of this compound (39). Early recognition of the symptoms of juvenile dermatomyositis and the prompt institution of aggressive therapy have decreased the occurrence of this serious complication. A prolonged disease course as well as the presence of pathologic calcinosis is associated with increased mononuclear cell production of tumor necrosis factor (TNF)-a and TNF-a–308A polymorphism at –308 in the promoter region of the TNFa allele in white children with juvenile dermatomyositis (68).

Inclusion Body Myositis

IBM is a recently described form of IIM affecting primarily older white men. It is characterized by: an insidious onset and progression; more distal and asymmetric muscle weakness than that seen in polymyositis; a tendency for atrophy of the anterior thigh muscles, which results in frequent falling; little extramuscular involvement; and a poor response to therapy (12,13,41). Patients with IBM generally meet the criteria for definite IIM, although the serum CK levels are seldom more than 5 to 6 times the upper limits of normal, and the EMGs often show evidence of both myopathy and neuropathy. The diagnosis is confirmed by the histologic finding of distinctive rimmed vacuoles in myofibers (Fig. 42.3). Without therapy, these patients gradually become restricted to the use of a wheelchair, and soon thereafter, they are bed bound. Possibly because they do not have the severe cardiac and pulmonary disease of other patients with myositis, deaths directly as a result of this disease are infrequent. As is the case for polymyositis and dermatomyositis, a few families appear to have a familial form of IBM (76). Another myopathy, not a member of the IIM group, is a related illness without muscle inflammation known as inclusion body myopathy. The inclusion body myopathy syndromes can be either sporadic or familial, and in the latter case, genetic diagnostic methods may be available in the not too distant future (15).

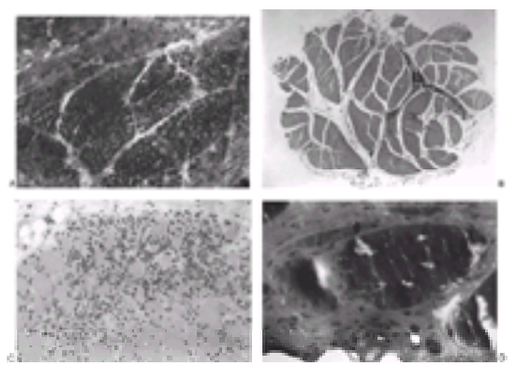

Figure 42.3. Histopathologic findings in muscle in the idiopathic inflammatory myopathies. **A:** A biopsy specimen of skeletal muscle from a child with dermatomyositis (trichrome stain) demonstrating perifascicular atrophy, fiber-size variation, vascular occlusion, and a perivascular infiltrate composed primarily of mononuclear cells. **B:** Adult dermatomyositis (trichrome stain) showing variation of myofiber size (reflecting degeneration and regeneration), perifascicular atrophy, increased interstitium, and scattered perivascular mononuclear infiltrate. **C:** Adult polymyositis (hematoxylin and eosin stain) showing variation of myofiber size and focal endomysial inflammation. **D:** Inclusion body myositis (high power, trichrome) showing the characteristic, purplish staining, rimmed vacuoles within the myocyte. (From Farid NR, Bona CA, eds. *The molecular aspects of autoimmunity.* Langhorne, PA: Harwood Academic, 1990.)

Myositis Associated with Antisynthetase Autoantibodies

Patients with autoantibodies directed against one of five aminoacyl-tRNA synthetases, enzymes that bind amino acids to tRNA in one of the early steps in protein synthesis, have been associated with what is known as the *antisynthetase syndrome*. The antisynthetase syndrome is characterized by myositis in association with a high frequency of interstitial lung disease, symmetric polyarthritis (which frequently mimics rheumatoid arthritis and involves the small joints of the hands, the wrists, and the knees), fevers, Raynaud phenomenon, and mechanic's hands (a scaling and cracking rash over the lateral and palmar aspects of the fingers; see Fig. 42.1). These patients tend to have an acute onset in the spring (38), a moderate response to therapy, but persistent disease and a flare of disease with taper of therapy, and a relatively poor prognosis (41). These patients can have polymyositis, dermatomyositis, or overlap myositis. Anti-Jo-1 autoantibodies, which are directed against histidyl-tRNA synthetase and are found in 20% to 30% of myositis patients (Table 42.5), are the most common antisynthetase autoantibodies.

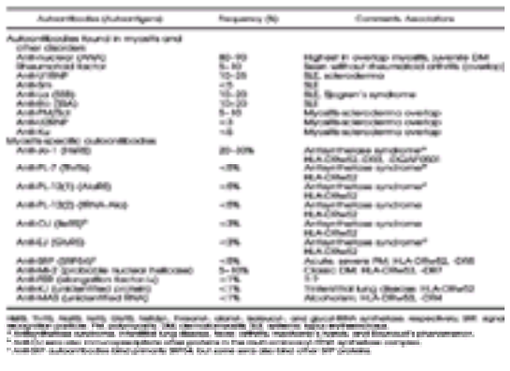

TABLE 42.5. Serologic Abnormalities in the Idiopathic Inflammatory Myopathies

Myositis Associated with Anti–Signal-Recognition Particle Autoantibodies

A different MSA binds to proteins of the SRP, a complex of six proteins and 7SL RNA that interacts with newly formed polypeptides on the polyribosome and moves them to the endoplasmic reticulum for processing. As a group, patients with anti-SRP autoantibodies have the most acute and severe myositis, usually beginning in the autumn, and the poorest response to therapy. These patients tend to have severe myalgias and, partly because of the high frequency of cardiac involvement, the poorest prognosis of all the serologic subsets (41). All patients with anti-SRP autoantibodies reported to date have been categorized in the clinical group of polymyositis (86).

Myositis Associated with Anti–Mi-2 Autoantibodies

In contrast to those of patients in the synthetase or SRP groups, the anti–Mi-2 autoantibody is directed against a nuclear protein, which is a helicase that is involved in regulating transcription, rather than a cytoplasmic protein involved in translation (83). They also differ from the other MSA groups in having relatively mild disease, with a good response to therapy. Patients in the Mi-2 group tend to have an acute disease onset; classic dermatomyositis clinically; an increased incidence of the V-sign rash, shawl sign rash, and cuticular overgrowth; a good response to therapy; and little extramuscular involvement (41).

CLINICAL EVALUATION

Because the differential diagnosis of muscle pain and weakness is extensive, it is important to be aware of a wide range of medical disorders that can result in muscle symptoms when evaluating these patients. First, it is important to define clearly the patient's problems. Is the patient actually weak or suffering from fatigue or pain that prevents performance of daily tasks? The characteristics and distribution of the weakness are often helpful. Is the weakness of acute to subacute onset, proximal, and

symmetric, as is usually seen in polymyositis and dermatomyositis; or slowly progressive and distal, as seen in several dystrophies and inclusion body myositis; or more focal and asymmetric, as can occur in other dystrophies? Is the weakness relatively constant, as in the IIMs, or is it intermittent and related to fasting or excessive activity, as in the case of certain metabolic myopathies? Weakness of the proximal leg muscles often results in difficulty climbing stairs, rising from a chair, and walking long distances. Proximal arm weakness usually causes problems in lifting objects, putting on heavy clothing, and combing or styling hair. The facial muscles are rarely involved in the IIMs affecting adults; in juvenile dermatomyositis, hoarseness, masseter involvement and atrophy can be seen (62), and the presence of difficulty in swallowing (often with penetration of the airway) indicates severe disease.

A review of systems may identify involvement of other organs, as manifested by pulmonary, cardiac, gastrointestinal, and skin symptoms. In children it is especially important to obtain a detailed family history to exclude the familial patterns associated with the various dystrophies and metabolic and mitochondrial myopathies. A careful history should be able to help the physician identify drug, toxin, or other exposures associated with myopathies (42). Symptoms and signs of endocrine disorders or infections should be confirmed by appropriate diagnostic testing.

The physical examination should focus on objectively defining the weakness and disability of the patient, by using a semiquantitative grading system. The cardiac, pulmonary, and gastrointestinal systems should be carefully examined for signs of involvement, and the skin and nail-beds checked for evidence of vasculitis or other abnormalities. The presence of pain on muscle compression should be noted.

Laboratory testing should include a complete blood cell count, measurement of electrolytes and CK and aldolase, as well as a general chemical survey that includes aspartate aminotransferase (SGOT), LDH, alanine aminotransferase (SGPT), and urinalysis. Testing for autoantibodies is often useful in evaluating patients with muscle weakness. The majority of adult patients with IIM have one or more autoantibodies (Table 42.5), and this distinguishes the IIMs from all the other forms of myopathy, in which autoantibodies are infrequent. ANAs are most common in IIM (41), usually seen in a coarse speckled nuclear pattern. In juvenile dermatomyositis, in addition to the positive speckled ANA, evidence of immune dysregulation is obtained by assessment of peripheral lymphocyte phenotype, which usually documents a lymphopenia, with an increased %$CD19^+$ B cells and increased CD4/CD8 ratio (60). EMG is frequently used to differentiate myopathic from neuropathic states.

A muscle biopsy with histochemical analysis is almost always indicated to confirm the diagnosis of IIM, as it provides critical information when the procedure is properly performed and the specimen properly processed. In addition to ruling out many of the mimics of the IIMs, different abnormalities and different distributions of inflammatory cells are seen in the various clinical groups. In dermatomyositis, the most characteristic finding is that the mononuclear cell infiltrate tends to be perivascular or perifascicular; in children with this disorder, occlusion of capillaries is prominent, and interfascicular fibrosis may be present. In both adults and children, atrophy of the myofibers at the periphery of the fascicle (perifascicular atrophy) is a characteristic finding, and in very severe cases, is associated with infarction of muscle. In polymyositis, in contrast, the leukocytes tend to be located within scattered, single myofibers, although sometimes the inflammatory infiltrate is extensive; perivascular inflammation and perifascicular atrophy are not usually present. Inclusion body myositis is diagnosed in a patient with myositis by the presence in trichrome-stained frozen muscle sections of characteristic reddish inclusions and vacuoles, which are rimmed by purplish granules (Fig. 42.3). Additional features include endomysial fibrosis, variation in myofiber diameter, and the presence of angulated fibers.

THERAPY

Corticosteroids are the mainstay of therapy for IIM. The timing, dose, and route of administration should be based on disease severity. Several factors are important in achieving responses to corticosteroids. In addition to the patient's clinical and autoantibody status, these include adequate initial dose (1 mg/kg/day or more) and continuation of prednisone until or after the serum CK level becomes normal in adults. In children, higher doses of corticosteroids are often used initially, and reduction of dose is guided by both the muscle enzymes and the immunologic data (normalization of neopterin, von Willebrand factor antigen, %CD19 cells). Children should be treated with a very slow rate of prednisone tapering. In adults, the reduction of prednisone therapy should average about 10 mg per month from the first reduction to the time that a maintenance dose is achieved (57). It is important to remember that corticosteroid responses in patients with myositis are generally slower and less dramatic than those seen in patients with systemic lupus erythematosus or rheumatoid arthritis, and improvement in strength may lag behind normalization of serum levels of muscle-derived enzymes by weeks to months. In the treatment of juvenile dermatomyositis, the dose, route, and duration of therapy are still controversial (44). Children with very active vasculitis often experience a decreased absorption of orally administered prednisolone, with less bioavailability compared with the same dose of methylprednisolone given by the intravenous route. Some data indicate that in this group of patients, in which vasculitis is a prominent feature, high-dose intravenous steroid therapy is associated with a more rapid resolution of disease and fewer adverse consequences of therapy (compression fracture, growth retardation, glaucoma). However, it remains unclear whether bolus corticosteroids and alternate-day steroid administration are useful in the majority of patients with IIM. The adverse effects of high-dose intermittent-pulse methylprednisolone have been identified in children and are variable (36). In children, osteopenia associated with decreased osteocalcin levels and bone mineral density (72) is found before therapy and can be exacerbated by administration of steroids, which suppress calcium absorption. In these children, administration of exogenous vitamin D and a calcium-sufficient diet, in addition to effective control of the inflammatory process, will increase bone mineral density and decrease the frequency of bone fracture (72). An eye examination is needed to evaluate the possibility of glaucoma, and to document the presence of cataracts, which may improve as the daily dose of corticosteroids is decreased.

Rehabilitation and physical therapy are very important in maintaining range of motion and preventing contractures during active disease. Exercise probably improves strength and endurance when initiated in a graded way during disease remission (29). Other immunosuppressive regimens have been tried in corticosteroid-resistant patients. Oral methotrexate and azathioprine are the major therapeutic options for corticosteroid-resistant patients. Which alternative is to be chosen is determined by the individual risk factors of each patient. Once patients are in remission, our experience with adults is that corticosteroid maintenance for periods of 6 to 12 months at 0.25 mg/kg every other day decreases the frequency of later flares of disease. In children with dermatomyositis, however, alternate-day steroid therapy does not appear effective either in the control of disease or in the prevention of flares. Methotrexate (intravenous or subcutaneously) given in conjunction with folic acid is helpful in moderately acute disease, whereas intravenous cyclophosphamide may be needed in critical conditions. Cyclosporine (28), intravenous gamma globulin (80), tacrolimus (58), or combinations of cytotoxics, particularly methotrexate and azathioprine (59), may be beneficial in some patients. A randomized double-blind, sham-controlled trial of apheresis failed to show any significant benefit after plasma exchange or leukapheresis in steroid-resistant patients with myositis not taking cytotoxic drugs (52). In patients with cutaneous involvement, hydroxychloroquine (7 mg/kg/day; maximal dose, 400 mg/day) and sunscreens with a sun protection factor (SPF) higher than 15, along with decreased sun exposure, can be useful for the treatment of the rash of dermatomyositis and the prevention of sun-induced disease exacerbation. No specific treatment besides therapy for the underlying myositis is known to improve the other systemic manifestations of the IIM. Patients in poor prognostic groups (patients with delays in diagnosis, older patients with acute severe myositis, cardiopulmonary disease, or dysphagia associated with myositis; the clinical group with polymyositis; or the serologic groups with antisynthetase or anti-SRP autoantibodies) should be considered for earlier and more aggressive therapy.

Part of the difficulty in defining appropriate therapy for myositis patients relates to the few reliable and objective measures of disease activity and damage (79). Although serum CK levels are often associated with levels of the other myositis-associated enzymes (aldolase, LDH, transaminases), all of these enzymes may not be elevated at the same time, and do not always accurately reflect disease activity (66). For example, they are not highly associated with the degree of weakness, rash and other extramuscular manifestations, or inflammation on biopsy specimens in some patients. This may be due to circulating inhibitors of CK enzyme activity (33), the focal nature of the inflammation, the effects of prior episodes of myositis on residual strength, the concurrent development of another cause of muscle weakness, and differences in rates of change with time among these factors. Serum CK levels, for example, often increase a few weeks to months before myositis flares are clinically evident, and often decrease before clinical improvement is recognized. The development of validated disease activity and damage-assessment tools for the IIM is an important step toward better future therapies (43).

ETIOLOGY AND PATHOGENESIS

The etiology of the IIM is by definition unknown, and the pathogeneses are still poorly understood, but intriguing findings suggest that environmental factors acting on groups of genetically predisposed individuals lead to immunologic activation and subsequent tissue damage. Predisposing genetic factors for all clinical forms of IIM in whites include human leukocyte antigen (HLA)-DR3 and -*DQA1*0501* (41,51,73,74). The relative risk for juvenile dermatomyositis is 3.8 for the white population with DR3 and 18.2 for those with *DQA1*0501*(73) and is similar for adult IIM (41, 51). The presence of TNF-a-308A in whites with juvenile dermatomyositis is associated with disease of a longer duration, and an increased frequency of pathologic calcifications (68). Immunogenetic risk factors, however, appear to differ in the different autoantibody groups (Table 42.5) (41) and in some ethnic groups (78). Further evidence for the importance of genetic susceptibility comes from the rare cases of monozygotic twins, siblings, and other family members with myositis, who can even share a common clinical group and autoantibody response (51). Newer molecular genetic methods that involve whole genome scanning (3), combined with family linkage studies (23), suggest that perhaps the same 20 or more regions located over the entire genome may be common risk factors for many autoimmune diseases. It is likely that these regions encode polymorphic genes regulating immune responses (immunogenetic markers) and the metabolism of environmental exposures (pharmacogenetic markers).

Anecdotal reports of the clustering in onset of myositis cases, as well as temporal associations with a growing list of infectious and noninfectious agents, have suggested strong environmental influences in the development of IIM. Environmental factors of interest include picornaviruses, for which there are supporting serologic, ultrastructural, animal model, and unconfirmed molecular hybridization data, and retroviruses, notably human immunodeficiency virus (HIV) and human T-cell lymphotropic virus type I (HTLV-I) (70). The different seasons of myositis onset in different clinical and autoantibody groups also suggest different environmental influences in different subgroups of patients (38). In juvenile dermatomyositis, case-control data document the presence of antecedent illness in the 3 months before the first definite symptom (rash/weakness) of disease (66); streptococcal exposure can induce disease flares (46). A growing list of noninfectious agents, including

foods and dietary supplements, drugs, biologic agents, excessive ultraviolet light exposure, and a number of occupational and other exposures have been reported in individual cases to be temporally associated with the onset of myositis. Much more work is needed in this area to confirm a true etiologic role of any of these agents (42). Cases of drug-associated myositis are the best-documented examples of environmentally associated myositis syndromes. The prototypic example of penicillamine-induced myositis is well-described entity that can exhibit similar clinical, serologic, laboratory, and pathologic findings as idiopathic myositis and usually resolves after discontinuing the medication. Of interest, the immunogenetic risk factors for the development of myositis after penicillamine exposure appear to differ from those of idiopathic myositis, suggesting that gene/environment interactions are likely important in the development of the environmental myositis syndromes (42).

Whatever the primary genetic and environmental risk factors are for the initiation of myositis, a growing body of evidence suggests that immunologic processes are central to the later aspects of pathogenesis. Many lines of evidence point to both humoral and cellular immune abnormalities in IIM patients (Table 42.6). Pathogenic mechanisms may be different in the different clinical groups. Muscle biopsy specimens from dermatomyositis patients show an early invasion of mononuclear cells, with prominent B cells present, with associated changes in the vascular endothelia, occlusion of capillaries, and deposition of components of the complement membrane attack complex (35). In contrast, patients with polymyositis or inclusion body myositis are characterized by higher proportions of activated T cells in their systemic circulation and within myofibers in muscle specimens (18,53). Both $CD8^+$ (suppressor/cytotoxic) T cells and DR^+ (activated) cells are seen surrounding and invading otherwise normal-appearing myocytes. However, there is evidence of peripheral blood T-cell activation [hypoxanthine-guanine phosphoribosyltransferase (HPRT) studies] (1) and oligoclonality of CRD3 region of the Vb8 T-cell receptor in muscle biopsies of untreated children (69), suggesting an antigen-driven response.

Humoral abnormalities
Autoantibodies (see Table 42.6)
Immunoglobulin and complement deposition in muscle
Hypergammaglobulinemia, hypogammaglobulinemia, and agammaglobulinemia
Circulating immune complexes
Cellular abnormalities
Activated MNCs and T cells expressing restricted receptors (TCR) in muscle
Altered peripheral MNC immunophenotypes
Altered peripheral MNC trafficking to muscle
Decreased autologous mixed lymphocyte and MNC mitogenic response
Proliferative responses of MNCs to autologous muscle
Increased cytokine expression in the periphery and target organs
Increased circulating levels of soluble cell markers (sIL-2R, sCD4, sCD8)
Altered expression of costimulatory molecules in target tissues

MNC, mononuclear cell; TCR, T-cell receptor; IL, interleukin.

TABLE 42.6. Immunologic Abnormalities in the Idiopathic Inflammatory Myopathies

Additional evidence for the role of T cells in the pathogenesis of IIM comes from *in vitro* stimulation studies that show that T cells from myositis subjects preferentially respond to autologous muscle. Studies of T-cell receptors in muscle biopsies demonstrate expression of restricted families of receptors with common motifs in different forms of IIM. Investigations of costimulatory molecules on the surface of myocytes suggest that the target cells themselves may act as efficient antigen-presenting cells for T cells that appear to traffic to and invade histologically normal myocytes (4,55,59,69).

The roles of the wide array of autoantibodies seen in myositis patients (Table 42.5) remain unclear, and there is no direct evidence to support a pathogenic role. However, indirect evidence for the involvement of some MSAs in disease pathogenesis includes the findings that they can be detected months before myositis onset, appear to be induced and sustained by conformational epitopes on the human autoantigen they target, and vary in titer with disease activity, sometimes becoming negative after prolonged remission (54).

Animal models are imperfect in reproducing the human IIM syndromes and provide limited insight into the susceptibility and pathogenesis of IIM. Familial canine dermatomyositis, first reported in 1984, affects collies and Shetland sheep dogs (25); skin and muscle involvement occurs in young animals and is preceded by circulating IgG-containing immune complexes (27). These dogs do not develop ANAs, but muscle specimens show a vasculitis and crystalline structures compatible with enterovirus in endothelial cells (26). Genetic susceptibility is suggested by some preference for DLA-D15 in these animals (27). In some murine strains, newborn animals develop myositis after injection with coxsackievirus B (32,70). This model is dependent on the presence of live virus and an intact immune response involving T cells (93). In addition, when myocarditis secondary to coxsackievirus was investigated, focal lesions were produced by $CD4^+$ T helper cells (9); the genetic background of the host appears to modulate the extent of damage seen on passive transfer (40). A model of adult polymyositis has been developed by using a myopathic variant of the encephalomyocarditis virus, a member of the cardiovirus subgroup of picornavirus (10).

CURRENT PROBLEMS AND FUTURE DIRECTIONS

Whereas advances in technology have afforded new insights into the pathophysiology of IIMs, they also have raised questions about the diagnostic criteria and classifications of these syndromes. For example, because the MSAs appear to define more homogeneous subsets of IIM patients, should these tests be added as an additional diagnostic criterion, and should they be performed routinely as part of the clinical evaluation? Should MRI also be part of the diagnostic evaluation of myositis patients? This radiologic approach permits a noninvasive *in vivo* functional analysis of large areas of muscle not possible by other methods and may help in defining clinical subgroups and in directing the site of biopsy (21). If so, what is the level of sensitivity and specificity of this procedure? The rate of change on MRI scans with clinical response to therapy and the correlation of MRI data with muscle histologic findings remain to be determined. MR spectroscopy, a noninvasive technology related to MRI, can measure the *in vivo* concentration of compounds in a given tissue. By analysis of the distribution of phosphate groups on different molecules involved in energy metabolism in muscle, biochemical abnormalities can be quantitated. These may relate to disease activity and may be different in various clinical subsets of patients.

Although both human and animal model data suggest that histocompatibility antigens play a role in disease susceptibility as well as in severity, there is no prospective evidence that disease severity or expression is also under immunogenetic control. If immunogenetics does play a role, then to what extent can this information be used in defining risk factors and potential immunotherapeutic approaches? Finally, at the heart of a current controversy, are infectious or noninfectious environmental agents responsible for IIM in some patients, and what is the relationship of these agents to the cardiomyopathies in humans? Studies of molecular mimicry in other conditions raise the question of whether shared epitopes between these environmental agents and muscle or skin targets in myositis might incite immunoreactivity.

We need to have better ways to assess disease activity, persistence, and outcome. In the future, various mediators of inflammation such as cytokines, cell-surface activation markers, hormones, and receptor levels may prove useful as adjuncts to traditional disease measures. We need better measures of disease outcome. Additionally, the effect of this group of diseases on socioeconomic, emotional, educational, and marital status has not yet been evaluated.

The establishment of national or international registries of newly diagnosed cases could provide important data on these rare diseases and could serve as a basis for multicenter studies. A registry including serum and tissue banking would add significantly to information available in areas ranging from genetic susceptibility to immune responses. As new knowledge emerges, this database could be accessed to validate and test new concepts and hypotheses.

Effective therapeutic options are quite limited. The optimal dose, route, and duration of therapy for all the current agents have yet to be well studied. Biologic agents may be useful in future therapy if inflammatory pathways in each disease subset can be characterized. Newer therapeutic approaches are urgently needed in these diseases that cause significant morbidity and mortality. These future modalities may include biologic mediators, monoclonal and antiidiotypic antibodies, cytokines, peptides, or autoantigen therapy.

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43 THE SPONDYLOARTHROPATHIES

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The spondyloarthropathies are a group of disorders that have certain clinical features in common and are all associated with the expression of the human leukocyte antigen (HLA)-B27 gene product (1,2,3,4,5,6,7 and 8). The similar clinical manifestations and genetic association suggest that these conditions are related and may share common pathogenic mechanisms. The spondyloarthropathies include ankylosing spondylitis, Reiter syndrome (reactive arthritis), the arthritis associated with inflammatory bowel disease, the spondylitis associated with psoriasis, juvenile-onset spondyloarthropathy, and the less clearly defined undifferentiated spondyloarthropathy. The prototypic spondyloarthropathies are on the one hand, ankylosing spondylitis, which is a chronic condition characterized by an inflammation of joints and periarticular tissues of the axial skeleton, and on the other hand, Reiter syndrome, which is a more acute inflammatory process involving primarily peripheral joints. Both conditions, as well as the other spondyloarthropathies, are notable for the tendency of those affected to develop inflammation of sacroiliac joints and a variety of extraarticular manifestations.

The spondyloarthropathies have affected human beings since antiquity, with evidence of disease noted in ancient Egyptian mummies (9). There is strong evidence that a condition similar to the spondyloarthropathies also affects nonhuman primates, including apes and Old World monkeys (10). The first clear descriptions of ankylosing spondylitis as a distinct clinical entity date from the latter part of the 19th century, whereas reactive arthritis was first described in the 18th century (11). A number of prominent historical figures, including Christopher Columbus and Benvenuto Cellini, are thought to have had reactive arthritis (12,13).

CLASSIFICATION AND DISEASE DEFINITION

The distinguishing features of the spondyloarthropathies include (a) sacroiliitis with or without associated spondylitis, (b) inflammatory asymmetric arthritis, (c) association with HLA-B27, (d) ocular inflammation, and (e) absence of autoantibodies associated with other rheumatic diseases.

The development of a set of criteria with which to capture the diverse clinical spectrum of the spondyloarthropathies has been challenging. The European Spondyloarthropathy Study Group (ESSG) proposed classification criteria for all patients with spondyloarthropathies (Table 43.1). These criteria exhibit a sensitivity of 86% and a specificity of 87% (14). The so-called Amor criteria encompass a somewhat wider spectrum of clinical features than the ESSG criteria, and include the presence of HLA-B27 as a criterion (15). The ESSG had found that inclusion of HLA-B27 did not improve the overall performance of the criteria set. These two broad-based spondyloarthropathy criteria sets have been compared in populations of patients with definite spondyloarthropathies, and both perform well (14). Their performance in heterogeneous cohorts of patients with a recent onset of musculoskeletal symptoms is less certain.

One of the following:
- A. Inflammatory back pain
- B. Synovitis
  - 1. Asymmetric
  - 2. Predominantly involving the lower extremities

Plus any one of the following:
- A. Family history
- B. Psoriasis
- C. Inflammatory bowel disease
- D. Urethritis, cervicitis, or acute diarrhea within 1 month before arthritis
- E. Alternate buttock pain
- F. Enthesopathy
- G. Sacroiliitis

TABLE 43.1. Criteria for Classification of Spondyloarthropathy Proposed By the European Spondyloarthropathy Study Group

Ankylosing Spondylitis

Criteria for the diagnosis of ankylosing spondylitis have been established (16). Although the diagnosis of long-standing disease with typical deformities is straightforward, diagnosis of early disease before the development of irreversible deformity can be difficult. Currently, the widely used criteria for diagnosis include the following: (a) a history of inflammatory back pain, (b) limitation of motion of the lumbar spine in both the sagittal and frontal planes, (c) limited chest expansion, and (d) definite radiographic evidence of sacroiliitis. The presence of radiographic sacroiliitis and any one of the other three criteria is sufficient to establish a diagnosis of definite ankylosing spondylitis (Table 43.2).

A. Criteria
- 1. Clinical
  - a. Low back pain and stiffness for >3 months, which improves with exercise but is not relieved by rest
  - b. Limitation of motion of the lumbar spine in both the sagittal and frontal planes
  - c. Limitation of chest expansion relative to normal values corrected for age and sex
- 2. Radiologic
  - a. Sacroiliitis grade ≤2 bilaterally or sacroiliitis grade 3–4 unilaterally

B. Diagnosis
- 1. Definite ankylosing spondylitis if the radiologic criterion is associated with at least one clinical criterion
- 2. Probable ankylosing spondylitis if
  - a. Three clinical criteria are present
  - b. The radiologic criterion is present without any signs or symptoms satisfying the clinical criteria

Radiologic grading of sacroiliitis: 0, normal; 1, suggestive; 2, minimal abnormality (small localized areas of erosion or sclerosis without alteration in the joint width); 3, unequivocal abnormality (erosions, sclerosis, change in joint width or partial ankylosis); 4, severe abnormality (total ankylosis).

TABLE 43.2. Modified Diagnostic Criteria for Ankylosing Spondylitis

A group of individuals has been described who are HLA-B27 positive and manifest typical symptoms of ankylosing spondylitis but who lack definite radiographic evidence of sacroiliitis (17,18). In many of these patients, radiographic changes of sacroiliitis eventually develop. However, diagnosis of these patients can be difficult, because they may not meet the criteria for classification early in the disease course. The use of more sensitive imaging modalities such as computerized tomography and magnetic resonance imaging may enhance the ability to diagnose sacroiliitis in such patients. Although more than 90% of patients with ankylosing spondylitis express HLA-B27, testing for the presence of this HLA allele is useful only as an adjunct to diagnosis, as B27 is not present in all patients, and only a very small percentage of B27-positive patients will have ankylosing spondylitis. Documentation of the presence of B27 is most helpful in patients with early disease in whom radiographic evidence of sacroiliitis has not yet developed.

Ankylosing spondylitis must be differentiated from other conditions that cause low back pain. The inflammatory back pain of ankylosing spondylitis is usually distinguished by the following features: (a) age at onset younger than 40 years, (b) insidious onset, (c) duration longer than 3 months before medical attention is sought, (d) morning stiffness, and (e) improvement with exercise or activity. The most common causes of back pain other than ankylosing spondylitis are primarily mechanical or degenerative rather than inflammatory, and do not manifest these features. It is important to note that the noninflammatory causes of back pain are substantially more prevalent than is ankylosing spondylitis, and indeed may coexist in patients with this diagnosis.

Reiter Syndrome

The classic triad of Reiter syndrome consists of arthritis, urethritis, and conjunctivitis. However, less stringent criteria for the classification of Reiter syndrome have been established by the American College of Rheumatology (ACR) because as many as two thirds of patients who appear to have Reiter syndrome do not manifest the classic triad (11). ACR criteria require the presence of peripheral arthritis of more than 1 month's duration, occurring in association with urethritis, cervicitis, or both (19). The ACR criteria exhibit a sensitivity of 84.3% and a specificity of 98.2% when used to delineate patients with Reiter syndrome from those with ankylosing spondylitis, psoriatic arthritis, seronegative rheumatoid arthritis (RA), and gonococcal arthritis. However, these criteria have not been universally accepted because a substantial number of patients, especially at the onset of disease, do not meet ACR criteria or exhibit the diagnostic triad (5,20). Attempts have been made to develop means to identify and classify those patients who exhibit lower extremity or axial inflammatory arthropathy and are often HLA-B27 positive, but who fail to meet the ACR criteria for Reiter syndrome. Proposed categories have included “incomplete Reiter” (21), “undifferentiated” (18) or “overlap” spondyloarthropathy (22), “sexually acquired reactive arthritis” (SARA) (23), “BASE syndrome” (B27, arthritis, sacroiliitis, and extraarticular inflammation) (24), and the hereditary multifocal, relapsing inflammation (HEMRI) syndrome (25). The clinical usefulness, validity, and prognostic value of these categorizations have not been proven. Indeed, a review of the published literature on “Reiter syndrome” and “reactive arthritis” indicated that 22.5% of the studies did not use any published criteria for this diagnosis (26).

EPIDEMIOLOGY

Ankylosing Spondylitis

Family studies have shown a very strong genetic component to all of the spondyloarthropathies, this being the highest for ankylosing spondylitis. The degree of disease clustering in families can be estimated by the risk ratio known as I_s (the disease frequency in relatives of affected individuals divided by the disease frequency in the general population). The value for I_s for ankylosing spondylitis is estimated to be approximately 50, with most of this genetic risk being in the major histocompatibility complex (MHC) (27). Ankylosing spondylitis shows a striking correlation with the histocompatibility antigen HLA-B27 (2,7) and occurs worldwide in approximate proportion to the prevalence of B27 in the population (28,29 and 30). In most of the populations studied, more than 90% of patients with ankylosing spondylitis are HLA-B27 positive. In the North American white population, ankylosing spondylitis has been estimated to have a prevalence of approximately 0.1%, whereas HLA-B27 has a prevalence of approximately 7%. Population surveys have suggested that 1% to 2% of adults inheriting HLA-B27 have ankylosing spondylitis (30,31). In contrast, in families of patients with ankylosing spondylitis, 10% to 20% of adult first-degree relatives inheriting HLA-B27 have the disease (32,33). Interestingly, a study comparing HLA-B27–positive and –negative blood donors in Germany found that 19 (13.6%) of 140 HLA-B27–positive blood donors had a clinically detectable spondyloarthropathy, nine of whom had ankylosing spondylitis (34). This study estimated the prevalence of ankylosing spondylitis to be 0.85% in the overall Berlin population, and 6.4% in HLA-B27–positive individuals. A similar risk was defined in Norway (35). In contrast to the 5% to 10% prevalence rate of HLA-B27 in white populations, several Native North American Indian tribes have a much higher prevalence of this HLA antigen (36). Indeed, approximately 50% of the Haida Indians living on the west coast of Canada are estimated to be HLA-B27 positive, and 10% of the adult population is estimated to have sacroiliitis (37). The prevalence of HLA-B27 is generally quite low in Asian and African populations, as is the prevalence of ankylosing spondylitis (30).

The presence of HLA-B27 does not appear to be related to the severity of the clinical manifestations of ankylosing spondylitis, although there has been some suggestion that HLA-B27 is associated with more severe radiographic disease (34). HLA-B27–negative ankylosing spondylitis is usually somewhat later in onset, is less frequently associated with acute anterior uveitis, is more frequently associated with psoriasis or inflammatory bowel disease, and is less frequently aggregated in families (38,39 and 40). It was recently shown that HLA-B39 has an increased prevalence in HLA-B27–negative patients with spondyloarthropathies in Japan (41).

Ankylosing spondylitis is uncommon before puberty, but then increases in frequency to reach a peak during young adulthood (33,42). In sporadic cases, male outnumber female subjects by about 5:1 (43,44). In contrast, in families with involvement of first-degree relatives, the male predominance is much less (1.5:1). Similarly, the aggregation of cases is greater in families with affected female members (45). These results imply that age and sex play permissive roles in the development of ankylosing spondylitis.

Reiter Syndrome

Estimates of the prevalence or incidence of Reiter syndrome are difficult to determine because of the lack of consensus regarding diagnostic criteria, the underreporting of venereal disease, and the asymptomatic or milder course in affected women. Despite these restrictions, epidemiologic information is available from military populations in which Reiter syndrome is the most common cause of inflammatory arthritis (8). Results from epidemics of dysentery in the military suggest that the incidence of Reiter syndrome is approximately four cases per 1,000 soldiers per year (46). Other studies of epidemic dysentery secondary to arthritogenic bacterial strains suggest that Reiter syndrome develops in 2% to 3% of infected individuals, whereas in nearly 20% of the HLA-B27–positive infected individuals, arthritis may develop (1,47,48). Similarly, in 1% to 3% of patients with nongonococcal urethritis secondary to infection with *Chlamydia trachomatis* a chronic arthritis will develop (4). Finally, a report from Rochester, Minnesota, identified 16 cases of Reiter syndrome between 1950 and 1980. The age-adjusted incidence rate for male patients younger than 50 years was 3.5 cases per 100,000 and was comparable to the incidence of RA (3.0 per 100,000) (49).

Reiter syndrome is a disease of young people, with its peak onset during the third decade of life. However, it also has been reported to occur in children and the aged (4,5). Male subjects are most commonly affected, with some estimates suggesting a male-to-female ratio of more than 25:1 (4,49). However, the male predominance is likely to be overestimated because Reiter syndrome in women may be associated with occult genitourinary disease. It was shown that 9% of United States female military recruits had evidence of occult genitourinary disease (50). Moreover, disease expression is usually less severe in women (4,51). Recent estimates suggest that the male/female ratio for Reiter syndrome is closer to 5:1 or 6:1 (1,4,20,21). Postvenereal Reiter syndrome is more common in men, whereas postdysenteric Reiter syndrome affects men and women equally (4,48).

HLA-B27 is found in 50% to 80% of patients with Reiter syndrome, with variations related to the frequency of the gene in the population at risk, and the organism involved (1,4,52). Thus 75% to 80% of white patients express this antigen, whereas it is seen in less than 50% of African-Americans with Reiter syndrome. Approximately 50% of patients with *Salmonella-, Campylobacter-,* and *Chlamydia*-induced Reiter syndrome are HLA-B27 positive; whereas 60% to 80% of patients with *Yersinia* and *Shigella* are positive for this antigen. HLA-B27 not only confers disease susceptibility but also seems to influence disease severity and expression. Patients with Reiter syndrome who are HLA-B27 positive are more likely to develop more prolonged and chronic disease (53). They also exhibit an increased frequency of axial involvement and systematic manifestations, such as fever, uveitis, balanitis, and oral ulcerations. These manifestations tend to be severe (25,53). In patients with psoriasis or inflammatory bowel disease, the presence of HLA-B27 or one of the cross-reactive MHC antigens, such as HLA-B7, appears to predispose to the expression of axial arthropathy (1). Likewise, arthropathies related to acquired immunodeficiency syndrome (AIDS) tend to be more severe in HLA-B27–positive individuals (54,55).

Despite the clear indication that HLA-B27 plays an integral role in the pathogenesis of Reiter syndrome, additional features of the host appear to contribute to disease susceptibility. There is a relatively low attack rate of Reiter syndrome in HLA-B27–positive individuals. Thus in epidemics of dysentery caused by known arthritogenic bacteria, only 20% or fewer of the HLA-B27–positive infected people will develop an incomplete syndrome, whereas even fewer will develop classic Reiter syndrome (1,4). Moreover, in some epidemics of dysentery with arthritogenic bacteria, the majority of Reiter syndrome has been noted in individuals who do not express HLA-B27 (56). Additionally, the observation that postvenereal Reiter syndrome predominantly occurs in men, despite B27 status, suggests that gender, as well as HLA-B27,

plays a role in its pathogenesis. Reiter syndrome is most frequent in early adulthood, with a lower incidence in other age groups, suggesting that maturational status also plays a role in disease susceptibility. Finally, studies of monozygotic HLA-B27–positive twins showed less than 50% concordance (57), indicating that host features besides this MHC allele play a role in disease pathogenesis. Epidemiologic studies of specific populations also have supported the conclusion that genetic influences other than HLA-B27 play a role in disease susceptibility. Thus, the Haida and Pima Indians have a higher frequency of HLA-B27 expression and an increased incidence of ankylosing spondylitis; however, Reiter syndrome is distinctly uncommon in these populations (58,59) but is quite common in Navajo and Eskimo populations (22). The explanations for these findings are unclear but indicate that characteristics of the target population other than B27 play a pivotal role in disease susceptibility. Despite this, the clear-cut association of HLA-B27 with the development of the spondyloarthropathies indicates a central role of this gene product in the pathogenesis of these conditions. It has been recently proposed that reactive arthritis be grouped into two categories, one that includes Reiter syndrome and is highly associated with B27, and a second that is not associated with B27. Only the former would be considered a spondyloarthropathy (60).

HLA-B27

Class I molecules of the MHC are 44-kd polymorphic proteins that are noncovalently associated with an invariant protein, b_2-microglobulin, and expressed as a heterodimer on the surface of many cell types (61). Allelic variations in the amino acids of these molecules have classically been identified by antisera. HLA-B27 is a serologically defined allele of the HLA-B locus, one of the three classic loci encoding class I MHC molecules (62). Examination of HLA-B gene products that react with HLA-B27 typing alloantisera has revealed a family of allelic subtypes. Twelve of these have been designated by the World Health Organization HLA Nomenclature Committee as *HLA-B*2701* through *HLA-B*2712* (63,64 and 65). This order is based on charge, determined by isoelectric focusing and not frequency. The prevalence of these gene products in various populations is shown in Table 43.3. *HLA-B*2705* is the predominant subtype in all populations. About 90% of HLA-B27 whites are *B*2705.* The other subtypes of B27 appear to have arisen from *B*2705* by point mutations (64). The amino acids that vary among these different subtypes are shown in Table 43.4.

Subtype	AS Association	Distribution
*B*2701*	Not known	Whites (rare)
*B*2702*	Yes	Whites (10%–15%)
*B*2703*	Yes	Blacks (west Africans)
*B*2704*	Yes	Asians (50%–70%)
*B*2705*	Yes	Whites (85%–90%) Asians (20%–50%) Amerindians (80%–100%)
*B*2706*	No	Asians (rare)
*B*2707*	Yes	Rare
*B*2708*	Not known	Rare
*B*2709*	No	Rare
*B*2710*	Yes	Rare
*B*2711*	Not known	Rare
*B*2712*	Not known	Rare

AS, ankylosing spondylitis.

TABLE 43.3. Hla-B27 Subtypes and Association with Ankylosing Spondylitis

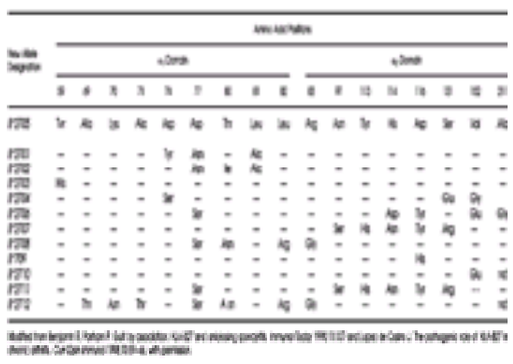

TABLE 43.4. Nomenclature and Amino Acid Differences of the Hla-B27 Subtypes

An association between many, but not all, of the HLA-B27 subtypes and the spondyloarthropathies has been demonstrated. This association has been most comprehensively analyzed for ankylosing spondylitis, as shown in Table 43.3. A number of the HLA-B27 subtypes, including *B*2701*and *B*2707* have been found only in a few individuals (63,66), and thus have not been amenable to population studies. However, *B*2701* was originally identified in an individual with advanced juvenile-onset ankylosing spondylitis (67). In general, the distribution of these B27 subtypes in patients with ankylosing spondylitis does not differ from that in healthy controls. Although it was initially shown that there was not an association between *HLA-B*2703* and ankylosing spondylitis in African blacks from Gambia (68), more recently this subtype was shown to be associated with this disease in West Africans (69). This is of interest because *HLA-B*2703* is a very unusual HLA-B molecule, containing a histidine at position 59, rather than the tyrosine that is present at this position in all other HLA class I molecules. Despite the unusual structure of *HLA-B*2703,* there appears to be an association with ankylosing spondylitis. Also, *HLA-B*2706*, an Asian subtype that was not associated with disease in Thailand (70), was subsequently detected in three Chinese patients with ankylosing spondylitis (69). With the possible exception of *HLA-B*2709*, it is likely, therefore, that ankylosing spondylitis is associated with each of the other HLA-B27 subtypes. Whether all HLA-B27 alleles are also associated with the other spondyloarthropathies is currently unknown but appears to be a likely possibility.

Transgenic animal models have provided strong evidence confirming that HLA-B27 itself is a direct disease-susceptibility factor, not merely a marker for a disease-susceptibility gene in close linkage disequilibrium with HLA-B27. Previously, indirect evidence from clinical epidemiology strongly suggested that HLA-B27 itself was the disease-susceptibility gene (71). Direct evidence that the HLA-B27 molecule itself can predispose to the spondyloarthropathies has come from studies of transgenic rats expressing *HLA-B*2705* and human b_2-microglobulin. These animals spontaneously develop a broad spectrum of disease manifestations closely resembling human HLA-B27–associated disease (72). These features include peripheral arthropathy; gastrointestinal, genitourinary tract, and cutaneous inflammation; and spondylitis. Moreover, HLA-B27 transgenic mice also have been shown to develop spontaneous inflammatory arthritis, in the absence of human b_2-microglobulin (73). These findings support the conclusion that the HLA-B27 molecule itself is the disease-susceptibility factor in the spondyloarthropathies. Therefore the association of the HLA-B27 molecule and the susceptibility to the development of spondyloarthropathy is likely to relate to one or more unique features of the structure or function of this class I MHC molecule.

Structure

The three-dimensional structure of three HLA class I molecules, HLA-A2, HLA-Aw68, and*B*2705*, has been determined by x-ray crystallography (74,75,76 and 77). The structure of HLA-B27 appears to be generally similar to that of HLA-A2 and -Aw68. The extracellular domains of these molecules consist of a membrane proximal immunoglobulin (Ig)-like structure encoded by the a_3 domain of the molecule and a receptor-like peptide-binding domain encoded by the polymorphic a_1 and a_2 domains. The a_3 domain is similar in all class I MHC molecules and is involved in the association with b_2-microglobulin. The a_1 and a_2 domains are folded into a peptide-binding cleft, with a helical regions from each domain creating the two walls and antiparallel b pleated sheets from these two domains forming the floor (Fig. 43.1). One unique feature of HLA-B27 relates to the fact that the peptide-binding cleft of this molecule is closed at both ends, creating a concave surface. This configuration has been hypothesized to have a role in the formation of tetrameric class I molecules on the cell surface (78). The unique features of each HLA-A, -B, and -C allele are, in general, determined by the amino acids of the a_1 and a_2 domains of the molecule that appear to alter the peptide-binding capacity of the molecule. Most of the polymorphic amino acid residues in class I MHC molecules are clustered along this cleft, projecting into it either from the two a helices or from the floor of the b pleated sheet. Pockets (designated A ® F) project from the cleft under the a helices (77,79). These pockets are created by amino acid residues outside the peptide-binding cleft itself. These pockets also are thought to play an important role in peptide binding. For HLA-B27, an acidic, hydrophilic pocket projecting under the superior a helix to residue 45, the so-called 45 pocket or B pocket, is thought to play a critical role in the binding of peptide (79).

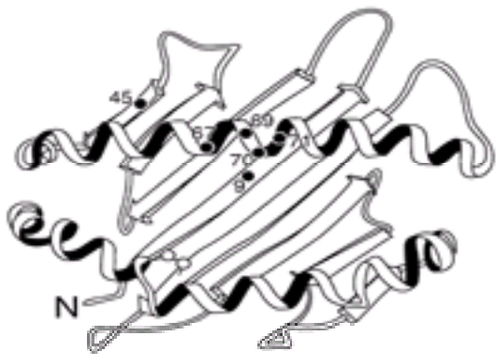

Figure 43.1. Structure of human leukocyte antigen (HLA)-B27. Highlighted are the amino acids thought to be involved in the binding of antigenic peptides. N, amino terminus. (Modified from Rothschild BM, Woods RJ. Spondyloarthropathy as an Old World phenomenon. *Semin Arthritis Rheum* 1992;21:306–316, and Chen JH, et al. A *Yersinia pseudotuberculosis* protein which cross-reacts with HLA-B27. *J Immunol* 1987;139:3003–3011, with permission.)

Class I MHC molecules bind antigenic peptides, largely derived from intracellular protein synthesis (80). Such peptides, which are generated from partial proteolytic cleavage of endogenously synthesized proteins, bind to class I MHC molecules in the endoplasmic reticulum and are transported, along with b_2-microglobulin, as a trimolecular complex to the cell surface. A number of studies have indicated that peptides consisting of nine amino acids are bound by class I MHC molecules (81,82 and 83). The amino acid sequences of peptides eluted from the HLA-B27 molecule suggest that they may potentially be derived from abundant cytosolic or nuclear proteins, such as histone, ribosomal proteins, and members of the 90-kd heat-shock protein family (82). The amino acids of the polymorphic regions of the a_1 and a_2 domains of class I MHC molecules are primarily responsible for the capacity of different class I MHC molecules to bind various peptides. After reaching the cell surface, endogenously synthesized peptide bound in the peptide-binding cleft of class I MHC molecules is presented to $CD8^+$ T lymphocytes expressing specific clonally distributed ab antigen receptors capable of recognizing the combination of polymorphic MHC molecule plus antigenic peptide (Fig. 43.2) (80).

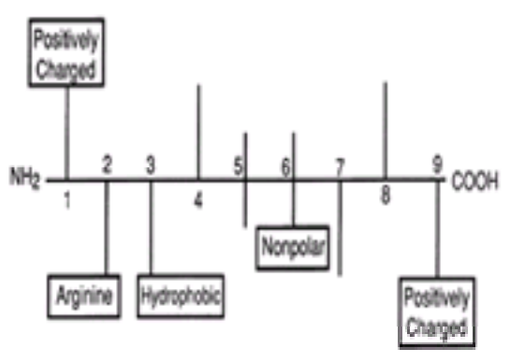

Figure 43.2. Generic structure of peptides that bind human leukocyte antigen (HLA)-B27. Nonamers with the indicated structure have been identified in the peptide-binding groove of HLA-B27. Amino acids that are pointed downward have their side chains directed into the pockets of the HLA-B27 molecule, whereas amino acids pointed upward have their side chains pointed out of the HLA-B27 peptide-binding groove. Amino acids that are centered have their side chains lying across the peptide-binding groove. (Modified from Jardetzky TS, et al. Identification of self peptides bound to purified HLA-B27. *Nature* 1991;353:326, with permission.)

The known HLA-B27 subtypes differ at specific amino acids, which are likely to be involved in the binding of specific antigenic peptides (Fig. 43.1). Of importance is the observation that susceptibility to the spondyloarthropathies does not appear to be related to the portions of the molecules that differ among the various B27 subtypes; therefore the residues that differ among the B27 subtypes are not likely to be involved in binding an arthritogenic peptide. The observation that the single amino acid change in *HLA-B*2703* (Tyr ® His at position 59) alters susceptibility to the spondyloarthropathies in some populations suggests that this residue could be important in disease pathogenesis. However, because all other class I MHC molecules possess a Tyr at position 59, it is unlikely that this amino acid plays a role in disease susceptibility. Rather, it is possible that the amino acid substitution at this position distorts the molecule in a manner to make other HLA-B27–specific amino acids unavailable for peptide binding. However, presentation of all peptides is not precluded by the change of Tyr to His at position 59, because *B*2705*-restricted, influenza-specific, cytotoxic T cells recognize infected *B*2703* cells (84). Binding of putative arthritogenic peptides, however, might be precluded by this nonconservative change in position 59 of the molecule.

Comparison of the HLA-B27 subtypes with other HLA class I sequences indicates that one amino acid residue is unique to all of the B27 subtypes (66,85,86). This is the Lys at position 70, located in the a helix formed by the a_1 domain of the molecule (Fig. 43.1). The Lys at position 70 points into the peptide-binding cleft, and, therefore, is a candidate for involvement in peptide binding. The consensus HLA-B27 sequence also shares a cluster of six amino acids within the peptide-binding cleft, including the HLA-B27 unique residue Lys70, as well as His9, Glu45, Cys67, Ala69, and Ala71. Besides HLA-B27, no other known class I HLA sequence possesses more than two of these residues. It is likely, therefore, that this portion of the HLA-B27 molecule is the disease-susceptibility element. Because these amino acids are arrayed in the antigen-binding cleft and the “45 pocket” of the molecule, it is reasonable to conclude that disease susceptibility relates to the unique capacity of these amino acids to permit HLA-B27 to bind a peptide capable of triggering or propagating the disease. The identity of such an arthritogenic peptide remains to be delineated.

A number of peptides that bind to HLA-B27 have been identified (47,65,82,87,88). In general, these peptides are positively charged and nine amino acids in length. Although an Arg residue at position 2 (P2) extending into the “45 pocket” is the best anchor for the binding of the peptides, other P2 residues such as Gln2 (65,89), as well as others (88) have been shown to be permissive for peptide binding. Other features of these peptides are shown in Fig. 43.2 and define a motif of B27-binding peptides. Because the carboxy-terminal residues interact with HLA-B27 “pockets” that feature more subtype polymorphism, it is less likely that they are relevant to disease predisposition. Interestingly, peptide ligands that bind the nonsusceptible *B*2706* (90) and *B*2709* (91) subtypes lack a carboxy-terminal tyrosine motif compared with peptides that bind susceptible subtypes such as *B*2705* and *B*2704*. Whether these differences are relevant to disease pathogenesis is still unclear. It should be pointed out that an estimated 13 million peptides might bind into the groove of HLA-B27.

Function: Peptide Binding and T-Cell Repertoire Selection

The biosynthesis of HLA-B27 is likely to be similar to that of other class I HLA molecules. The 44-kd heavy chain is synthesized in the endoplasmic reticulum, where it undergoes association with the invariant 11.5-kd b_2-microglobulin protein (92,93). Posttranslational modification of the molecule occurs in the Golgi, from which it is transported to the cell surface; there it is expressed as an integral transmembrane surface protein by most nucleated cells (61). Under physiologic circumstances, intracellularly synthesized and processed antigenic peptides become bound in the class I MHC–binding cleft within the endoplasmic reticulum. In most circumstances, the heavy chain, b_2-microglobulin, and antigenic peptide form a trimolecular complex within the endoplasmic reticulum that permits stable association on the cell surface (94). Interestingly, mice that are transgenic for human HLA-B27, but lack b_2-microglobulin, develop a spontaneous inflammatory arthritis with many of the features of spondyloarthropathies. As with the HLA-B27 transgenic rats, the arthropathy in these mice develops only after transfer from a pathogen-free environment. The fact that the mice do not express HLA-B27 on the cell surface has suggested that aberrant assembly, transport, and expression of HLA-B27 may play a role in the pathogenesis of human spondyloarthropathies (95).

Most peptides that are presented by class I MHC molecules are synthesized within the antigen-presenting cell and are encoded either by endogenous genes or by intracellular viruses or bacteria (80,96). It is the peptide/MHC molecular complex expressed on the cell surface that is recognized by $CD8^+$ T lymphocytes (80). Class I MHC molecules expressed within the thymic cortex also influence the T-cell repertoire by exerting both positive and negative selective influences on the $CD4^+$ $CD8^+$ thymocyte precursors of mature T cells (97). Thus class I MHC molecules play a dual role with regard to antigen recognition by T cells. On the one hand, they serve to select the T-cell receptor (TCR) repertoire of $CD8^+$ T cells in the thymus, and on the other, they function to bind and present antigen to $CD8^+$ T cells in peripheral tissues.

HLA-B27 and Molecular Mimicry

Several hypotheses can be constructed to account for the role of HLA-B27 in the pathogenesis of spondyloarthropathies. Although the most straightforward hypothesis is that HLA-B27 acts to bind a microbially derived peptide and that B27-restricted recognition of such a peptide leads to the pathogenic consequences recognized clinically as spondyloarthropathy, there are other potential hypotheses. One invokes the concept of molecular mimicry, which suggests that a small region of a human antigen is identical to amino acid sequences of proteins encoded by the triggering microorganism (98,99). Mimicry may be of a structural gene product or the HLA-B27 molecule itself (90,100,101). Although it is not clear how mimicry of a class I MHC molecule would lead to the anatomically localized disease characteristic of the spondyloarthropathies, there is considerable evidence for the sharing of antigenic determinants between HLA-B27 and different bacterial products.

Monoclonal antibodies against HLA-B27, B27M1, and B27M2 react with bacterial cell envelope glycoproteins from *Shigella flexneri, Klebsiella pneumoniae*, and *Yersinia enterocolitica* O:9 (102). Similarly, B27M2 identifies two cross-reactive proteins of 80 kd and 60 kd from *K. pneumoniae* (103). Another monoclonal antibody, Ye-2, derived from a mouse immunized with both HLA-B27–positive cells and with *Yersinia pseudotuberculosis*, recognizes a 19-kd envelope protein from *Y. pseudotuberculosis* as well as HLA-B27 itself (104). The monoclonal antibody failed to react with several other bacteria, including arthritogenic *Salmonella* and *Shigella* strains. Both B27M1 and B27M2 identify molecules of 23 and 36 kd from several bacterial species, including *S. flexneri, Shigella sonnei, Salmonella typhimurium, K. pneumoniae*, and *Escherichia coli* (105). Moreover, monoclonal antibodies against an isolate of *S. flexneri* cross-react with cells bearing HLA-B27 and -B7 (106). The antibodies detected bacterial antigens of 36 and 19 kd. Monoclonal antibodies against a peptide derived from amino acids 63 through 83 of *HLA-B*2705* were noted to bind HLA-B27–positive and HLA-B7–positive cell lines and appeared to bind the class I heavy chain as well as the 36- and 19-kd bacterial proteins (107). These results imply that the a_1 polymorphic region of HLA-B27 is antigenically cross-reactive with bacterial envelope proteins. However, amino acid sequencing of these bacterial proteins has indicated that there is no homology with the HLA-B27 molecules; therefore cross-reactivity is explained by the presence of similar conformational determinants or chance occurrence of particular amino acids. Inasmuch as $CD8^+$ T cells recognize linear sequences of amino acids, the significance of these antibody cross-reactivities remains unclear.

Computer searches have identified bacterial proteins with amino acid sequence homology to HLA-B27 (101,108). The nitrogenase reductase enzyme in *K. pneumoniae* contains a six–amino acid region that is homologous with *HLA-B*2705* residues 72 through 77. Moreover, a rabbit antipeptide antiserum was shown to react with intact HLA-B27, a synthetic peptide of HLA-B27 amino acids 63 through 84, the intact *K. pneumoniae* nitrogenase protein, and a synthetic peptide derived from the *K. pneumoniae* protein (98). As many as 54% of patients with reactive arthritis and 30% of patients with ankylosing spondylitis were claimed to have elevated levels of antibodies against an HLA-B27–derived peptide containing the region of homology with *K. pneumoniae* (108,109), although other investigators found such antibodies only rarely in patients with ankylosing spondylitis (110). T-cell reactivity to these peptides has not been examined. The results clearly indicate that there is amino acid sequence homology between HLA-B27 and the *K. pneumoniae* nitrogenase reductase gene product, but its relevance to disease pathogenesis is not at all clear.

Whereas several species of bacteria are capable of triggering reactive arthritis, not all isolates within a bacterial species are arthritogenic. From this observation, it was reasoned that the factor conferring arthritogenicity to a given bacterium might be a mobile genetic element, such as a plasmid. A 2-Md plasmid, termed *pHS-2*, was identified in several arthritogenic *S. flexneri* isolates, but was absent from nonarthritogenic *Shigella* (111). The nucleotide sequence of this plasmid revealed a predicted open reading frame encoding a 22-amino acid peptide potentially encoding an eight-amino acid stretch (Ala-Gln-Thr-Asp-Arg-His-Ser-Leu), seven residues of which are identical to residues 71 through 78 of the *HLA-B*2704* and *HLA-B*2706* sequences (Ala-Gln-Thr-Asp-Arg-Glu- Ser-Leu). Residues 1 through 5 and 8 of this sequence are shared by all known HLA-B27 subtypes; residue 77, this sequence corresponds to HLA-B27 residue 77, which is the most variable residue among the HLA-B27 subtypes (Table 43.4). Although the significance of this homology remains to be determined, antibodies showing high-affinity binding to the pHS-2–derived peptide, but not to the *K. pneumoniae* nitrogenase peptide, have been found in patients with ankylosing spondylitis or Reiter syndrome (112). The antibodies cross-reacted with both an HLA-B27–derived peptide and with the native HLA-B27 molecule. T-cell reactivity to this peptide has not been measured.

More recently, two independent groups isolated an RRYLENGKETL peptide from B^*2701, B^*2704, and B^*2706, a sequence that spans residues 169 to 179 of HLA-B27 (90,113). As with a very similar peptide previously identified (101), the peptide identified in these studies was demonstrated to have sequence homology with bacterial peptides. Although it can be postulated on the basis of these data that this B-27–derived peptide that mimics peptides derived from disease-associated enterobacteria can break self-tolerance, there was no correlation in these studies between presentation of this peptide and subtype association with ankylosing spondylitis. As in the case of the association with the *K. pneumoniae* nitrogenase reductase gene product, the relevance of this association to disease pathogenesis remains speculative.

ROLE OF T CELLS IN THE PATHOGENESIS OF THE SPONDYLOARTHROPATHIES

The role of antigen-specific and autoreactive T lymphocytes in the pathogenesis of spondyloarthropathies has been extensively explored, primarily in the context of reactive arthritis, in which the spectrum of antigens derived from inciting pathogens is better defined. The peripheral T-cell responses to the triggering microorganism appear to be impaired in reactive arthritis (114,115). These patients also tend to have persistent IgA antibodies to the organism (115,116 and 117). In B27-positive ankylosing spondylitis patients, the peripheral blood T-cell responses to *K. pneumoniae* were found to be impaired compared with B27-positive healthy controls (118,119). In general, though, the specificity and characteristics of the peripheral blood lymphocytes in reactive arthritis and ankylosing spondylitis have not been found to be either distinctive or informative. In contrast, the analysis of synovial tissue and synovial fluid lymphocytes has been much more informative. Both $CD4^+$ and $CD8^+$ T cells isolated from the joints of patients with reactive arthritis have been shown to respond specifically to antigens derived from the inciting microorganisms and, as has recently been shown, some clones of synovial $CD4^+$ and $CD8^+$ T cells derived from the same joint appear to respond to the same bacterial sequence (120).

$CD4^+$ T cells

The involvement of antigen-specific, class II restricted $CD4^+$ cells in the joints of patients with reactive arthritis has been demonstrated by a number of groups (120,121 and 122). Bacterial heat-shock proteins, particularly hsp60, are immunodominant antigens in these responses (123,124 and 125). A second group of immunodominant antigens includes positively charged proteins such as the *Yersinia* urease b-subunit and ribosomal protein L23 (126,127), and *Chlamydia* histone-like protein HCL (124). It is possible that these cationic proteins are bound to negatively charged structures in the joint, such as proteoglycans in the synovial lining layer and the cartilage. In ankylosing spondylitis, one study demonstrated the presence of synovial T-cell clones specifically recognizing *K. pneumoniae* antigens (118).

The cytokine patterns of both mouse and human $CD4^+$ T cells have been broadly classified as Th1 [interferon (IFN)-g] or Th2 (IL-4, IL-10). Th1 cytokines are associated with cell-mediated cytotoxicity and elimination of intracellular bacteria, whereas Th2 cytokines mediate humoral immune responses. The ratio of Th1 to Th2 responses has been explored in the synovial $CD4^+$ T cells of patients with reactive arthritis. One group of investigators who have focused on this question initially demonstrated a predominantly Th1 pattern of cytokine expression by synovial fluid T cells from patients with *Chlamydia*-induced reactive arthritis (128). Subsequently this group showed that the synovial membrane T cells from a spectrum of reactive arthritis patients tended to produce more Th2 cytokines (129,130). A particularly important role for IL-10 was noted, and it was proposed that the presence of high levels of this cytokine favors intraarticular persistence of the pathogens. It is not clear whether the discrepancies in the studies reflect differences between synovial fluid and synovial tissue T cells in various forms of reactive arthritis, or whether this simply reflects sampling bias.

$CD8^+$ T Cells

The association of HLA-B27 with the spondyloarthropathies implies a role for $CD8^+$ T cells in the pathogenesis of these conditions, as the only known functions of the polymorphic portions of class I MHC molecules are selection of the TCR repertoire of $CD8^+$ T cells in the thymus and presentation of antigen to $CD8^+$ T cells in the periphery. Bacteria-specific B27-restricted cytolytic T lymphocytes (CTLs) have been detected in the synovial fluid of ankylosing spondylitis and reactive arthritis patients (131,132 and 133). CTLs that were isolated and expanded from a number of patients with *Yersinia* reactive arthritis all reacted to a single nonamer peptide (321–329) derived from *Yersinia* hsp60, and the peptide required HLA-B27 for presentation (131). Subsequently it was shown that there are highly conserved amino acid sequences in the CDR3 region of the synovial T cells of reactive arthritis patients, despite differences in Vb. chain use (134). A $CD8^+$ synovial cell line expressing one of the shared clonotypes reacted toward several B^*2705 lymphoblastoid cell lines. Together these studies strongly suggest that $CD8^+$ T lymphocytes are responding to bacterial antigens such as hsp60 presented in the context of HLA-B27 in the synovial microenvironment. It also is possible that the $CD8^+$ T lymphocytes may be responding to autoantigens such as collagen (135).

Direct evidence to support the conclusion that $CD8^+$ T cells are involved in the pathogenesis of the spondyloarthropathies also has come from the observations made in individuals with AIDS. Despite suppression of $CD4^+$ T cells, these individuals are capable of developing reactive arthritis (55,136,137). The development of ankylosing spondylitis has not been reported. Reiter syndrome arthritis in patients with AIDS frequently follows a gastrointestinal or genitourinary infection, is usually associated with HLA-B27, and can be extremely aggressive. $CD8^+$ T-cell function is usually normal, although the number and function of $CD4^+$ T cells can be severely depleted. These observations are consistent with the conclusion that reactive arthritis and perhaps the other spondyloarthropathies are mediated by $CD8^+$ T cells responding to an antigenic peptide in the context of HLA-B27. Moreover, the finding that reactive arthritis is extremely aggressive in AIDS patients, even though it may not occur at increased frequency, suggests that $CD4^+$ T cells may function to suppress the development of reactive arthritis.

As proposed in Fig. 43.3, the spondyloarthropathies appear to be triggered by $CD8^+$ T cells responding to a bacterially derived antigenic peptide bound to HLA-B27. In this model, $CD4^+$ T cells regulate this response by recognizing other bacterially encoded antigenic peptides presented in the context of class II MHC molecules. As a result of this response, $CD4^+$ T cells may produce high levels of IFN-g and other cytokines that activate the macrophages, and thereby limit intracellular growth of the triggering microorganisms and the production of bacterially derived peptides that can associate with HLA-B27 and initiate arthritogenic responses of $CD8^+$ T cells. A relative shift toward a Th2 response in this context may favor bacterial persistence and ongoing antigen presentation to the $CD8^+$ T cells. A potential role for gd T cells also has been proposed in these disorders (138,139).

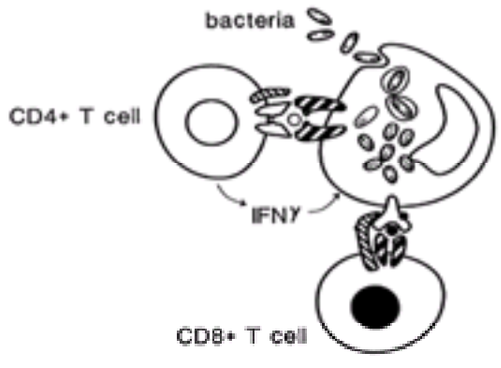

Figure 43.3. Model of cellular interactions involved in the recognition of bacterial peptides by $CD8^+$ T cells. IFN-g, interferon-g.

TRANSGENIC ANIMALS EXPRESSING HLA-B27

HLA-B27 Transgenic Mice

One means to test directly the role of HLA-B27 in the pathogenesis of the spondyloarthropathies is to generate transgenic animals that express the HLA-B27 gene product. A number of groups have generated HLA-B27–positive transgenic mice. Despite the high levels of HLA-B27 expression, spontaneous disease did not develop in these animals (140). Moreover, challenging HLA-B27–positive transgenic mice with a number of putative arthritogenic bacteria or bacterial products, including *Yersinia, Shigella*, or streptococcal cell walls, did not induce reactive arthritis, although the B27 transgenic mice appeared to be more susceptible to *Yersinia* infection than did their wild-type littermates (140). The explanation for the failure of HLA-B27–positive transgenic mice to develop reactive arthritis is unclear, especially because the human transgene is functional as an alloantigen and as an antigen-presenting structure in mice (141). More recently, it was shown that male HLA-B27 transgenic mice lacking endogenous b_2-microglobulin developed spontaneous inflammatory arthritis affecting the hind paws and resulting in joint ankylosis (95). These mice do not express B27 on the cell surface, have very low numbers of $CD8^+$ T cells, and develop the arthropathy only after transfer from a pathogen-free colony. When double transgenic mice were generated having both B27 and human b_2-microglobulin, but no mouse b_2-microglobulin, a similar phenotype developed, which also required the presence of bacterial flora. In this case, the animals did express B27 on the cell surface (142). Free B27 heavy chains were expressed by thymic epithelial cells and a subset of peripheral blood leukocytes. Treatment with antibodies to these heavy chains delayed disease development. Experiments in which the B27 transgenic mice were bred with MHC class II knock-out mice indicated that the development of disease was not dependent on MHC class II (143). These results militate against a mechanism whereby HLA-B27–derived peptides are presented by MHC class II molecules, and are in keeping with a primary role for HLA-B27 in the presentation of a relevant antigen(s).

HLA-B27 Transgenic Rats

Transgenic rats expressing the human *HLA-B*2705* gene product and human b_2 microglobulin spontaneously develop an inflammatory condition that resembles the spondyloarthropathies (72). This phenotype requires a high level of expression of the transgene on the cell surface (144). The disease features include peripheral and axial arthritis, gastrointestinal inflammation and diarrhea, psoriasiform skin changes, and male urogenital inflammation. Histologically, the joint, gut, and skin lesions closely resemble the lesions seen in HLA-B27–associated disease in humans. A number of transgenic rats expressing HLA-A2 or HLA-B7 fail to develop inflammatory disease, indicating that HLA-B27 is likely to be specifically involved in the development of this inflammatory disease. Importantly, when the rats were raised in a germ-free state, they developed neither the gut nor the joint inflammation (145). The gastrointestinal inflammation was an early and consistent finding that precedes the other disease manifestations. When normal gut bacteria were gradually introduced into the germfree B27 transgenic rats, the colitis and arthritis subsequently developed, and there appeared to be a particularly important role for *Bacteroides* species (146). Together, these observations are consistent with the hypothesis that gastrointestinal and articular inflammation are intimately related in the spondyloarthropathies, and that the commensal gut flora may play a role in the pathogenesis of these disorders.

Immunologic studies of the B27 transgenic rats have indicated that disease development requires expression of B27 on bone marrow–derived hematopoietic precursors and on peripheral $CD4^+$ T cells, but not in the thymus (147,148). Recently it was shown that male B27 transgenic rats whose B27 molecules were loaded with a high-affinity peptide derived from an influenza nucleoprotein were less likely to develop arthritis, although they did continue to develop colitis (149). Moreover, it was determined that two peptides derived from the male-specific HY minor histocompatibility antigens, which lacked an anchoring arginine residue in position 2, were specifically presented by HLA-B27 in the transgenic rats. These data support the hypothesis that B27-related arthritis requires binding of specific peptides to B27.

ROLE OF HLA-B27 IN THE IMMUNOPATHOGENESIS OF THE SPONDYLOARTHROPATHIES

Although the role of the HLA-B27 molecule in the pathogenesis of the spondyloarthropathies is not completely known, an understanding of its function suggests hypotheses about its involvement in these conditions (Fig. 43.4). One hypothesis is that infection with particular gastrointestinal or genitourinary microorganisms triggers an immune response by $CD8^+$ T cells, as these organisms are intracellular pathogens and would therefore generate peptides within infected host antigen-presenting cells. The arthritogenic response could result from the generation of a response to one or more bacterial peptides that bind to HLA-B27 and are presented to $CD8^+$ T cells. In addition to triggering immune responses, there might be dissemination of microbial antigens, including those restricted by HLA-B27, to distant tissue, such as the synovium, which is richly endowed with phagocytic cells. There is evidence that *Chlamydia, Salmonella*, and *Yersinia* antigens can be found in the synovial fluid and tissues of patients with reactive arthritis triggered by these different organisms (150,151,152 and 153). In this model, joint inflammation would result from persistent reactivity to those slowly cleared microbial products.

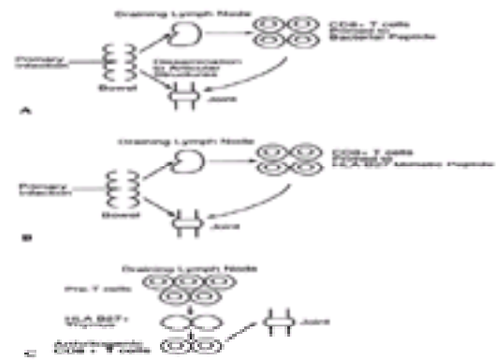

Figure 43.4. Potential immunopathogenic mechanisms in the spondyloarthropathies. The primary infectious process is shown in the bowel, although it could involve the genitourinary tract as well. **A:** Dissemination of bacterial products. **B:** Molecular mimicry. **C:** Human leukocyte antigen (HLA)-B27–mediated selection of arthritogenic T cells.

The role of HLA-B27 in bacterial invasion has been explored by a number of investigators with contradictory results (154,155,156 and 157). One group transfected murine L cells with a number of human HLA-B alleles, including B27. In contrast to all other HLA-B alleles, B27 specifically resulted in decreased invasion of a spectrum of enteric arthritogenic pathogens (154). This effect was associated with the ME1 epitope on B27, which includes a critical cysteine residue in position 67 (155). Other studies have suggested that, rather than impairing bacterial invasion, B27 was associated with enhanced intracellular survival and persistence of arthritogenic organisms such as *Salmonella enteriditis* (156,157). Thus the role of B27 in bacterial invasion and persistence remains to be clarified.

An alternative hypothesis is that the pathogenesis of the spondyloarthropathies involves the induction of autoreactivity to self-antigens because of molecular mimicry between antigens of the infecting microorganism and host tissues. In this model, infection with a bacterium expressing an HLA-B27 mimetic peptide might result in the priming of $CD8^+$ T cells to the endogenous HLA-B27–derived amino acid sequences. During the infection with the microorganism, an immune response to a variety of other bacterial antigens might provide sufficient help to overcome normal tolerance to self-peptides. Subsequently, endogenous HLA-B27 itself could be the source of the antigenic peptide. This could be produced within the synovium in increased amounts after a variety of traumatic or stressful insults that result in the local production of cytokines and increased expression of MHC molecules. This might account for the tendency of the disease to be exacerbated and to remit. In this model, HLA-B27 or another class I MHC molecule could serve as the presenting element. The critical role of HLA-B27 in this model would be to provide the endogenously derived HLA-B27 peptide.

It also is possible that the role of the HLA-B27 in the development of the spondyloarthropathies is less direct. HLA-B27 might function at the level of the thymus to select a repertoire of TCRs expressed by $CD8^+$ T cells that includes receptors uniquely capable of responding to microbial antigens in a pathogenic manner.

Additional hypotheses have been suggested. B27 might serve as a receptor for a bacterial product that alters the B27-expressing cell in a way that makes it prone to immunologic attack (76). It has been suggested that the free sulfhydryl side chain of the position 67 Cys may be altered by oxidative attack and thereby be rendered immunogenic (158,159). This unpaired cysteine residue, located in the antigen-binding B pocket of the B27 molecule, also was shown to be susceptible to modification by homocysteine, and as a result of the homocysteine modification was recognized by cytolytic T cells from patients with ankylosing spondylitis and reactive arthritis (160). An alternative hypothesis has suggested that free HLA-B27 chains can form a homodimer linked by a stable disulfide bond at the free Cys-67 (161). HLA-B27 homodimer formation has been observed at higher levels in spondyloarthropathy patients than in normal HLA-B27–positive individuals. It is hypothesized that such as homodimer may enhance the ability to present peptides. In each of these models, B27 either indirectly or directly facilitates the development of altered self-molecules that can induce persistent immunologic attack.

Each of these hypotheses is tenable, is consistent with the known structure and functions of the HLA-B27 molecule, and could explain the role of this class I MHC molecule in the pathogenesis of the spondyloarthropathies. Moreover, none is mutually exclusive of the others. Therefore, some combination of the hypothesized events might be involved in the development of the spondyloarthropathies. These hypotheses do not address a number of issues, including the unique tissue distribution of the disease; the differences in clinical manifestations between ankylosing spondylitis, Reiter syndrome, and the other spondyloarthropathies; and the immunopathogenesis of the spondyloarthropathies in HLA-B27–negative individuals.

OTHER GENETIC FACTORS PREDISPOSING TO SPONDYLOARTHROPATHIES

It is clear that the greatest genetic contribution to spondyloarthropathies comes from HLA-B27. Other HLA class I alleles have been associated with this group of diseases (162). Two studies have indicated an association between ankylosing spondylitis and HLA-B60, this being present in both HLA-B27–positive and HLA-B27–negative individuals (163, 164). HLA-B39, which has an antigen-binding B pocket similar to that of HLA-B27, has been associated with spondyloarthropathies in Japan (41).

A potential role for MHC class II alleles has been suggested (165). In a white population of ankylosing spondylitis patients in Britain, DR1 and DR8 were both found to be modestly increased, whereas DR12 was reduced in frequency compared with the frequency in a control population (27). These associations were calculated to be independent of HLA-B27. An HLA study in a heterogeneous cohort of patients with early synovitis demonstrated an association between HLA-B27 and both DR1 and DR4, although this was not specific for spondyloarthropathies (166). It should be noted that B27 and DR1 are part of an extended haplotype that is relatively common in white populations. In view of the large size of the genomic interval between the B and the DR locus, an association involving such an extended haplotype implies a potential role for a large number of other MHC genes in the susceptibility to spondyloarthropathies. It is possible that epistatic gene interactions that are unsuspected on the basis of analyzing individual MHC alleles may ultimately be quite relevant to disease pathogenesis.

Of potential importance in the class II region are genes involved in antigen processing and transport. Components of the large multifunctional protease (LMP) are encoded in this region. This complex is involved in the regulation of proteosome function at sites of inflammation, and may alter the repertoire of peptide antigens generated by the proteosome. In turn, this may affect the binding of these peptides to HLA-B27. These genes have been shown to exhibit limited polymorphism. One polymorphism in the LMP 2 gene was shown to increase the prevalence of iritis in HLA-B27–positive patients with ankylosing spondylitis, but this was not confirmed in other studies (167). Near the LMP locus are the transporters associated with antigen-processing (TAP) genes, which encode for proteins involved in the transport of peptide antigens to nascent HLA class I molecules in the cytosol. The Tap1C and Tap2A alleles have been shown to be increased in patients with B27-linked Reiter syndrome (168). Tap1B was increased in patients with ankylosing spondylitis (169). Together these findings suggest that genes involved in the processing and transport of peptides may play a role in the susceptibility to spondyloarthropathies, possibly by modifying antigen presentation by HLA-B27.

Another candidate locus that has received considerable attention is the TNF locus, which lies in the class III region between the class I and class II regions of the MHC. Several lines of investigation have indicated that TNF-a is involved in the pathogenesis of chronic inflammatory arthropathies, including the spondyloarthropathies (170,171). Considerable polymorphism has been demonstrated in the promoter region of the TNF-a locus. In particular, a polymorphism at position –308 of the TNF-a promoter has been shown to be associated with markedly increased transcription of this gene (172). Several recent studies have evaluated TNF-a polymorphisms in ankylosing spondylitis, with inconsistent results (173,174 and 175).

It has been noted in family studies that the calculated l_s for the HLA region has a value of 3.2 compared with a l_s of 50 for the overall genetic risk (165). The MHC is estimated to contribute approximately 36% of the overall genetic risk. This suggests that genes outside the HLA region make a substantial contribution to disease risk. In view of this, genome-wide linkage studies have been used to identify novel susceptibility genes outside the MHC. One study evaluated 105 British families by using 254 polymorphic microsatellite markers to identify chromosomal regions that potentially contribute to susceptibility to spondyloarthropathies (27). Although this study was rather limited in statistical power, it did reveal several genomic regions outside the MHC that showed evidence of highly significant linkage with ankylosing spondylitis. Of these, the strongest region showing linkage was located in 16q23, having a logarithm of odds (LOD) score of 2.6 compared with an LOD score of 3.8 for the MHC. Genome-wide scans of patients with psoriasis (176) and inflammatory bowel disease (177) have indicated susceptibility loci in 16q, near those associated with ankylosing spondylitis. Further identification of candidate genes will be facilitated by the completion of the Human Genome Project and the application of DNA microarray technologies.

ETIOLOGY

Ankylosing Spondylitis

Despite the association with HLA-B27, the etiology of ankylosing spondylitis is unknown. A number of features of the disease implicate immune-mediated mechanisms, including elevated serum levels of IgA (178,179 and 180) and acute-phase reactants (181), inflammatory histology (182), and close association with HLA-B27 (2,7). No specific event or exogenous agent that triggers the onset of the disease has been identified, although overlapping features with reactive arthritis and inflammatory bowel disease suggest that enteric bacteria might play a role. The possibility that enteric colonization with *Klebsiella* species plays a role in the development of ankylosing spondylitis has been suggested (183) but has not been confirmed (184). Although multiple studies have suggested the presence of specific antibody responses to *Klebsiella* in the sera of patients with ankylosing spondylitis, so far no direct association with the organism has been demonstrated. Evidence of antigenic interrelatedness between HLA-B27 and certain enteric bacteria (98,99) suggests a role for molecular mimicry in the disease, but it is not yet known whether this contributes to the pathogenesis of ankylosing spondylitis.

Reiter Syndrome

Reiter syndrome often occurs after gastrointestinal or genitourinary infection. In most instances, no identifiable infectious etiology can be identified. The most common microbial pathogens inducing Reiter syndrome are *Shigella, Salmonella, Yersinia, Campylobacter*, and *Chlamydia* (1,4,51,185,186). Although the syndromes induced by these pathogens are similar, there are differences in the pathogenic consequences, including disease severity, presence of presumed antigenic material in the synovium, and the association with HLA-B27. Whether the arthritis induced by each of these microorganisms is reactive or is related to the dissemination of microbial material to the joint is unclear because the arthritides associated with *Chlamydia, Yersinia*, and *Salmonella* are associated with the presence of microbial antigens within the synovium (150,151,152 and 153,187,188 and 189). The overall similarity of the conditions induced by these various microorganisms suggests that at least

some features of the immunopathogenesis may be common to all.

SHIGELLA

The increased incidence of Reiter syndrome after epidemics of *Shigella* dysentery has documented the potential arthritogenicity of this organism (46). Several reports suggested that 0.2% to 2.0% of infected individuals develop Reiter syndrome after epidemic shigellosis (48,49,190). Infections with *S. flexneri*2a and 1b trigger Reiter syndrome, whereas infection with the more frequent *S. sonnei* does not (4). Arthritis most commonly affects HLA-B27–positive infected individuals and has its peak onset 10 to 30 days after the onset of dysentery. In most cases, the diarrheal illness resolves before the articular inflammation appears. *S. flexneri* carries a 2-Md plasmid, pHS-2, encoding for an HLA-B27–mimetic epitope, and antibodies directed toward B27 cross-react with a peptide encoded by this sequence (112,191). This suggests that molecular mimicry may play a role in the pathogenesis of *Shigella* reactive arthritis. Interestingly, a group working a mouse model have recently shown a potential role for MHC class Ib molecules in infection-induced autoimmune recognition (192).

SALMONELLA

S. typhimurium is the most common species of *Salmonella* giving rise to Reiter syndrome. In as many as 1% to 6% of infected individuals, a sterile arthropathy develops after *Salmonella* outbreaks (48,193,194 and 195). Reactive arthritis usually develops within 3 weeks of *S. typhimurium* infection, and in some patients, the symptoms can persist for several years after the infection (195). Reactive arthritis also has been associated with infections secondary to *S. paratyphi, S. enteritis, S. heidelberg, S. infantis*, and *S. bovismorbificans* (4,193,196,197). In some patients, *Salmonella* antigens can be found in synovial tissue and fluid (150,198), although bacterial DNA was not detected (198). Like *Shigella*-associated disease, the Reiter syndrome that follows salmonellosis is associated with HLA-B27. Many of the HLA-B27–negative patients with Reiter syndrome express one of the other B27 cross-reactive antigens: B7, Bw22, B40, B42, and B60 (48,193). In some series of epidemic salmonellosis and Reiter syndrome, the incidence of B7 is greater than that of B27 (193). No important clinical differences between *Shigella*- and *Salmonella*-induced Reiter syndrome have been observed.

YERSINIA

Yersinia is a common cause of reactive arthritis in certain endemic areas, especially the Scandinavian countries (185,199,200 and 201). *Y. enterocolitica* is the most frequent isolate in northern Europe but is rarely encountered in England or America. *Y. enterocolitica* O:3 has most commonly been associated with reactive arthritis, but arthritis also has been described after infections with serotypes O:9 and O:8. *Y. pseudotuberculosis* has been reported outside of the Scandinavian countries but is still an uncommon pathogen (202). *Yersinia* infection produces an acute, mild, self-limited enteritis and mesenteric lymphadenitis, which may be associated with joint complaints in nearly 50% of patients (185,203). Men and women are equally affected. *Yersinia* arthritis is most commonly observed in young adults, although children and the elderly also have been affected.

HLA-B27 is present in 60% to 80% of patients with *Yersinia* arthritis (201,203). The arthritis is predominantly oligoarticular, affecting the lower extremities and hands. The arthritis runs a chronic or relapsing course in some patients, with chronic low back pain and chronic sacroiliitis developing in one third of patients. Five percent of patients demonstrate recurrent attacks of arthritis, often resulting from infections with other arthritogenic microorganisms such as *Salmonella* or *Chlamydia* (203). Rarely these patients progress to a clinical picture similar to that of ankylosing spondylitis. Extraarticular features, which include urethritis, ocular inflammation, mucocutaneous disease, and carditis, occur in 20% to 30% of individuals and appear most often in those who are HLA-B27 positive. Other features, including erythema nodosum and glomerulonephritis, are more common in HLA-B27–negative individuals. Active infection is associated with elevated IgA or IgG antibodies. Sustained elevations of IgA antibody titers correlate with persistent infection, chronic arthritis, and occult enteritis (204,205 and 206). *Yersinia* is commonly cultured from the stool during the acute infection. However, attempts to culture the organism from synovial fluid or tissue have been uniformly unsuccessful. *Yersinia* antigens have nonetheless been identified in both the synovial fluid (187) and tissue (151), suggesting that a persistent immune response to this material might play a role in the induction of inflammation. *Yersinia* antigens such as LPS and hsp are detectable in peripheral blood and synovial fluid leukocytes years after the initial infection (207). Moreover, it was shown that *Yersinia* 16s ribosomal RNA was present in the synovial fluid of a patient with reactive arthritis, which had occurred months after the initial episode of *Yersinia* infection (208). These data suggest that persistent subclinical infection with this pathogen may account for the chronicity of this arthropathy in some patients.

Yersinia arthritis is usually mild, and there are few long-term sequelae. The disease is more severe in a small number of patients. The presence of HLA-B27 may be of value in the identification of these individuals, as chronicity, severity, sacroiliitis, and ocular inflammation are more likely in HLA-B27–positive persons (203). In patients with persistently positive stool cultures for *Yersinia*, appropriate antibiotic therapy should be used.

CHLAMYDIA

Chlamydia trachomatis is a common urogenital pathogen that can trigger Reiter syndrome. Indeed, a study of 13,204 female army recruits in the United States indicated an overall prevalence of chlamydial infection equaling 9.2%, this prevalence being even higher in the teenage recruits (50). More than 50% of patients with Reiter syndrome have antibodies to *C. trachomatis*, although positive cultures of material from patients with active disease are seldom observed (4,23,209). An equivalent percentage of patients diagnosed with sexually acquired reactive arthritis or nongonococcal urethritis possess antichlamydial antibodies (209). These antibodies are presumably directed at *Chlamydia* antigens (chlamydial elementary bodies) that have been identified in the synovial fluid and synovial tissue of patients with Reiter syndrome, particularly in early disease (152,153,210). It also has been possible to detect chlamydial DNA in the joints of some patients with sexually acquired reactive arthritis (153,189,211,212), with synovial tissue having a higher sensitivity than synovial fluid (213). Importantly, there was a lack of correlation between antichlamydial antibodies, synovial fluid lymphocyte responses, and the presence of chlamydial DNA in the synovial fluid (212). DNA from chlamydial plasmid, major outer membrane protein (MOMP), and 16s ribosomal RNA has been detected in the synovial fluid and synovial tissue of a spectrum of arthropathies (211,212,214), and even from normal synovium (215).

In patients with sexually acquired Reiter syndrome, synovial lymphocytes have been shown to proliferate in response to chlamydial antigens (216). These responses involve both $CD4^+$ and $CD8^+$ T cells. The cytokine profile of the synovial T cells demonstrates high levels of both IFN-g and IL-10 (130,217). This has led to the speculation that this cytokine profile may have a permissive effect for the synovial persistence of *Chlamydia trachomatis*. An aberrant chlamydial gene-expression profile may result with attenuation of outer membrane proteins (218,219). This has been demonstrated *in vitro* (220). Moreover, an animal model has shown that synoviocytes can act as a reservoir for *C. trachomatis* and precipitate a chronic aseptic arthritis (221). In this model, *C. trachomatis* antigens, but not DNA, are detectable in the joint after 21 days. Studies in an HLA-B27 transgenic mouse demonstrated the recognition of chlamydial antigens by B27-restricted CTLs (222). Together these results suggest that a T cell–mediated, chlamydial antigen-specific immune response within the affected synovia may play a role in the immunopathogenesis of the disease and, unlike other forms of reactive arthritis, whole *C. trachomatis*organisms may persist in the joints of affected patients.

Chlamydial infection is thought to be responsible for as much as 10% of all cases of early inflammatory arthritis (223). Of those individuals with *Chlamydia* urethritis, in as many as 1% to 3%, arthritis ultimately develops. Of the *Chlamydia* species responsible for Reiter syndrome, *C. trachomatis* (serotypes D through K) is the most common, with primary infection involving either the urogenital or ocular tract in men and women. *C. psittaci* also produces a reactive arthropathy (224). Diagnosis is suggested by the presence of persistent arthritis in at least one joint, symptoms of genitourinary infection, detection of IgG or IgA antichlamydial antibodies, the demonstration of *Chlamydia* in genitourinary swabs or urine culture, and exclusion of other diseases (223). Recently *Chlamydia* pneumonia has been identified in the synovial tissues and synovial fluid of some patients with reactive arthritis and undifferentiated arthritis (214,225), and a number of patients with reactive arthritis demonstrated specific antibody evidence of recent infection with the pathogen (226).

The rheumatic manifestations of *Chlamydia* infections are similar to those described for classic Reiter syndrome (i.e., asymmetric oligoarthritis, enthesitis, ocular inflammation, low back pain, and sacroiliitis). However, only 20% of patients meet the criteria for the diagnosis of Reiter syndrome, and up to 15% have no urogenital manifestations at all, especially women (223). In more than half of the patients, a chronic arthropathy develops, with nearly one third having inflammatory low back pain and radiographic sacroiliitis or enthesopathy. In many patients, clinical activity is paralleled by the titer of IgA antichlamydial antibodies; sustained elevation of IgA titers suggests persistent infection with *Chlamydia* (227).

Despite similarities with classic Reiter syndrome, *Chlamydia*-induced arthritis differs in several ways. First is the identification of chlamydial antigens and even whole organisms at sites of tissue inflammation (152,153,210). Second, fewer than 50% of patients with documented disease are HLA-B27 positive (223). Finally, patients with *Chlamydia*-induced arthritis may respond to appropriate antibiotics (228,229).

OTHER AGENTS

Ureaplasma urealyticum has occasionally been implicated as a pathogenic trigger in Reiter syndrome, especially in those patients with nonchlamydial sexually acquired reactive arthritis (230,231). Gastrointestinal infections with *Campylobacter jejuni* and *C. fetus* also are associated with the development of typical Reiter syndrome (4,48).

PATHOLOGY AND IMMUNOPATHOLOGY

Ankylosing Spondylitis

Sacroiliitis is usually one of the earliest manifestations of ankylosing spondylitis. Most often, the thinner iliac fibrocartilage is eroded first, and then the thicker sacral hyaline cartilage (232). The irregularly eroded, sclerotic margins of the joint are gradually replaced by fibrocartilage and then by ossification. Ultimately, the joint may be totally obliterated. The early lesion consists of subchondral granulation tissue containing lymphocytes, plasma cells, mast cells, macrophages, and chondrocytes (233). Recently a detailed study of computed tomography (CT)-guided sacroiliac biopsies was undertaken by using immunohistology and *in situ* hybridization (234). This study demonstrated the presence of a mononuclear cell infiltrate predominated by $CD4^+$ T cells and $CD14^+$ macrophages, along with high levels of TNF-a mRNA. This picture is quite similar to that seen in rheumatoid synovitis. Small amounts of TGF-b were detected close to areas of new bone formation. A more extensive study by the same group of investigators subsequently showed that T cells become less prominent as the disease becomes more chronic, whereas numbers of macrophages continue to be high (235). Importantly, the degree of cellularity correlated with the degree of enhancement as determined by dynamic magnetic resonance imaging (MRI). This finding suggests that this imaging modality can be used to evaluate noninvasively the inflammatory process in the sacroiliac joints.

The initial lesion in the spine consists of inflammatory granulation tissue at the junction of the annulus fibrosus of the disc cartilage and the margin of vertebral bone (233,236,237 and 238). The outer annular fibers are eroded and eventually replaced by bone, forming the beginning of a bony excrescence called a *syndesmophyte*, which then grows by continued enchondral ossification, ultimately bridging the adjacent vertebral bodies. Ascending progression of this process leads to the “bamboo spine” observed radiographically. Other lesions in the spine include diffuse osteoporosis, erosion of vertebral bodies at the disc margin, “squaring” of vertebrae, and inflammation and destruction of the disc/bone border. Inflammatory arthritis of the apophyseal joints is common. Early in the course, this is characterized by the formation of pannus, which erodes cartilage. Subsequently, bony ankylosis develops.

The pathology of peripheral joint arthritis in ankylosing spondylitis shows hyperplasia of the lining layer, infiltration with lymphocytes and plasma cells, and pannus formation; however, the process is usually, but not always, less intense than that seen in RA, particularly with respect to lining-layer hyperplasia (182,239,240). The synovial lesions in ankylosing spondylitis were found to have a lower CD4/CD8 ratio than that found in RA synovial samples (241). Central cartilaginous erosions caused by proliferation of subchondral granulation tissue are common in ankylosing spondylitis but rare in RA

Reiter Syndrome

The characteristic pathologic findings in Reiter syndrome include an inflammatory synovitis, inflammation and erosions at the insertion of ligaments and tendons, the excessive production of heterotopic bone at sites of inflammation, and cutaneous changes similar to those observed in psoriasis (4). Pathologic changes in the synovial membrane are not specific, but include edema, vascular changes, and infiltration with lymphocytes, polymorphonuclear leukocytes, and plasma cells. These findings are not unique to Reiter syndrome, but are observed in many of the inflammatory arthropathies. Immunohistologic studies comparing Reiter synovitis with rheumatoid synovitis have suggested that the RA lesions tend to have higher numbers of plasma cells, cells expressing granzyme B, and cells expressing IFNg, although the differences were quantitatively modest (242). Extensive pannus formation is rare, and bone erosions are uncommon and are observed only with persistent inflammation. The overlying synovial fluid is inflammatory, with a predominance of polymorphonuclear leukocytes. There is no local complement consumption, as evidenced by the finding that synovial fluid complement levels often approach serum levels (243). The entheses are infiltrated with inflammatory cells and may be accompanied by erosions and eburnation of subligamentous bone (244,245). Bony proliferation results from subchondral osseous hyperplasia and periosteal new bone formation (245). The mucocutaneous lesions of Reiter syndrome are indistinguishable from those seen in psoriasis, with thickening of the horny layer, parakeratosis, and acanthosis (4). The outer layer of the corium is infiltrated with lymphocytes and plasma cells. Epidermal vesicles are filled with epithelial and inflammatory cells and may resemble microabscesses. Oral and genital mucosal lesions resemble cutaneous lesions with the absence of keratosis.

The intestine is characterized by subclinical inflammation in the terminal ileum and colon in approximately 60% of patients (246,247,248,249 and 250). These changes have been found regardless of the presence of HLA-B27, type of onset, clinical manifestations, or administration of nonsteroidal antiinflammatory drugs (247). Acute intestinal lesions resemble those associated with bacterial enteritis, whereas chronic lesions are similar to those observed with inflammatory bowel disease. Acute pathology is most often associated with enteropathic reactive arthritis, and chronic lesions are more commonly observed in patients with ankylosing spondylitis. Even patients with a postvenereal onset of disease develop intestinal pathology, although it is milder and less frequent (247). Follow-up studies have demonstrated that the degree of intestinal inflammation closely parallels the patient's clinical status and response to therapy (247,249). These findings, along with the demonstration of altered intestinal permeability (250,251) and elevated secretory IgA titers (4,251) in patients with active disease, suggest that occult intestinal inflammation may be central to the perpetuation of disease.

CONCLUDING REMARKS

The spondyloarthropathies represent an important opportunity to examine the interface between host genetic factors and microbial agents, and how this interface precipitates and perpetuates human pathology. The pathogenesis of conditions such as Reiter syndrome potentially encompasses a spectrum of immunologic mechanisms that includes ongoing responses to persistent pathogens and molecular mimicry between endogenous and microbial antigens. The role of HLA-B27 is at this point indisputable, and unique to this group of disorders. A more complete understanding of the role of HLA-B27 in the pathogenesis of spondyloarthropathies will be further complemented by an evolving understanding of the role played by other genes within, and outside of, the MHC.

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44 RHEUMATIC FEVER AND OTHER CARDIOVASCULAR DISEASE

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There is considerable evidence that immune-mediated tissue injury is an important pathophysiologic factor in a variety of disorders affecting the cardiovascular system including rheumatic fever, collagen vascular diseases, the vasculitides, myopericarditis, endocarditis, the hypereosinophilic syndrome, and the postcardiac injury syndrome (PCIS). Cardiac tissue damage in these disorders may occur by antibody-dependent complement-mediated cytolysis or phagocytosis, effector cell chemotaxis followed by release of toxic substances, or direct T-cell–mediated cytotoxicity. Moreover, secreted cytokines such as tumor necrosis factor-a, interleukin-8 (IL-8), and IL-6 have been implicated in the pathogenesis of rheumatic fever. Whereas the inciting immune stimulus in cell injury often remains obscure, brisk B- and T-cell responses to foreign antigens having shared epitopes with host cardiac cells represent a popular theory of immune-mediated tissue destruction. Expression of foreign cell surface antigens in virus-infected cells and the development of autoimmunity to host surface and cytoplasmic molecules may also serve to induce immunologically mediated injury. In addition, the ability of certain “superantigens” to stimulate intense clonal T-cell responses may in part explain immunologic mechanisms in the expression of certain diseases such as rheumatic fever. Bacterial superantigens such as streptococcal pyrogenic toxin A act as potent exotoxins and have been shown to produce responses in both $CD4^+$ and $CD8^+$ T cells, and this may, in part, explain the immunologic mechanisms involved in rheumatic fever. Whatever the mechanisms involved, it seems clear that a derangement in immune regulation is an essential component of these autoimmune disease phenomena.

Although many of the classic collagen vascular disorders such as systemic lupus erythematosus, ankylosing spondylitis, rheumatoid arthritis, and the vasculitides have important effects on the heart and blood vessels, it is beyond the province of this chapter to discuss these diseases in detail. These disorders are specifically addressed in other chapters of this book. This chapter focuses on selected clinical syndromes producing significant heart involvement with manifestations believed to be mediated in part by immunologic mechanisms.

RHEUMATIC FEVER

Rheumatic fever is an acute inflammatory disease involving primarily the heart, joints, central nervous system, and subcutaneous tissues. It is generally a disease of children and young adults, but it may be seen in older patients (9). Rheumatic fever and acute poststreptococcal glomerulonephritis represent the nonsuppurative sequelae of pharyngitis caused by group A streptococci *(Streptococcus pyogenes),* usually after a 1- to 5-week period in immunologically and genetically susceptible persons. It is classified among the rheumatic diseases because destruction of connective tissue, which may in part be immune mediated, is a prominent feature of this disorder. It has been said that rheumatic fever “licks the joints|.|.|. but bites the heart” because of its potentially serious immediate and long-term cardiac effects and its relatively transient and reversible joint involvement. The incidence of rheumatic fever in the United States is low; approximately 1 case per 200,000 children per year (73). However, regional sporadic outbreaks have been noted (15,58). Segregated populations with limited access to medical care and overcrowded living conditions result in widely disparate trends even in developed nations. The prevalence rate of rheumatic heart disease reported in isolated, rural communities of Aborigines in northern Australia is one of the highest in the world, ranging from 9.9 to 44.1 per 1,000 (22). Overall, rheumatic fever remains a major health problem in developing and economically depressed countries.

History

The earliest descriptions of acute rheumatic fever date back to the sixteenth century when the French physician Guillaume de Baillou coined the term *rheumatism* to distinguish acute arthritis from gout. The seventeenth-century writings of Thomas Sydenham described chorea but failed to associate it with the other systemic manifestations of acute rheumatic fever. In the landmark studies of Jean-Baptiste Bouillard in 1836 and Walter B. Cheadle in 1889, clear association was drawn between rheumatic arthritis and carditis (115). In 1904, Ludwig Aschoff described the specific pathologic myocardial rheumatic lesion that now bears his name. J.K. Fowler first described the association of antecedent sore throat and rheumatic fever in 1880. Detailed clinical, bacteriologic, and epidemiologic studies by Coburn, Collis, Rammelkamp, and Stollerman during the first half of the twentieth century clearly established the causative role of group A streptococcus infection in rheumatic fever (115).

Epidemiology

Rheumatic fever remains a disease of the young, affecting primarily school-aged children at a mean age of approximately 9.8 years (130). Rheumatic fever in infants is distinctly rare, and many of the cases reported in adults may be recurrences in patients not receiving prophylaxis (84). Rheumatic fever and rheumatic heart disease display no particular gender predilection, although mitral stenosis and Sydenham chorea are reportedly more common in postpubertal women, whereas aortic stenosis is more common in men. Available data demonstrate no significant difference in the susceptibility of different races to rheumatic fever. High attack rates among various ethnic groups relate more to low socioeconomic status, conditions of overcrowding, and unavailability of health care.

Rheumatic fever remains a major worldwide health problem even though its incidence has been sharply declining in the United States. Rheumatic heart disease remains the most common form of acquired heart disease in children and young adults and is an important cause of all heart disease seen in adults. Acute rheumatic fever and rheumatic heart disease are two of the most common worldwide causes of death in young people and are responsible for 25% to 40% of all cardiovascular disease. Thirty-three to 50% of all hospital admissions for heart disease are for rheumatic heart disease (49,78). Annual attack rates as high as 140 per 100,000 population have been reported in developing countries (9). This is in stark contrast to the situation seen in the United States and Western Europe, where the occurrence of rheumatic fever has been declining since the early 1900s (Fig. 44.1). Regional data from Tennessee revealed an annual incidence of rheumatic fever of 0.5 per 100,000 among white suburban children (73). The steady decline in the prevalence of rheumatic fever has been interrupted by intermittent sporadic outbreaks beginning in the mid-1980s (42,52,84,130,137). Despite this development, rheumatic fever in the United States is largely seen only in lower socioeconomic groups. The major environmental risk factor for the development of rheumatic fever is crowding. Rheumatic fever has persisted in low-income, inner-city neighborhoods where the population density is high and crowded living conditions persist (42).

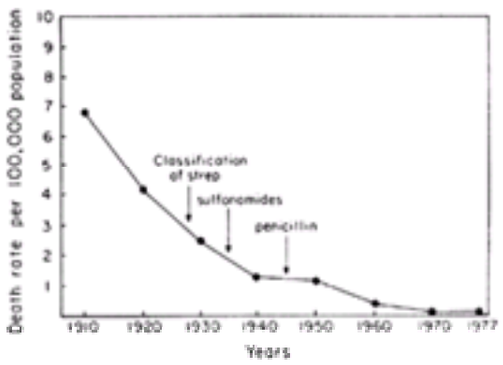

Figure 44.1. Crude death rates from rheumatic fever in the United States, 1910 to 1977. (From Gordis L. The virtual disappearance of rheumatic fever in the United States: lessons in the rise and fall of disease. *Circulation* 1985;72:1155. Copyright 1985, American Heart Association, with permission.)

As best as can be determined, all cases of acute rheumatic fever follow group A streptococcal infection of the upper respiratory tract. The attack rate after unrecognized or untreated group A streptococcal exudative pharyngitis is consistently 2% to 3% (100). Factors influencing the attack rate include (a) the severity of the antecedent pharyngeal streptococcal infection, (b) pharyngitis caused by more virulent "rheumatogenic" M protein serotypes (16,43), (c) the magnitude of the host immune response to streptococcal antigens coupled with genetic predisposing factors (132), (d) prolonged convalescent streptococcal carriage (109), and (e) a previous attack of rheumatic fever. Rheumatic fever has a recurrence rate of up to 50% during the first year after the initial attack and then decreases until 4 to 5 years later, when it levels off at approximately 10%.

Despite the high prevalence of group A streptococcal pharyngitis, the attack rate for rheumatic fever even in the presence of severe infection is only 2% to 3%. This finding implies variability in host predisposition to rheumatic fever. Genetic factors have been postulated to be important in determining susceptibility patterns to rheumatic fever. Monozygotic twins have a higher concordance rate for rheumatic fever than do dizygotic twins (36). In some studies, class II human histocompatibility leukocyte antigens (HLA) have been encountered in higher frequency in patients with rheumatic fever. The strongest associations correlate with the HLA-DR2, HLA-DR4, and HLA-DR7 phenotypes (7,54,99). No similar association has been found for the HLA class I antigens. In addition, monoclonal antibodies directed against a human B-cell alloantigen were shown to be present on the B cells of 92% of all patients with rheumatic fever in India. In contrast, this antigen occurred in only low frequency in patients without rheumatic fever (97,136). Various monoclonal antibodies developed against human B-cell alloantigen have different positive predictive values in different population groups around the world. Monoclonal D8/17, found to be 100% specific for rheumatic fever and rheumatic heart disease in patients from New York, correctly identified only 62% to 68% of north Indian patients. Thus, standardized immunochemical assays developed for specific subgroups may provide the optimal diagnostic sensitivity, specificity, and positive predictive value (72).

The reasons for the gradual decline of rheumatic fever in the United States remain obscure but are believed to be multifactorial. Certainly, the broad availability and use of antibiotics and the aggressive treatment of streptococcal pharyngitis have had a dramatic impact on the decreasing attack rate of rheumatic fever. However, because the decline of rheumatic fever predates the advent of the antibiotic era, this factor alone cannot account for the dramatic decrease in the incidence of this disease. Other possibilities include changes in the virulence of the group A streptococcal organism with a disappearance of the M serotypes associated with rheumatic fever, improvement in urban crowding conditions, and improved health care delivery (14).

The reasons for the sporadic outbreak of rheumatic fever noted in the mid-1980s remain a topic of intense debate. Most students of the field believe that these outbreaks correlate with the appearance of highly mucoid strains of group A streptococcus belonging to the previously recognized rheumatogenic M serotypes (e.g., types 1, 3, 5, 6, and 18).

Clinical Signs and Symptoms

Despite its prominence as a worldwide health problem and years of intensive basic and clinical research, rheumatic fever remains devoid of pathognomonic signs and symptoms as well as laboratory or other diagnostic tests. It is a multisystemic disease in which the clinical manifestations vary considerably not only from patient to patient, but also according to the organ systems involved, the severity of the lesions, and at what point during the course of the illness the patient presents to the physician. Rheumatic fever presents with various signs and symptoms that may occur alone, in succession, or in combination. The most important of these, in terms of diagnosis, are referred to as the *major manifestations* and consist of carditis, polyarticular arthritis, chorea, subcutaneous nodules, and erythema marginatum. Other minor manifestations are frequently present and aid in recognizing and diagnosing the disease, but they are considered too nonspecific to be of major diagnostic importance. These are referred to as *minor manifestations* and include fever, arthralgia, heart block, acute-phase reactants (C-reactive protein, erythrocyte sedimentation rate, increased total leukocyte count), and a history of an episode of rheumatic fever or rheumatic heart disease.

Upper respiratory tract infection with group A streptococcus is believed to be the necessary antecedent to the development of rheumatic fever. Nonetheless, a review of the literature revealed that up to 42% of patients had no recollection of previous pharyngitis (27). The crucial period between the time of onset of pharyngitis and the first appearance of symptoms of acute rheumatic fever averages 18.6 days, with a range of approximately 1 to 5 weeks.

The mode of onset after the latent period is highly variable. At presentation, patients may appear ill, with the onset of an acute febrile illness associated with severe, polyarticular arthritis or with the mild insidious onset of isolated carditis. With this caveat in mind, most attacks of rheumatic fever begin with polyarthritis, which may be preceded by abdominal pain. Polyarthritis and carditis constitute the most common major manifestations, occurring in approximately 77% and 54% of patients, respectively (42,105). If carditis appears, it usually does so early in the course of the illness. Sydenham chorea, subcutaneous nodules, and erythema marginatum are each present in fewer than 10% of patients (Fig. 44.2). The expression of major manifestations depends on age; carditis more commonly affects younger children, and the incidence of arthritis increases with the age of the patient.

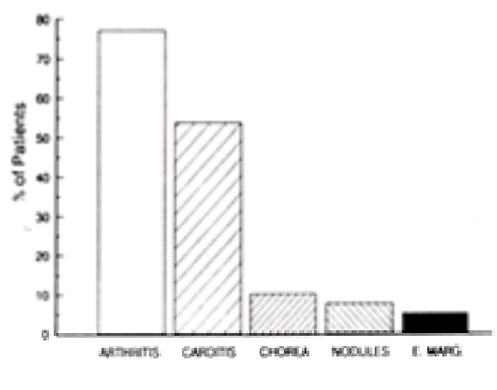

Figure 44.2. Major clinical manifestations in 39 patients with fever. E. MARG, erythema marginatum. (From Ferguson GW, Shultz JM, Bisno AL. Epidemiology of acute rheumatic fever in a multiethnic, multiracial urban community: the Miami-Dade County experience. *J Infect Dis* 1991;164:720, with permission.)

The arthritis of rheumatic fever is usually polyarticular, most commonly affecting the large joints such as the knees, ankles, elbows, and wrists. The small joints of the hands and feet are less frequently involved, and the hips and spine are rarely affected. Joint involvement ranges from mild arthralgias to severe arthritis with heat, swelling, redness, and intense pain. Severe cases of arthritis are rarely associated with the development of carditis. In the most classic presentation, several joints are usually affected in rapid succession, to create the picture of migrating polyarthritis. Once a particular joint is involved, inflammation begins to subside within a few days to a week and completely resolves within 2 to 3 weeks. The temporal course of individual joint inflammation is such that multiple joints are usually involved at any point. In most cases, the arthritis is severe for a week and completely resolves within 4 weeks without any residual long-lasting joint damage.

In contrast to polyarthritis, carditis is the most serious major manifestation of rheumatic fever because of its potential to cause death or long-term disability. Rheumatic fever frequently presents as a pancarditis with involvement of the myocardium, the endocardial structures, and the pericardium. Rheumatic carditis, in the absence of overt heart failure, acute pericarditis, or constitutional symptoms, is usually asymptomatic. Carditis, when it appears, usually does so within the first 3 weeks of the

rheumatic attack. Carditis presenting after this period is distinctly rare. The four major criteria for the clinical diagnosis of rheumatic carditis include (a) development of new organic heart murmurs not previously present, (b) cardiac enlargement, (c) congestive heart failure, and (d) pericardial friction rubs or signs of effusion (116). The presence of any one of these criteria in a patient with acute rheumatic fever justifies the diagnosis of rheumatic carditis.

In the beginning of the twentieth century, severe acute rheumatic carditis with myocarditis leading to fulminant heart failure and death was the leading medical cause of death in school-aged children (117). Fortunately, this severe form of acute rheumatic carditis has become extremely rare. More often, one sees a low-grade inflammatory process involving the myocardium and endocardium that leads in some cases to the delayed appearance of chronic rheumatic heart disease. The severity of acute rheumatic carditis does not correlate with the future development of rheumatic heart disease. During acute rheumatic endocarditis, the mitral valve is most frequently involved. Mitral valvulitis with edema and thickening of the leaflets leads to the characteristic high-pitched blowing holosystolic murmur of mitral regurgitation. This is often associated with a transient, low-pitched, apical, middiastolic flow murmur (Carey Coombs murmur). With aortic valve involvement, one may hear the soft, high-pitched decrescendo murmur of aortic insufficiency occurring just after S_2 in the aortic area. The murmurs of aortic and mitral stenosis associated with rheumatic heart disease are not heard in the setting of acute rheumatic fever.

Congestive heart failure, the least common manifestation of rheumatic carditis, occurs in approximately 5% to 10% of patients during the first attack. It is more common in recurrent bouts of rheumatic carditis. Acute pericarditis occurs in approximately 5% to 10% of patients with rheumatic carditis. It is not the sole manifestation of rheumatic carditis, but it is usually found in association with rheumatic pancarditis. Chest pain associated with pericardial friction rubs and effusions should alert the physician to the presence of pericarditis. Although pericardial effusions are common, tamponade is rare. Delayed atrioventricular (AV) conduction manifested as varying degrees of AV block is almost always transient, resolves spontaneously, and is easily reversed with atropine.

Subcutaneous nodules, although one of the major manifestations of rheumatic fever, are not pathognomonic for the disease. Similar findings are found in patients with collagen vascular disorders such as rheumatoid arthritis and systemic lupus erythematosus. Subcutaneous nodules rarely occur as an isolated finding. They most often occur in patients with severe rheumatic carditis, usually several weeks after its onset. The nodules themselves are firm, round, and painless, measuring from 0.5 to 2.0 cm. They occur in crops of one to four dozen and are usually located over bony surfaces, prominences, or tendons. Common sites are the extensor surfaces of the fingers and toes and the flexor surfaces of the wrists and ankles, as well as overlying the spinous processes of the vertebrae. They usually persist for 1 to 2 weeks, rarely more than 1 month. Erythema marginatum is an uncommon manifestation of rheumatic fever. It presents as a nonpruritic, nonpainful, macular, blanching light pink skin eruption most commonly found on the trunk and proximal portions of the extremities. Lesions rarely extend beyond the elbows and knees, and the face is generally spared. The margins spread outward in serpiginous fashion with central clearing. The lesions may change in a manner of minutes and for this reason have been likened to “smoke rings.” Although individual lesions may wax and wane, the process generally resolves within 4 weeks.

Chorea (Sydenham chorea, St. Vitus dance) is a neurologic disorder seen in fewer than 10% of patients with acute rheumatic fever. The chorea consists of involuntary, uncoordinated, purposeless, rapid movements that are associated with generalized muscular weakness and emotional lability. The choreiform moments tend to disappear with sleep and may be suppressed by sedation. Sydenham chorea often occurs together with rheumatic carditis but hardly ever with arthritis. In postpubertal women, chorea may rarely be the sole manifestation of rheumatic fever. Patients with “pure chorea” reportedly have a high incidence of rheumatic heart disease.

Chorea appears only after a relatively long latent period, as long as 1 to 6 months after the antecedent streptococcal infection. Chorea may last anywhere from 1 week to more than 2 years, but it usually is approximately 8 to 15 weeks in duration. In patients with “pure chorea,” minor disease manifestations of acute rheumatic fever such as elevated erythrocyte sedimentation rate or C-reactive protein are notably absent, most likely as a result of the long lag period before the onset of the disorder.

Pathobiology

The streptococcal origin of acute rheumatic fever is firmly established. However, proof of this relationship is indirect because group A streptococci are not recovered from the lesions of rheumatic fever, and no experimental model of the disease has been developed. Nevertheless, compelling epidemiologic, clinical, immunologic, and prophylactic treatment data strongly support a causal relationship between group A streptococcal infection of the upper respiratory tract and rheumatic fever. Epidemics of streptococcal pharyngitis and scarlet fever have a close temporal relationship with epidemics of rheumatic fever. Patients with rheumatic fever usually give a history of sore throat, and those who do not almost always have serologic evidence of recent group A streptococcal infection. Previous studies demonstrated that both initial and recurrent bouts of rheumatic fever did not occur without a significant streptococcal antibody response, the magnitude of which correlated well with the risk of subsequently developing rheumatic fever (100,109). Finally, possibly the most convincing evidence resides in the observation that initial and recurrent attacks of rheumatic fever can be prevented by either penicillin administration in the case of streptococcal pharyngitis or continuous chemoprophylaxis against future streptococcal infection in those patients with a history of rheumatic fever (35,44).

Investigators generally accept that the antecedent group A streptococcal infection leading to subsequent rheumatic fever must be located in the upper respiratory tract. Streptococcal skin infections such as pyoderma or impetigo rarely, if ever, lead to rheumatic fever (117,128). Moreover, streptococcal strains causing acute rheumatic fever and acute glomerulonephritis in the same population are different in terms of their M protein serotype (16). This finding has fostered the notion of the existence of “rheumatogenic” streptococcal strains, with the M protein serotype representing a significant virulence factor in terms of rheumatogenic potential. The hyaluronic acid capsule also represents a significant virulence factor such that highly mucoid strains of streptococci rich in M protein (e.g., types 1, 3, 5, 6, and 18) are most commonly associated with epidemics of rheumatic fever. In addition, lipoteichoic acid present on the surface of the group A streptococcus functions as an adhesion molecule by facilitating the binding of the bacterial organism to pharyngeal epithelial cell surface membranes and initiating the infection process. However, the M protein is considered to be the major virulence factor of the group A streptococcus, and more than 80 different group A streptococcal serotypes exist based on antigenic differences in the M protein molecule. Strains lacking M protein are avirulent. It is present in the cell wall of the streptococcal organism and has been shown to protrude from the cell surface as a component of the hairlike fimbriae. The M protein functions as a virulence factor by conferring resistance to phagocytosis. This is accomplished by diminishing complement activation by the alternative pathway and thus preventing activated complement from being deposited on the bacterial surface. In addition, one study demonstrated purified protein M5 to be a potent human T-cell mitogen in a process involving binding of M protein fragments to antigen-presenting cells using class II major histocompatibility complex (MHC) molecules (124). Subsequent clonal T-cell expansion then occurs in a manner similar to that described for other “superantigens” such as streptococcal enterotoxin B (13,116). This finding has implications for the autoimmune origin of rheumatic fever because clonal T-cell subsets responding to M protein may have shared specificity for cardiac or joint tissue resulting in autoimmune injury.

Despite abundant evidence of group A streptococcus as the etiologic agent in rheumatic fever, the exact pathophysiologic mechanism in which the pharyngeal infection initiates the rheumatic disease process remains unclear. Through the years, several theories have been postulated. These include (a) direct invasion by group A streptococcal organisms, (b) toxic effects of somatic and extracellular streptococcal products, (c) an immune complex–mediated serum sickness–like reaction, and (d) autoimmune phenomena through mechanisms of molecular mimicry involving cross-reactivity of streptococcal antibodies and host tissue.

The possibility that the lesions of rheumatic fever are a manifestation of direct streptococcal tissue invasion has always been an attractive hypothesis but has not been supported by experimental and clinical data. Streptococci are not visible histologically, nor can the organisms be reliably cultured from carefully prepared specimens isolated from patients with rheumatic fever. In addition, long-term antibiotic therapy administered in an effort to eradicate the streptococcal organism has not been shown to alter the course of acute rheumatic fever or the subsequent development of rheumatic heart disease.

Group A streptococcus elaborates many extracellular products including the following: streptolysins S and O; deoxyribonucleases (DNases) A, B, C, and D; hyaluronidase; streptokinase; proteinase; nicotinamide adenine deaminase; amylase; and esterase. Most of these substances are antigenic. Indeed, the most familiar of these, streptolysin O, DNase B, hyaluronidase, and streptokinase, stimulate specific antibodies that are used to obtain serologic evidence of recent streptococcal infection in patients thought to have acute rheumatic fever or glomerulonephritis. Some of these extracellular products, such as streptolysin, are capable of inducing tissue injury. However, because of the lack of an acceptable experimental model of rheumatic fever, theories concerning the direct action of toxins in the pathogenesis of rheumatic fever have been extremely difficult to confirm. Nonetheless, many students of rheumatic fever believe that some form of toxic injury to the heart and joints does play an important role during initiation of the disease process. Indeed, secreted cytokines such as tumor necrosis factor-a IL-8, and IL-6 may play a significant role in the pathophysiology of rheumatic fever (133).

The most popular theory for the pathogenesis of rheumatic fever, a theory supported by a considerable body of indirect laboratory and clinical evidence, proposes that immunologic mechanisms play a central role in the disease process. Rheumatic fever is thought to be caused by a hyperimmune host reaction to streptococcal antigenic challenge involving both the humoral and cell-mediated arms of the immune system. Production of antibodies directed against epitopes common to both streptococcal antigens and human tissue is believed to result in host cellular injury (43). Indeed, although the precise mechanisms remain unclear, rheumatic fever is believed to represent the prototypic postinfectious autoimmune disorder.

Rheumatic fever does not occur without a significant streptococcal antibody response. The mean antibody titer to just about every streptococcal antigen is elevated during an attack of rheumatic fever. Formation of immune complexes leading to a serum sickness–like syndrome with polyarthritis, rash, fever, hematuria, and elevated acute-phase reactants is postulated to occur. Indeed, the signs and symptoms of rheumatic fever bear similarities to those of other immune complex disorders such as systemic lupus erythematosus and rheumatoid arthritis. Circulating immune complexes were detected in 89% of acute-phase sera taken from patients with rheumatic fever (134). However, in contrast to other immune complex disorders, complement levels in patients with rheumatic fever tend to be normal. Although immune complex

deposition may be a contributing factor, this mechanism seems inadequate in explaining all the clinical and pathologic manifestations of rheumatic fever.

Since 1970, much attention has focused on autoimmune phenomena associated with rheumatic fever. Interest in this area arose from the finding that sera from some, but not all, patients with rheumatic fever or rheumatic heart disease contain antibodies that are reactive with human heart tissue (64). However, these antibodies are also present in low titers in the sera of patients with uncomplicated streptococcal pharyngitis. Furthermore, similar antibodies are also identified in patients with other systemic inflammatory disorders involving the heart, such as the postcardiotomy and postmyocardial infarction syndromes. Despite these problems, the observation that the myocardium of patients with severe rheumatic carditis contains surface-bound immunoglobulins and complement is irrefutable and lends credence to the importance of circulating heart-reactive antibodies in the pathogenesis of the disease process (66).

Heart-reactive antibodies appear to be directed against a variety of different antigenic determinants in heart tissue. Many of these antibodies display cross-reactivity with group A streptococcal antigens. Kaplan demonstrated that antisera to streptococcal cell wall components raised in rabbits cross-reacted with the sarcolemma and subsarcolemmal sarcoplasm of human cardiac myofibers as well as skeletal muscle and vascular smooth muscle (63). Similarly, goat antisera raised against human heart tissue showed cross-reactivity with streptococcal cell walls (67). Moreover, immunoreactivity as determined by immunofluorescence was abolished by prior absorption with streptococcal cell wall preparations. Antigenic determinants responsible for the production of heart-reactive antibodies appear to reside in the cell wall or cell membrane of the group A streptococcus (65,135). Streptococcal membrane polypeptides recognized by heart-reactive antibodies have been purified (126).

Emphasis has shifted to the epitopes residing within the M protein molecule as the source of heart-reactive antibodies possibly responsible for autoimmune tissue injury. Indeed, antisera directed against type-specific M protein recognized antigenic determinants shared by cardiac tissue. These antigenic determinants were reportedly found residing within myosin, actin, and DNA molecules of cardiac cells (30). Besides stimulation of the formation of heart-reactive antibodies, studies demonstrated cross-reactivity of M protein antisera with joint tissue. Rabbit antisera directed against purified protein M5 as well as M protein–specific antibodies from patients with rheumatic fever were found to cross-react with murine chondrocytes, cartilage, and synovium. Antibody binding was associated with complement fixation and provides further evidence that M protein cross-reactive antibodies may potentially be involved in the pathogenesis of rheumatic fever and arthritis (10). Cardiac myosin has been recognized as one of the major antigens recognized by the cross-reactive antistreptococcal autoantibodies (26). One study showed that antistreptococcal antibodies cross reactive with *N*-acetyl-D-glucosamine and myosin were cytotoxic for human valvular endothelial cells and reacted with human valvular endothelium and underlying basement membrane (46).

In addition to M protein antibodies, antibodies directed against streptococcal polysaccharide and glycoproteins have been shown to cross-react with heart valve glycoprotein (48). This finding may be significant as patients with rheumatic valvulitis have persistently elevated titers of antibodies directed against group A streptococcal carbohydrate moieties, titers that decrease after valve resection (38). Finally, sera from patients with chorea have been demonstrated by immunofluorescence to contain antibodies that cross-react with cytoplasm of subthalamic and caudate nucleus neurons; sera from patients with uncomplicated streptococcal pharyngitis were nonreactive (60).

Pathologic Findings

The acute phase of rheumatic fever is characterized by diffuse, exudative, and proliferative inflammatory reactions involving the heart, joints, and skin. The magnitude of histologic changes on tissue specimens does not correlate well with the severity of clinical manifestations. During the early stages of the disease, one sees disruption of collagen fibers, inflammatory cellular infiltrate, and principally lymphocytic and fibrinoid degeneration. It is shortly after this point that the only pathognomonic lesion of rheumatic fever appears: the myocardial Aschoff nodule (6). The Aschoff nodule is a perivascular inflammatory focus consisting of large mononuclear cells and multinucleated giant cells surrounding a central necrotic zone. The importance of Aschoff nodules is unclear because they persist for years after a rheumatic attack and do not seem to correlate with the severity of rheumatic carditis. Besides the Aschoff nodule, findings of myopericarditis and endocarditis may be present. Valvular involvement begins as swelling and inflammatory cellular infiltration of the leaflets and chordal structures with small verrucae along the lines of cusp closure. With time, the valve becomes thickened, deformed, and rigid. Chordal structure becomes shortened with fusion of valve leaflets. The end result of the process is development of valvular stenosis or insufficiency and rheumatic heart disease.

Joint involvement during the acute phase of rheumatic fever consists of swelling and edema of the articular and periarticular structures with serous effusion into the joint spaces but without erosion of the joint surface, pannus formation, or joint destruction. Histologically, one sees focal cellular infiltrates consisting of lymphocytes and polymorphonuclear leukocytes as well as fibrinoid lesions. The subcutaneous nodules consist of a central zone of fibrinoid necrotic material surrounded by histiocytes, fibroblasts, and perivascular collections of inflammatory cells. These lesions appear to resemble myocardial Aschoff bodies. Central nervous system changes include arteritis, cellular degeneration, perivascular inflammatory cell infiltration, and occasional petechial hemorrhages. These changes are generally unimpressive and are not correlated with the presence of chorea.

Diagnosis

The correct diagnosis in patients with suspected acute rheumatic fever is essential because of important implications for both prognosis and short- and long-term treatment. The diagnosis is made more difficult by the absence of pathognomonic clinical or diagnostic findings in rheumatic fever. The diffuse inflammatory nature of this disease has clinical features in common with various other disorders including systemic lupus erythematosus, serum sickness, rheumatoid arthritis, infectious arthritis, infective endocarditis, myocarditis, sickle cell crisis, Lyme disease, and drug sensitivity. The criteria for the diagnosis of acute rheumatic fever, originally formulated by T. Duckett Jones in 1944 (62) and most recently revised by the committee of the American Heart Association (93), use the major and minor disease manifestations along with documented evidence of recent streptococcal infection. These criteria are summarized in Table 44.1. If there is evidence of preceding group A streptococcal infection, then the existence of two major manifestations or of one major and two minor manifestations indicates a high probability of the presence of rheumatic fever. Because of the similarity of rheumatic fever with other systemic inflammatory disorders, the importance of documenting recent group A streptococcal infection cannot be overemphasized. The absence of such supporting evidence makes the diagnosis doubtful, except when rheumatic fever is discovered only after a long latent period from the antecedent infection (e.g., Sydenham chorea or indolent carditis) or in rare cases of suspected recurrent rheumatic fever. In patients with a reliable history of rheumatic fever or rheumatic heart disease, a presumptive diagnosis of rheumatic fever can be made with a single major manifestation or several minor manifestations as long as supporting evidence for recent group A streptococcal infection is provided.

Major Manifestations	Minor Manifestations
Carditis	Clinical
Polyarthritis	Fever
Chorea	Arthralgias
Erythema marginatum	
Subcutaneous nodules	Laboratory
	Elevated acute-phase reactants
	Erythrocyte sedimentation rate
	C-reactive protein
	Prolonged PR interval
Plus supporting evidence of antecedent group A streptococcal infection: positive throat culture or rapid streptococcal antigen test showing elevated or rising streptococcal antibody titer	

From Special Writing Group of the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. Guidelines for the diagnosis of initial attack of rheumatic fever. JAMA 1992;268: 2069. Copyright 1992, American Medical Association, with permission.

TABLE 44.1. Revised Jones Criteria for the Diagnosis of Rheumatic Fever

Evidence of streptococcal infection may include a positive finding on throat culture or rapid antigen test for group A streptococci or the demonstration of elevated or rising streptococcal antibody titers. Culture evidence for pharyngeal group A streptococcal infection is unreliable because only about 25% of patients have a positive culture at the time of the diagnosis of rheumatic fever. Furthermore, information from cultures does not distinguish between recent infection, which may be a prelude to rheumatic fever, and chronic nonpathologic pharyngeal colonization. Therefore, supporting evidence for recent streptococcal infection usually rests with the demonstration of elevated or rising antibody titers to streptococcal antigens, most commonly streptolysin O, DNase B, streptokinase, and hyaluronidase. Streptococcal pharyngitis elicits an antibody response to these substances in most patients. The antibody titer reaches a peak 2 to 3 weeks after the acute infection, remains at a plateau for 3 to 6 months, and then gradually declines, reaching normal levels in another 3 to 6 months (Fig. 44.3).

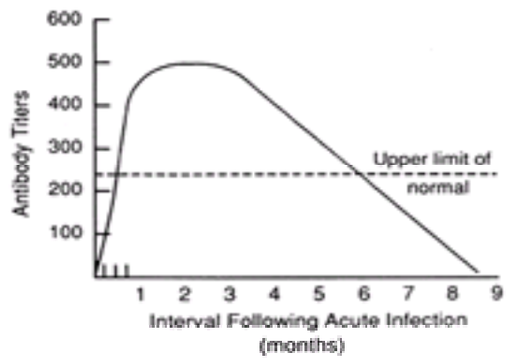

Figure 44.3. Temporal pattern of antibody response to group A streptococcal antigens. (From Ayoub E. Immune response to group A streptococcal infections. *Pediatr Infect Dis J* 1991;10:515, with permission.)

Serum samples obtained at the time of presentation usually display elevated streptococcal antibody titers. A significant increase in antibody is usually defined as a rise in titer of two or more dilution increments between acute-phase and convalescent-phase samples, regardless of the absolute magnitude of the antibody titer. The antistreptolysin O test is the most widely used and best standardized test. Titers of at least 250 Todd units in adults and 333 Todd units in children should be considered to be elevated. With this assay, approximately 80% of patients with acute rheumatic fever have an elevated antistreptolysin O titer. If a second confirmatory antistreptococcal antibody assay is used, this percentage increases to 90%. If a series of three antibody assays is used, the figure exceeds 95%.

More recently, a slide hemagglutination test using five group A streptococcal antigens attached to sheep erythrocytes was developed. Widespread availability and ease of performance have made this assay a popular office-based test for the determination of recent streptococcal infection. However, positive test results should be confirmed by more conventional antistreptococcal assays. A novel imaging technique using radiolabeled Fab fragments of monoclonal antimyosin antibodies for the detection of active rheumatic myocarditis has shown promising results but is limited by its lack of specificity (89).

Treatment

The management of patients with rheumatic fever is in part determined by the severity of the clinical manifestations of the disease. Therapy is directed toward eradicating residual group A streptococcal organisms, interrupting and suppressing the inflammatory process, and treating signs and symptoms of congestive heart failure, if they exist. None of these measures alters the course of the rheumatic attack or the probability of the subsequent development of rheumatic heart disease. However, good supportive care can affect morbidity and mortality in a positive manner.

Once the diagnosis of rheumatic fever is made, antimicrobial therapy should be instituted to eradicate any residual group A streptococcal organisms, even if cultures are negative. Parenteral therapy is preferred. A single intramuscular injection of 1.2 million units of penicillin benzathine or of 600,000 units of procaine penicillin each day for a 10-day course is considered an effective regimen. An erythromycin-based drug can be used in penicillin-allergic patients. Initial treatment should be followed by continuous long-term chemoprophylaxis to provide protection from reinfection.

All patients should be kept on bed rest for the first 3 weeks of the illness. After this initial period, the degree of physical activity should be tailored to the severity of the disease manifestations. Patients with carditis without cardiomyopathy or heart failure should be continued on bed rest for a minimum of 4 weeks after the carditis is detected. More prolonged periods of activity restriction are required in patients with severe carditis with overt signs of heart failure. The mainstay of therapy for patients with heart failure consists of the usual measures of fluid and salt restriction, supplemental oxygen, diuretics, digitalis, and probably afterload reduction.

Antiinflammatory therapy for acute rheumatic fever generally consists of salicylates and corticosteroids. The selection of a particular agent is not critical to the outcome of the rheumatic attack. Both classes of drugs are effective means of controlling the toxic manifestations of the disease and contributing to the comfort of the patient. A study comparing prednisone and acetylsalicylic acid for acute rheumatic fever did not demonstrate a significant therapeutic advantage of one agent over another (25). Patients with only mild arthralgias or arthritis without evidence of carditis are best treated with analgesics alone such as codeine or propoxyphene. Patients with more severe constitutional symptoms, arthritis, or mild carditis without overt heart failure should be treated with salicylates. A starting dose of 100 to 125 mg/ kg per day in children and 6 to 9 g per day in adults weighing 70 kg or more given in four or five divided doses is recommended. This dose of salicylate should be continued until an acceptable clinical response is achieved. Thereafter, the dose may be slowly decreased while the clinical and laboratory indices of inflammatory disease activity are closely monitored. Patients who do not obtain adequate relief with salicylates or who cannot tolerate the medication because of gastric irritation, gastrointestinal bleeding, or symptoms of salicylism should be switched to corticosteroids.

Patients with more severe forms of carditis as determined by chest x-ray studies, endomyocardial biopsy, echocardiography, or gated blood pool scanning as well as clinical signs and symptoms of heart failure should be treated immediately with corticosteroids. Starting daily doses of 40 to 120 mg of prednisone in four divided doses should be given to all patients regardless of age and should be adjusted according to clinical response. Parenteral preparations such as methylprednisolone or dexamethasone should be dose adjusted according to relative potency.

Once the clinical manifestations of the inflammatory process are under control with salicylates or corticosteroids, therapy should continue until laboratory indices of disease activity (i.e., erythrocyte sedimentation rate) approach normal. In several weeks, therapy may be slowly tapered. During this period, clinical or laboratory evidence suggesting reactivation of the inflammatory process may occur. This “rebound” phenomenon is seen more commonly with corticosteroid administration. Rebounds generally occur within 2 weeks of cessation of the antiinflammatory drugs. Rebounds occurring more than 5 weeks after cessation of therapy must be distinguished from rheumatic disease recurrence.

Patients with Sydenham chorea respond poorly to antirheumatic therapy. The use of haloperidol has been successful in some cases, and most patients respond to an initial dose of 0.5 to 1 mg per day, with increases of 0.5 to 1 mg per day every 3 days up to a maximum of 5 mg per day (123). Management of these patients is directed more toward supportive measures such as providing a quiet, nonstressful, restful environment.

Prognosis and Prevention

The natural course of rheumatic fever is highly unpredictable, and it is impossible to predict during the early stages of the disease. Approximately 75% of rheumatic attacks resolve within 6 weeks, 90% resolve within 12 weeks, and fewer than 6% persist more than 6 months. Those patients with a prolonged course of illness lasting more than 6 months usually have more slowly resolving chorea or chronic unremitting carditis (122). After a period of 2 months has elapsed after normalization of clinical and laboratory indices of disease activity and cessation of antiinflammatory therapy, rheumatic fever does not recur without recurrent streptococcal infection.

The prognosis is excellent for patients without carditis during the initial attack. Rheumatic heart disease is generally not observed in patients who did not develop carditis during the initial bout of rheumatic fever (125).

The best therapy for rheumatic fever is prevention. The goal of primary prevention is the accurate diagnosis of group A streptococcal infection and the use of appropriate antibiotics for eradicating the organism. Convincing clinical data demonstrate that acute rheumatic fever can be prevented by prompt treatment of group A streptococcal tonsillopharyngitis (44,129). Primary prevention for high-risk communities with a high prevalence of rheumatic fever can be instituted with an intramuscular injection of penicillin G benzathine as the first treatment of choice because of the poor compliance with the 10-day protocols for oral penicillin (28). A single intramuscular injection of benzathine benzylpenicillin, 600,000 units (in patients weighing less than 27 kg) or 1.2 million units (in patients weighing more than 27 kg), or oral phenoxymethylpenicillin (penicillin V), 250 mg two to three times daily for children or 500 mg two to three times daily for adolescents and adults, is effective (28). Erythromycin may be given for patients allergic to penicillin. Throat culture or tests for group A streptococcal antigen may provide a reliable negative predictive test in patients at sufficiently low clinical risk and may preclude the need for unnecessary antibiotics. False-positive tests are the result of subclinical colonizing strains or non–group A streptococci (groups C and G). Follow-up cultures are not recommended when appropriate treatment and compliance are documented (118).

Patients who have experienced a bout of rheumatic fever are at high risk of recurrent bouts of rheumatic fever. Recurrence rates decline with the length of time since the last attack (121,122). Nevertheless, patients with a history of rheumatic fever are at increased risk of recurrence well into adult life. Features influencing rheumatic fever recurrences include the presence of residual rheumatic heart disease and the magnitude of the immune response to the antecedent streptococcal infection. The committee of the American College of Cardiology/American Heart Association recommends the following for the duration of secondary rheumatic fever antibiotic prophylaxis: (a) rheumatic fever with carditis and persistent valvular disease: more than 10 years since last episode of rheumatic fever and at least until age 40 years of age, occasionally lifelong prophylaxis if at high risk and likely to come into contact with populations with increased prevalence of streptococcal infection (i.e., day care

workers, teachers); (b) rheumatic fever with carditis but no residual valvular disease: 10 years since last episode or well into adulthood, whichever is longer; and (c) rheumatic fever without carditis: 5 years or until age 21, whichever is longer (11,18). Continuous long-term antimicrobial prophylactic therapy may be instituted using one of the regimens shown in Table 44.2. The decision to withdraw antibiotics should be carefully individualized after assessment of the patients' overall risk for repetitive exposures. This prophylactic regimen for the prevention of recurrent rheumatic fever, however, does not substitute for the standard bacterial endocarditis prophylaxis required for patients with documented valvular rheumatic heart disease (29).

Agent	Dose	Mode
Penicillin G benzathine	1,200,000 units	intramuscula every 4 wk
	or	
Penicillin V	250 mg b.i.d.	Oral
	or	
Sulfadiazine	0.5 g q.d. for patients <60 lb; 1.0 g q.d. for patients >60 lb	Oral
For patients allergic to penicillin and sulfadiazine:		
Erythromycin	250 mg b.i.d.	Oral

Dajani A, Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease of the American Heart Association. Treatment of acute streptococcal pharyngitis and prevention of rheumatic fever: a statement for health professionals. *Pediatrics* 1995;96:748–754, with permission.

TABLE 44.2. Secondary Prevention of Rheumatic Fever

Work continues on identifying rheumatogenic strains of group A streptococcus in an effort to develop an effective multivalent vaccine. Research has focused on identifying and characterizing the streptococcal M protein, the antigenic stimulus that produces antibodies that confers immunity to the streptococcus organism (119). However, the development of an effective vaccine has been hindered by the vast number of M protein serotypes and the potential for producing possible adverse autoimmune reactions. Emerging strategies for overcoming these problems include identifying and combining multiple epitopes from the N terminus and the conserved carboxy-terminal regions of the M protein, and eliminating host cross-reactive epitopes (21). Identifying a genetic marker for rheumatic fever susceptibility is also on the horizon, and this would be useful in identifying those patients at highest risk for acquiring this disease and those who would benefit most from aggressive prophylactic regimens (80).

MYOCARDITIS

Myocarditis is defined as an inflammatory process involving the heart. The inflammation may be restricted to the heart, or, more commonly, it represents the cardiovascular component of a systemic disease process. Inflammation is often not restricted to the myocardium but may involve the endocardial structures or pericardium to varying degrees, in which case terms such as *pancarditis and myopericarditis* are occasionally applied.

The incidence, prevalence, and predisposing factors for myocarditis are difficult to ascertain for several reasons, including the following: (a) the existence of multiple, distinct, infectious and noninfectious causes; (b) the finding that most cases are mild and subclinical and are not brought to the attention of the physician; (c) the lack of highly sensitive and specific diagnostic criteria; (d) the unresolved antecedent relationship with the pathogenesis of cardiomyopathy; and (e) differing opinions on the histologic diagnosis of myocarditis. Nonetheless, retrospective analysis of unselected autopsy data suggests that myocarditis is far more prevalent than expected. Autopsy studies performed on young, healthy soldiers during the course of World War II demonstrated histologic evidence of myocarditis in 3.5% to 10.0% (50,106). More recent studies using more stringent histologic guidelines for the diagnosis of myocarditis supported a much lower prevalence (51). Studies of children experiencing sudden, unexpected, nonaccidental death revealed myocarditis in 17% to 21% (8,91).

Clinical Signs and Symptoms

The clinical presentation of myocarditis is variable and in part depends on the severity of myocardial involvement. Signs and symptoms range from asymptomatic to rapidly progressive florid congestive heart failure leading to death. Patients often complain of nonspecific, constitutional symptoms including fever, weakness, fatigue, dyspnea, palpitations, and chest discomfort. A history of viral prodrome during the preceding 1 to 3 weeks can sometimes be elicited. Chest pain usually reflects concomitant pericarditis. Occasionally, the clinical presentation may be confused with acute myocardial infarction.

On physical examination, fever and tachycardia are usually present. The first heart sound is often diminished and may be associated with an S_3 gallop and an apical systolic murmur. Signs consistent with right-sided or left-sided heart failure with pulsus alternans, pulmonary congestion, jugular venous distention, hepatomegaly, ascites, and peripheral edema may be present in patients with more severe disease. A pericardial friction rub may be audible with concomitant pericarditis. Pulmonary and systemic emboli have also been noted to occur. Arrhythmias may appear and are occasionally associated with sudden death.

Pathobiology

Unlike rheumatic fever, in which the group A streptococcus is the singular etiologic agent, myocarditis has been associated with many infectious agents as well as with collagen vascular disorders, drugs, heavy metals, toxins, and hypersensitivity reactions. Myocardial inflammation may well represent a final common pathway response to a host of different insults. The many potential causes of myocarditis are summarized in Table 44.3. In North America, viral agents are believed to be by far the most commonly identified causative agent in myocarditis. Moreover, myocarditis resulting from more recently identified agents such as human immunodeficiency virus (HIV) or *Borrelia burgdorferi* is becoming increasingly recognized. In South America, *Trypanosoma cruzi,* the causative agent of Chagas disease, is considered the most common cause of myocarditis and cardiomyopathy.

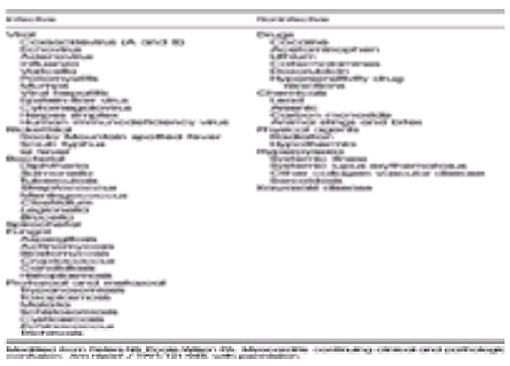

TABLE 44.3. Causes of Myocardial Inflammation

Of the viruses, the picornaviruses, notably the enteroviruses such as coxsackievirus A and B and echovirus account for most cases of myocarditis. Coxsackievirus B may cause up to 50% of all cases (53). Other viruses such as cytomegalovirus, influenza A and B, rubella, mumps, and varicella virus have also been associated with myocarditis. However, definitive evidence of a viral origin in individual patients is difficult to obtain and is usually based on the demonstration of rising titers of virus-specific antibodies. Isolation and culture of virus from tissue specimens are unusual, and immunohistochemical techniques attempting to localize viral antigens in myocardial tissue have proved unreliable. However, studies using recombinant DNA techniques have detected enteroviral RNA in a subset of patients with myocarditis and cardiomyopathy (20,61). For most cases of myocarditis, no specific etiologic agent can be identified. These cases, referred to as *idiopathic myocarditis* are presumed to be viral in origin (81). Myocarditis, unless referred to in more specific terms, generally implies a viral origin.

Theories concerning the pathogenesis of virus-induced myocardial inflammation and injury include (a) cardiotropic viral infection leading to viral replication and cell lysis, and (b) humoral and cell-mediated autoimmunity directed against both normal myocytes (through shared epitopes) and virus-infected cardiac cells expressing structurally altered antigens or neoantigens. Viral infection may lead to active intracellular viral replication, with ultimate cytolysis and myocyte necrosis. This

mechanism is believed to be particularly important during the early phases of the disease before specific immune responses occur. However, virus particles are hardly ever seen by electron microscopy, and the ability to culture virus or to detect virus-specific antigens from myocardial tissue of patients with myocarditis lacks reproducibility. Nevertheless, most investigators believe that the myocarditic process begins with a cardiotropic viral infection, replication, and cell lysis. Convincing animal data using a murine model of coxsackievirus B–induced myocarditis support this theory (59). During this initial phase of the disease, before mounting a significant immune response, inflammatory cellular infiltrates are absent. After the initial phase of the infection, the humoral and cell-mediated arms of the immune system are activated. Virus-specific antibodies are produced, resulting in clearance of virus through the monocyte-macrophage system. During this initial phase of the inflammatory process, the mechanisms of autoimmunity are believed to be implicated. Humoral and cell-mediated autoimmunity may be directed against normal and virus-infected myocytes. This results in mononuclear cell infiltration of the myocardium, and this, along with myocyte cell death, constitutes the histologic criteria for the diagnosis of myocarditis.

Sera taken from patients with myocarditis were found to contain antibodies reactive with both cardiocytes and viral antigens, and they were found to be cytotoxic in the presence of complement (76). In addition, suppressor T-cell activity is postulated to be reduced in these patients (39). More recently, sera from patients with myocarditis were found to have an autoantibody reactive with the adenosine diphosphate–adenosine triphosphate carrier protein of the inner mitochondrial membrane and were found to impair the performance of isolated guinea pig hearts (108). Further evidence for immune-mediated mechanisms is the demonstration of increased expression of the MHC antigens within the myocardium of patients with active myocarditis (56). Studies have implicated the role of cytokines such as tumor necrosis factor-a and ILs in the induction of apoptosis of myocardial cells (20).

The importance of T-lymphocyte–mediated autoimmunity in myocarditis is best demonstrated in the murine model. T-cell–deficient, athymic mice, or mice treated with antithymocyte serum, demonstrated less myocardial inflammation in response to injection of coxsackievirus B3 than did mice with normal T-cell function. T-cell–deficient mice reconstituted with coxsackievirus B3–sensitized T cells developed a more severe form of myocarditis (71).

Pathologic Findings

The pathologic findings in myocarditis are varied and depend on the etiologic agent and the severity of the inflammatory process. In patients with severe disease, the myocardium may be flabby with focal hemorrhages. In patients with chronic disease, the heart may be hypertrophied with four-chamber dilatation. Histologically, the presence of an inflammatory infiltrate associated with myocyte necrosis is characteristic of myocarditis (Fig. 44.4). The inflammatory infiltrate consists of a variety of cell types including polymorphonuclear cells, lymphocytes, macrophages, plasma cells, eosinophils, and giant cells. Myocyte damage may be manifested as necrosis with myocyte debris, degeneration, vacuolization, or cell disruption with infiltration of inflammatory cells (77).

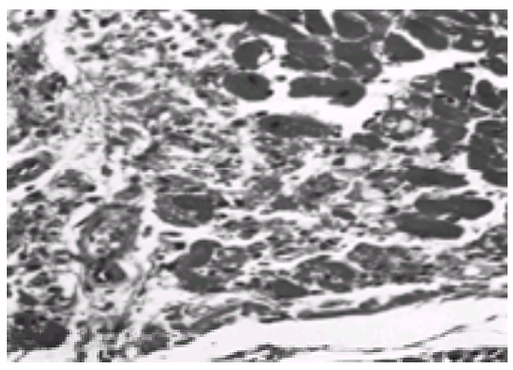

Figure 44.4. Hematoxylin and eosin–stained section from a patient with myocarditis. Note the extensive inflammatory cellular infiltrate with myocyte necrosis. (Original magnification ×250)

Diagnosis

Rigid criteria for the diagnosis of myocarditis based on clinical, laboratory, or diagnostic test findings do not exist. Such information serves to suggest the diagnosis but not to confirm it. Presentation of a patient with a compatible clinical syndrome of constitutional symptoms, weakness, fever, dyspnea, palpitations, and chest pain 1 to 3 weeks after a viral illness should alert the physician to the possibility of the diagnosis. Physical findings of tachycardia, new heart murmurs, a pericardial friction rub, or findings consistent with acute heart failure serve to support the diagnosis further.

Elevation in acute-phase reactants (erythrocyte sedimentation rate, total leukocyte count) is often noted. Serum levels of cardiac enzymes (creatine phosphokinase, lactic dehydrogenase) may be normal or elevated, depending on the extent of myocardial damage. Virus may rarely be isolated from various tissue or body fluid samples. A fourfold rise in viral type–specific neutralizing antibodies in paired acute-phase and convalescent-phase sera is sometimes observed. The chest x-ray film may show signs of cardiac enlargement or pulmonary vascular engorgement. The electrocardiogram usually reveals nonspecific changes in the ST segments and T waves. Arrhythmias and AV conduction disturbances are common, particularly in cases associated with severe heart failure. Echocardiography is helpful in assessing chamber enlargement, depression of ventricular performance, valvular involvement, and the presence of pericardial effusions.

Technetium-99m pyrophosphate and gallium-67 citrate scanning may occasionally demonstrate increased myocardial uptake. Newer nuclear imaging techniques using indium-111–radiolabeled Fab fragments of monoclonal antimyosin antibody preparations show promise in identifying patients with suspected myocarditis (32).

In the end, definite proof of the presence of myocarditis requires histologic examination of myocardial biopsy material. However, the sensitivity of endomyocardial biopsy in detecting suspected myocarditis has been questioned. In one study, definitive histologic evidence of myocarditis was found in only 30% of patients with clinical myocarditis (4). In another study, only 11% of patients with biopsy-proved myocarditis were suspected clinically to have myocarditis (90). The difficulties associated with the histologic diagnosis of myocarditis are believed to be related to the focal nature of the inflammation process and variability in histopathologic criteria used to establish the diagnosis. With general acceptance of the Dallas classification system, the histologic diagnosis of myocarditis can only be applied when an inflammatory cellular infiltrate is noted in the myocardium in association with myocyte necrosis or degeneration not typical of the ischemic damage associated with coronary artery disease (5).

Course

Most bouts of myocarditis of any cause either are subclinical or result in a self-limited illness that resolves without any long-term sequelae. Patients with more acute, fulminant myocarditis with heart failure either recover or die within weeks of the onset of the disease. Arrhythmias and progressive congestive heart failure are the usual causes of death in this group of patients.

A third group of patients, estimated to represent between 10% and 15% of patients who recover from diagnosed viral myocarditis, are believed to go on to develop chronic cardiac dysfunction indistinguishable from that of idiopathic dilated cardiomyopathy. Indeed, numerous studies of patients with dilated cardiomyopathy reported biopsy evidence of myocarditis in a significant number of these patients (86,95). Many investigators believe that autoimmune processes activated during the early phases of myocarditis are maintained through persistent and low-level viremia or periodic reactivation of latent virus infection, leading to expression of autoantigens, chronic autoimmune injury, and myocardial damage. Multiple bouts of reactivation myocarditis ultimately result in the development of a cardiomyopathic picture. Indeed, enterovirus RNA has been detected in myocardial biopsy specimens from patients with congestive cardiomyopathy using gene amplification by polymerase chain reaction (61). In summary, some patients with myocarditis develop cardiomyopathy, and some patients with cardiomyopathy have evidence of antecedent myocarditis. How frequently these two processes overlap awaits further study.

Treatment

The therapy for patients with myocarditis is largely supportive and is directed at the major manifestations of the disease process. Patients with myocarditis should have bed rest during the acute phase of this illness. Experimental evidence suggests that exercise worsens myocardial inflammation. Patients with congestive heart failure respond to conventional therapy consisting of supplemental oxygen, diuretics, and digitalis preparations. Animal studies suggested a beneficial effect of angiotensin-converting enzyme inhibitors (85). Rhythm disturbances should be treated with specific antiarrhythmic therapy. Patients with intractable New York Heart Association class IV failure may require inotropic agents (dobutamine, milrinone), and patients with the most severe disease should receive circulatory support devices.

Heart transplantation remains an option for patients with impending death from progressive heart failure or arrhythmias unresponsive to conventional therapy.

The role of antiinflammatory agents remains controversial. Nonsteroidal antiinflammatory agents such as indomethacin, salicylates, and cyclosporine have classically been contraindicated during the early phases of viral myocarditis because they worsen the myocardial damage. Many different immunosuppressive agents such as steroids, cyclosporine, FK-506, and cyclophosphamide have been examined in both animal models and human patients with varying results (69). Several small early studies suggested a beneficial effect in terms of improving clinical signs and symptoms of inflammation as well as histologic evidence of myocarditis on biopsy specimens (77,82). A prospective, randomized trial of patients with idiopathic dilated cardiomyopathy and severe left ventricular dysfunction revealed a modest improvement in left ventricular ejection fraction in those patients with cellular infiltrate observed in myocardial biopsy specimens who were treated with prednisone (96). This study suggested that some patients with cardiomyopathy and a cellular infiltrate in myocardial biopsy specimens have a corticosteroid-responsive inflammatory process in their myocardium. The largest controlled clinical trial to date has been the Myocarditis Treatment Trial, which was a randomized multicenter trial of immunosuppressive therapy for biopsy-proven myocarditis with prednisone and either cyclosporine or azathioprine versus conventional therapy for congestive heart failure. This study revealed no significant differences in left ventricular ejection fraction or left ventricular end-diastolic diameter at 28 or 52 weeks and no significant difference in survival during follow-up between the patients receiving conventional therapy and those receiving immunosuppressive therapy (83). However, this study had significant limitations in execution of its protocol and case selection.

Selected Specific Myocarditides

LYME DISEASE

Lyme disease is a multisystemic infectious disease caused by the spirochete *B. burgdorferi,* which is transmitted by the bite of the *Ixodes dammini* tick (113) (Chapter 79). It is endemic to most regions of the United States and is the most common vectorborne disease in the United States. From 1982 to 1996, more than 99,000 cases were reported to the United States Centers for Disease Control and Prevention, and during this time the incidence of reported cases increased by at least 32 times (23). Disease involvement includes the skin, joints, central and peripheral nervous systems, and the heart. The disease usually begins during the summer with a characteristic skin rash (erythema chronicum migrans), followed in days to months by signs and symptoms of disseminated infection including constitutional symptoms resembling a flulike illness with neurologic, arthritic, and cardiac manifestations. A persistent infection may be seen months to years after the onset of the illness. This late stage of Lyme disease expresses itself as chronic arthritis with persistent neurologic and cutaneous manifestations.

Carditis has been associated with Lyme disease in 4% to 10% of patients. It is usually seen in the second stage of the illness during the disseminated infection. Cardiac involvement may manifest itself as myopericarditis with varying degrees of AV block and myocardial dysfunction (87). AV block, often requiring temporary placement of a pacemaker, is the most common manifestation. Myocardial biopsy specimens may show areas of dense lymphocytic infiltrate with myocyte necrosis. Persistence of the spirochete organism within the myocardium is thought to be an important pathophysiologic mechanism in Lyme carditis. This concept is supported by identification of spirochetes in myocardial biopsy specimens of patients with Lyme carditis (33). In contrast, the late manifestations of Lyme disease including chronic arthritis and neurologic impairment are believed to be, in part, immune related. Studies demonstrated a significant association between class II histocompatibility molecules, particularly HLA-DR2 and HLA-DR4 haplotypes, and the development of chronic Lyme arthritis (112). Specific T-cell subsets directed against spirochetal antigens are postulated to be either clonally deleted, resulting in an ineffective immune response, or cross-reactive with shared tissue epitopes, resulting in autoimmune injury.

The diagnosis of Lyme disease relies on isolation of the spirochete organism or detection of increased or rising titers of specific antispirochetal antibodies (98). The prognosis in Lyme carditis is excellent, with complete resolution of conduction disturbances and cardiac dysfunction the rule. Administration of antibiotics to all patients with this form of carditis is generally recommended (98). Some authors recommend prednisone specifically for those patients with persistent heart block lasting more than 1 week, associated meningoencephalitis, or cardiomegaly. However most of the data to support this recommendation are anecdotal (88).

Vaccination has shown to be effective in preventing Lyme disease in several animal and human studies. In 1990, a whole-cell inactivated *B. burgdorferi* vaccine was licensed for use in dogs. In a 20-month postlicensure study, Lyme disease developed in (1%) of 1,969 vaccinated dogs versus 211 (4.7%) of 4,498 unvaccinated dogs (74). However, because whole-cell vaccines may be associated with severe local reaction in humans, a recombinant immunogenic *B. burgdorferi* protein was chosen for human trials. Antibodies against this protein in the outer coating of the organism (outer surface protein A or Osp A) have been found to be protective and active inoculation with Osp A have prevented *B. burgdorferi* infection. The results of the adult Osp vaccine trial were published in 1998 (114). In this large, multicenter, randomized, double-blind study involving 10,936 persons who lived in Lyme disease–endemic areas in the United States, administration of three injections of the vaccine at 0, 1, and 12 months prevented most definite cases of Lyme disease or asymptomatic *B. burgdorferi* infection. After the third injection, Lyme disease developed in 66 (1.2%) of 5,467 placebo recipients versus 16 (0.3%) of 5,469 vaccine recipients (p = .009), and the vaccine efficacy was 76% (95% confidence interval, 58%, 86%). The Advisory Committee in Immunization Practices has recommended that the Lyme disease vaccine should be considered for patients 15 to 70 years of age who live, work, or travel in moderate-risk to high-risk areas (24). The availability of the vaccine should not detract from the importance of personal precautions for tick bite prevention because the vaccine does not protect against other tickborne disease such as ehrlichiosis and babesiosis. The shortest and the most efficacious dosing schedule for vaccination including boosters has not been clearly defined, and accelerated dosing given in 3 to 6 months have shown encouraging results. Large trials using the vaccine in the pediatric population are currently under way.

CHAGAS DISEASE

Chagas disease (American trypanosomiasis) is the most common form of heart disease in Central and South America. It is also becoming increasingly recognized in the United States, largely because of increased numbers of infected immigrants from endemic regions (55,70). The disease is caused by the protozoan *T. cruzi,* which is transmitted to humans through the bite of an insect vector (reduviid bug). During the acute phase of illness, the patient may be asymptomatic or may experience constitutional symptoms such as fever, diaphoresis, myalgias, nausea, vomiting, diarrhea, dizziness, and palpitations. Signs of congestive heart failure, tachycardia, lymphadenopathy, hepatosplenomegaly, and meningeal irritation may be present. In a few patients, myocarditis develops and may be characterized by cardiomegaly or heart failure. Patients may experience progressive heart failure leading to death over a short period. In this setting, parasites are generally found in the myocardium.

Patients surviving acute infection may enter a latent phase. Up to 30% of patients eventually develop the chronic form of Chagas heart disease, leading ultimately to a clinical picture characterized by a dilated cardiomyopathy. Clinical manifestations of biventricular heart failure are frequently seen, with right ventricular failure usually dominating. Ventricular arrhythmias are frequently found and may result in syncope or sudden death. Classic electrocardiographic abnormalities of right bundle branch block and left anterior hemiblock are seen in up to 80% of patients (103). Examination of myocardial biopsy specimens usually reveals mononuclear cell infiltration and fibrosis, particularly in the area of the sinoatrial and AV nodes, and myocyte degeneration. Myocardial involvement may lead to the formation of left ventricular aneurysms at the apex or adjacent to the mitral annulus, associated with apical thrombus.

The slowly progressive, often indolent nature of Chagas cardiomyopathy that occurs in the absence of parasitic organisms is believed to be autoimmune mediated (104). Animal models have demonstrated the presence of self-reactive cytotoxic T cells after acute infection that were able to lyse normal noninfected heart cells (2).

The diagnosis of Chagas disease usually rests on demonstration of a positive result on complement fixation test or detection of the parasite in the blood during the acute phase of the illness. No specific therapy exists for Chagas cardiomyopathy, although antiparasitic agents such as nifurtimox and benznidazole may be helpful in select patients. The long-term prognosis correlates with the degree of impairment of ventricular function. Patients with mild ventricular dysfunction have a 5-year mortality of 20%. The mortality exceeds 60% in patients with more severe cardiac impairment.

HUMAN IMMUNODEFICIENCY VIRUS–RELATED HEART DISEASE

Cardiac involvement is being increasingly recognized in association with *HIV infection* in the setting of acquired immunodeficiency syndrome (AIDS) and has been extensively reviewed in the literature (1,68). Approximately 50% of patients with AIDS are estimated to experience some form of cardiac involvement during the course of their disease. Mortality from cardiac complications is estimated to be as high as 18% and is usually the result of progressive heart failure or arrhyth- mias. The spectrum of etiologic agents includes a large range of infectious, noninfectious, and neoplastic causes involving any of the myocardial, pericardial, or endocardial structures (Table 44.4). The common cardiac manifestations of AIDS include pericardial effusion, myocarditis, dilated cardiomyopathy, drug-related cardiotoxicity, endocarditis, pulmonary hypertension, and malignant neoplasms. All these cardiac sequelae are expected to increase in prevalence with the improved survival of HIV-infected patients receiving the newer antiretroviral medical regimens (102).

TABLE 44.4. Cardiac Involvement in Acquired Immunodeficiency Syndrome

Reports demonstrated myocarditis to be a common finding in patients with AIDS. Up to 46% of patients with AIDS were found to have histologic evidence of myocarditis at necropsy (101). Most of these patients were asymptomatic, and in most, no clear etiologic agent could be identified. Biopsy specimens usually reveal mononuclear cell infiltrates with focal myocyte necrosis. The origin of AIDS-related myocarditis is unclear. HIV-1 is not usually identified in biopsy tissue from patients with myocarditis by recombinant DNA techniques (12). Many investigators believe that myocarditis is not initiated by HIV-1 but rather by virus strains usually associated with myocarditis in non-HIV–infected individuals such as coxsackievirus B. Myocardial injury is believed to occur by the same mechanism of virus- and immune-mediated injury as described for other forms of viral myocarditis. Besides myocarditis, AIDS is associated with dilated cardiomyopathy. Current thought concerning the pathogenesis of AIDS-related cardiomyopathy suggests that it represents the end point of chronic persistent viral myocarditis and myocardial injury. Indeed, in some series, myocarditis was present in all cases of AIDS-related death with gross evidence of cardiac dilatation (3). Besides infectious myocarditis, antiretroviral drugs have also been implicated with the development of reversible cardiac dysfunction (57).

POSTCARDIAC INJURY SYNDROME

PCIS encompasses a group of disorders characterized by a pericarditis syndrome occurring several days to several months after a myocardial insult as a result of infarction (postmyocardial infarction syndrome, Dressler syndrome), surgery (postcardiotomy syndrome), or trauma (postcardiac trauma syndrome). A similar syndrome is occasionally observed after pulmonary embolism. PCIS was first reported in 1953 in patients undergoing mitral commissurotomy for rheumatic heart disease (110). It was later noted in patients undergoing a variety of cardiac surgical procedures. Investigators recognized that the syndrome did not occur without surgical manipulation of the pericardium. A similar syndrome in patients after myocardial infarction was described by Dressler in 1956 (37). Factors such as young age, previous episode of pericarditis, and a recent viral infection are said to predispose to the development of PCIS. The extent of myocardial injury does not correlate with PCIS. The incidence of PCIS is estimated to be 4% of patients after myocardial infarction and up to 30% of patients after cardiac surgery.

PCIS is characterized by the onset of constitutional symptoms, fever, and pleuritic chest pain occurring 2 to 3 weeks after the initial insult (infection, surgery, or trauma) with a range of 1 week to several months. Physical examination may often reveal pericardial and pleural friction rubs. In the postcardiac surgery setting in which pericardial friction rubs are the rule, the persistence of fever and chest pain suggests of PCIS. Pleural effusions, pulmonary infiltrates, and enlargement of the cardiac silhouette may be noted on chest x-ray studies. The electrocardiogram may reveal ST-segment elevation or T-wave inversions suggestive of pericarditis. Echocardiography is useful in identifying pericardial effusion and tamponade. Elevation of acute-phase reactants, particularly the erythrocyte sedimentation rate and total leukocyte count, is frequently observed.

The precise mechanisms responsible for the pathogenesis of PCIS are unknown. However, an immunologic component has been proposed. This is supported by the following: the presence of a latent period between the initial myocardial insult and the onset of symptoms; the often dramatic response to antiinflammatory drugs, particularly corticosteroids; and the occasional associated relapse after tapering or cessation of steroid therapy. Investigators have proposed that entry of blood into the pericardium induces cellular injury, which after a latent period triggers an autoimmune response to heart cell antigens that leads to release of cellular mediators of inflammation. This concept is supported by several studies demonstrating the occurrence of antiheart antibodies in patients who developed PCIS after infarction and cardiac surgery (40,127). Further studies identified reactive antigenic determinants residing within the major contractile proteins, actin and myosin (31). Finally, some investigators believe that reactivation of latent virus may provide an immune stimulus that serves to initiate the cascade of events leading to inflammation (40).

The diagnosis of PCIS relies largely on clinical criteria. Laboratory and diagnostic test data may provide supportive evidence. The syndrome is usually self-limited and resolves slowly over time, but it may recur. Oral anticoagulants should be discontinued because of the risk of pericardial hemorrhage. Patients with severe symptoms should be placed on bed rest and treated with aspirin or a nonsteroidal antiinflammatory agent such as indomethacin, ibuprofen, or naproxen. In patients with severe incapacitating chest pain or chest pain unresponsive to aspirin or nonsteroidal antiinflammatory agents, corticosteroids are the agents of choice. Patients treated with 60 mg of prednisone per day usually respond dramatically with rapid defervescence, relief of chest pain, and increased sense of well-being. The other indices of disease activity such as the erythrocyte sedimentation rate, white blood cell count, and pleural and pericardial effusions normalize. Recurrence may occur during steroid tapering and may be treated with nonsteroidal antiinflammatory agents or reinstitution of higher doses of corticosteroids.

The most serious complication of PCIS is cardiac tamponade, which occurs in approximately 0.1% to 6.0% of patients and should be treated with prompt pericardial drainage. Pulmonary infiltrates thought to be inflammatory pneumonitis are also reported. These patients usually require corticosteroid therapy.

EOSINOPHILIC HEART DISEASE

Loeffler endocarditis, first discovered in 1936 in two patients with new-onset heart failure and peripheral eosinophilia, is now thought to represent the cardiovascular manifestations of the *hypereosinophilic syndrome* (75). It has been referred to by a variety of terms including *eosinophilic leukemia, eosinophilic endocarditis, idiopathic collagen vascular disease,* and *idiopathic eosinophilia* and is believed by many investigators to be a variant of endomyocardial fibrosis, which has similar cardiac manifestations and primarily affects patients in tropical and subtropical Africa.

The hypereosinophilic syndrome is primarily a disease of men in their fourth decade. More than 75% of patients with this disorder experience heart failure. The principal clinical, noncardiac features of the disease include constitutional symptoms, weight loss, fever, cough, and skin rash. Cardiac involvement may be asymptomatic, but more often signs and symptoms of heart failure are present. The cardiovascular manifestations in 26 patients with documented hypereosinophilic syndrome are summarized in Table 44.5 (94). Common symptoms include cough, dyspnea, palpitations, and chest pain. Signs of congestive heart failure often associated with the murmur of mitral regurgitation are seen. Systemic emboli may occur. The chest x-ray film may reveal cardiac enlargement or pulmonary edema. The electrocardiogram may demonstrate left atrial enlargement, T-wave inversions, or first-degree AV block (94). The echocardiogram frequently shows increased ventricular wall thickness as a consequence of endocardial fibrosis, apical thrombus, or abnormal motion of the posterior leaflet of the mitral valve with mitral regurgitation. Dense endomyocardial fibrosis and scarring lead to a hemodynamic picture of restrictive cardiomyopathy with increased stiffness and reduced ventricular cavity size resulting in abnormal diastolic filling properties.

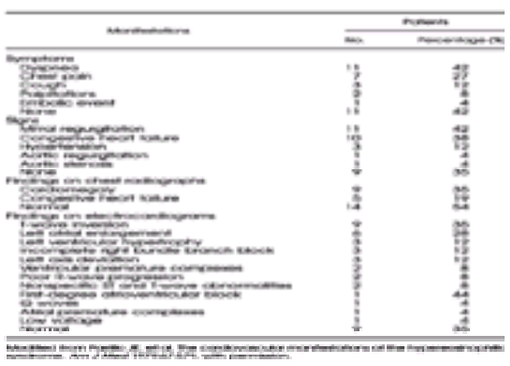

TABLE 44.5. Cardiovascular Manifestations of 26 Patients with the Hypereosinophilic Syndrome

Investigators have postulated myocardial involvement in the hypereosinophilic syndrome to consist of three stages (92). The first stage is an acute necrotic phase that is characterized by intense myocarditis with exuberant eosinophilic infiltration and associated arteritis and occurs within the first few months of the illness. It is followed by a thrombotic-necrotic stage occurring months to years after the initial presentation, at which time resolution of the myocarditic component with myocardial thickening and a variable amount of endocardial thrombus is seen. The third stage is that of late progressive endocardial fibrosis with findings consistent with restrictive cardiomyopathy (Fig. 44.5).

Figure 44.5. Extensive endocardial fibrosis and thickening of the left ventricular wall in a patient with eosinophilic heart disease.

Current thought concerning the origin of eosinophilic heart disease postulates a cardiotoxic effect produced by release of eosinophil granule products. The finding of intense eosinophilic infiltration in the setting of severe myocardial inflammation makes this an attractive theory. Major basic protein, the primary component of eosinophil granules, has been demonstrated to be cytotoxic to a variety of parasites and human cells *in vitro* (47). Eosinophilic infiltrates have been found associated with focal myocardial abscess formation and granuloma (107). More recently, DeMello et al. found deposits of eosinophil major basic protein within necrotic myocardium of two patients with the severe form of acute eosinophilic necrotizing myocarditis (34). The initial stimulus for the profound eosinophilic response in this disorder is unknown, but investigators have speculated that certain viral, occult parasitic, or allergic causes may be involved (92).

Patients with eosinophilic heart disease often have a beneficial response to the administration of corticosteroids or cytotoxic agents, particularly hydroxyurea. Such therapy may slow progression of the disease and may improve symptoms as well as survival (93). Such therapy may be combined with conventional therapy for heart failure including diuretics, digitalis, and afterload reduction. Long-term anticoagulation therapy should be considered when ventricular thrombi are noted. Patients with significant mitral or tricuspid regurgitation may benefit from endocardial resection and valve replacement. The course of the illness is variable. Patients with severe, acute necrotizing myocarditis may rapidly drift into severe heart failure and cardiogenic shock. Other patients may experience a more chronic, slowly progressive course of worsening heart failure.

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45 VASCULITIS

Paul Katz, M.D.

Vasculitis is the term applied to a large and heterogeneous group of clinical disorders characterized by inflammation and damage to blood vessels. The injury to the vascular lumen results in distal ischemia to the tissues supplied by a given involved vessel. This reduction in blood flow causes an array of symptoms and clinical expressions of the underlying syndrome. Depending on the pathologic entity, vasculitis can exist as the major manifestation of the disease process, or it may be present as a lesser feature of a variety of illnesses. For example, vasculitis is the most prominent feature of Wegener granulomatosis (WG), yet it may be a minor component of diseases such as rheumatoid arthritis or systemic lupus erythematosus. This chapter addresses primarily those clinicopathologic processes in which the sole or major feature of the disease is secondary to vasculitis. In addition, remarks are confined to those vasculitic syndromes that are systemic, that is, those characterized by multisystem disease involving organs other than or in addition to the skin. The reader is referred to Chapter XX for a discussion of those syndromes in which vasculitis is confined to the skin or is predominantly expressed in the skin and to Chapter 37 and Chapter 39 for in-depth reviews of those entities in which vessel inflammation may be a secondary process. The following entities in the systemic vasculitis group are discussed:

1. Systemic necrotizing vasculitis
   a. Polyarteritis nodosa (PAN)
   b. Allergic angiitis and granulomatosis (AAG) (Churg-Strauss syndrome)
   c. Polyangiitis "overlap" group
2. WG
3. Lymphomatoid granulomatosis (LG)
4. Giant cell arteritis
   a. Takayasu arteritis
   b. Temporal arteritis
5. Miscellaneous vasculitides
   a. Mucocutaneous lymph node syndrome (MLNS) (Kawasaki disease)
   b. Isolated central nervous system vasculitis
   c. Behçet disease

The American College of Rheumatology (ACR) published classification criteria for the vasculitides (1), and the Chapel Hill Conference in 1992 also proposed a scheme based on clinical and histologic aspects, vessel size, and autoantibodies (2). Any classification scheme, including the one used in this chapter, is probably imperfect. For example, Rao and colleagues used the ACR criteria to categorize 198 patients referred for evaluation of possible vasculitis (WG, giant cell arteritis, PAN, or hypersensitivity vasculitis) (3). The positive predictive value for these four syndromes using ACR criteria was only 29% to 75% for patients with the final, proven diagnosis of vasculitis. Nonetheless, classification schemes provide a framework for approaching patients with these syndromes.

PATHOGENESIS

Although blood vessel inflammation may be apparent in seemingly unrelated and distinct clinical disorders, the events leading to vasculitis are thought to be similar irrespective of the nature of the particular disease (4). Investigators generally believe that the primary immunopathogenic mechanism responsible for the development of most vasculitic syndromes is an immune complex model similar to that first noted in the Arthus reaction (5) and in experimental serum sickness (6). Based on the animal data of Cochrane and Dixon (7), one can construct a theoretic model of immune complex–mediated vasculitis, which appears applicable to many of these diseases in human patients. The extrapolation of these elegant animal models to human vasculitis is largely based on similarities in histopathologic features and the demonstration of circulating and tissue-bound immune complexes (8) (Chapter 25). Unfortunately, only rarely has a causative antigen been clearly implicated in any of these clinical syndromes, one exception being the association of some cases of PAN with hepatitis B antigenemia (9). Thus, although it is possible to demonstrate circulating and even deposited immune complexes in many of the vasculitic syndromes, the causal relationship of these complexes with the pathophysiologic events in most of the vasculitides remains speculative.

Investigators have postulated that circulating antigen-antibody complexes, formed in a state of relative antigen excess, are deposited in blood vessel walls. This deposition is enhanced by the release of vasoactive amines from platelets and immunoglobulin E (IgE)–triggered basophils. In this regard, the vasculitis of experimental models of serum sickness can be abrogated by pretreatment of the animals with antihistamines and serotonin antagonists (6,10). Additionally, complex deposition is augmented by mechanical factors such as blood flow turbulence and hydrostatic forces, which explain the preferential localization of immune complexes at vessel bifurcations and in the lower extremities, respectively.

After localization in the blood vessel wall, the antibody component of the complex activates the classical complement cascade, with the resultant generation of complement-derived chemoattractants, particularly C5a (4,11). After neutrophil egress into the blood vessel wall, these cells release a variety of lysosomal enzymes after cellular interaction with the immune complex (12). These highly damaging enzymes (e.g., collagenase, elastase) result in necrosis and compromise of the vessel lumen and lead to distal ischemia. The failure to demonstrate immune complex components in vasculitic tissue specimens or in the circulation may be explained on the basis of the following mechanisms: (a) circulating immune complexes may be present for only a brief time and may be cleared rapidly by the reticuloendothelial system, and (b) the Ig and complement components of immune complexes may only be detectable in tissue for 24 to 48 hours after deposition (13).

In addition to the immune complex models and, as discussed later, in some forms of vasculitis, circulating autoantibodies may contribute to the pathogenesis of blood vessel inflammation. Antineutrophil cytoplasmic autoantibodies (ANCAs) have been isolated from sera from patients with WG as well as from patients with other systemic vasculitic and nonvasculitic conditions (14). Apart from serving as possible diagnostic markers, these antibodies, which diffusely stain the cytoplasm or the perinuclear areas of neutrophils (C-ANCAs and P-ANCAs, respectively), may activate these inflammatory cells. C-ANCA reacts with proteinase 3 localized within primary granules. After cell activation, proteinase 3 may be expressed by neutrophils and monocytes. *In vitro,* C-ANCA can bind to proteinase 3 and initiates degranulation and stimulation of the respiratory burst (15), thereby injuring endothelial cells. ANCAs could participate in the pathogenesis of vessel inflammation by a variety of mechanisms including activation of leukocyte effector cells and alteration of the physiologic activity of the antibody-bound antigen. Both paths could lead to endothelial cell damage. Antibodies to myeloperoxidase (MPO), that is, although not specific for vasculitic syndromes, could also participate in this process. Like proteinase 3, MPO can be located in ANCA-positive tissue specimens.

Serum antiendothelial cell antibodies (AECAs) can be found in some vasculitic syndromes. Although the target antigen is uncertain, AECAs have been associated with various syndromes such as the small vessel vasculitis of rheumatoid arthritis, systemic lupus erythematosus, Kawasaki syndrome, and WG. Investigators have speculated that AECAs could injure endothelial cells through the complement cascade with the formation of the membrane attack complex, by antibody-dependent cellular cytotoxicity (ADCC), or by altering endothelial cell function (16). Endothelial cells may also be actively involved in immune-mediated injury, because these cells can secrete cytokines, such as interleukin-1 (IL-1), IL-6, and IL-8 and colony-stimulating factors, and they can express class I and II major histocompatibility complex (MHC) and adhesion molecules (17). Additional information regarding the possible contribution of endothelial cells to the pathogenesis of vasculitis is anticipated to

accrue.

Cell-mediated immune reactions may also contribute to the development of certain forms of vasculitis and, in particular, those characterized by concomitant granulomatous inflammation (e.g., WG). In this scheme, antigen-sensitized lymphocytes interact with circulating antigen and result in the release of soluble mediators or lymphokines. These mediators induce the accumulation and activation of monocytes in a manner similar to neutrophils, which may release vessel-damaging lysosomal enzymes. Additionally, these same activated macrophages could transform into epithelioid cells, which then evolve into the multinucleated giant cells characteristic of granulomas (18). These mechanisms may explain the mononuclear cell infiltration and perivascular granulomas observed in certain forms of vasculitis that also display features of immune complex–mediated diseases. Finally, immune complexes themselves can directly induce granuloma formation either by being phagocytosed by the macrophage or by activating this cell through surface membrane receptors for Ig. Both processes can trigger the cascade of epithelioid cell transformation and ultimate granuloma formation (8).

Additional examples exist in temporal arteritis. Histologic specimens of temporal arteries in affected patients show $CD4^+$ T cells, with associated monocytes and macrophages, as well as epithelioid and giant cells. This latter group of cells express adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), lymphocyte function antigen-1 (LFA-1) and LFA-3, and human leukocyte antigen (HLA)-DR. The $CD4^+$ T cells express IL-1 and interferon-g, typical of the Th1 T-helper cell type, as seen in cell-mediated immune reactions. Conceivably, interferon could activate macrophages and could lead to giant cell and granuloma formation.

CLASSIFICATION

As noted, this chapter focuses on those clinical entities in which vasculitis is the major and, in some cases, sole feature of the disease. The classification scheme used is based on the clinical, pathologic, and therapeutic characteristics of these syndromes (8). Excluded from the present chapter are the predominantly cutaneous vasculitides, which are often referred to as the *hypersensitivity vasculitides,* and vasculitis secondary to an underlying disorder.

Systemic vasculitis refers to those syndromes in which blood vessel inflammation will likely progress to significant and often life-threatening end-organ dysfunction without therapeutic intervention. This condition is in contradistinction to those diseases in which cutaneous vasculitis predominates and significant end-organ failure is unusual. For each subgroup of systemic vasculitis, the pertinent clinical characteristics, histologic features, laboratory features, and therapeutic approach are presented. Emphasis is placed on those characteristics that make each syndrome unique and clearly distinguishable from other types of vasculitis.

SYSTEMIC NECROTIZING VASCULITIS GROUP

This large group of disorders has perhaps presented the most difficulty in classification. Included in this category are classic PAN, AAG (Churg-Strauss syndrome), and the polyangiitis “overlap” syndrome (4,8).

Classic Polyarteritis Nodosa

The first description of necrotizing vasculitis was that of Kussmaul and Maier, who, in 1866, presented the case report of a patient with what is now called *classic PAN* (19). This term is now applied to those syndromes with necrotizing vasculitis of small and medium-sized muscular arteries in which the lesions tend to be segmental. There appears to be a predilection for this process to involve the bifurcation of vessels with some distal involvement of arterioles and adjacent veins (20).

This disease is truly multisystemic, often with nonspecific and protean early clinical manifestations. Initially, symptoms may include malaise, fatigue, fever, myalgias, muscle weakness, abdominal pain, and head and extremity pain. Less frequent signs include neurologic and joint abnormalities. Both the insidious nature of these symptoms and the rarity of this disorder may contribute to delays in establishing the diagnosis.

Renal disease is present in 70% of patients, it is the major cause of mortality, and it may be present as vasculitis with glomerular ischemia, glomerulonephritis, or a combination of both processes. Hypertension may accompany renal disease and may contribute to the underlying vasculitic process. Although investigators had thought that the hypertension of PAN was associated with the healing stages of renal disease (21), it is now apparent that this hypertension may occur early in the course of the illness and may, in fact, antedate apparent kidney involvement. Urinary findings may include hematuria, cellular casts, and proteinuria.

Apart from hypertensive cardiovascular disease, PAN may directly involve the coronary arteries and may be a major cause of death (22). Clinically, PAN-related cardiac disease is present in one third of patients and in two thirds of subjects at autopsy. Most commonly, this condition is manifested by heart failure secondary to coronary arteritis or hypertension. Additionally, evidence of myocardial infarction may be found at autopsy despite no clinical evidence of such an event (23). Other manifestations of PAN can include pericarditis, which is seen in up to 14% of patients (22), and involvement of the conduction system secondary to nutrient arteritis (24,25).

Gastrointestinal symptoms are reported in up to 75% of patients with PAN and are related to visceral artery vasculitis (4). This involvement may be manifested by abdominal pain, nausea, vomiting, diarrhea, ileus, bleeding, infarction, and perforation of visceral organs. Additional signs may include abdominal angina and steatorrhea related to superior mesenteric artery inflammation. Acute presentations of abdominal pain may resemble appendicitis, cholecystitis, or pancreatitis (26). At autopsy, gastrointestinal tract involvement is noted in 50% of subjects and may involve any segment of the bowel (4).

Liver disease in PAN secondary to hepatitis B antigenemia can range from mild, subclinical disease with minimal elevation of transaminases to chronic active hepatitis (9). Vasculitis of the liver may be seen in one half of patients in the absence of evidence of hepatitis infection and may lead to massive hepatic infarction and necrosis (27). Hepatitis C antigenemia can be found in some patients with PAN, although the precise role in pathogenesis, except when associated with cryoglobulinemia, is unclear (28).

Neurologic manifestations are observed in 80% of patients with PAN, with greater involvement of the peripheral rather than the central nervous system (29). Approximately two thirds of patients have peripheral neuropathy as mononeuritis multiplex, extensive mononeuritis, cutaneous neuropathy, or polyneuropathy. These conditions are thought to arise from inflammation of the vasa nervorum. Peripheral neuropathy may be relatively refractory to therapy and, in fact, may remain as some degree of functional impairment in patients otherwise in remission. Forty percent of subjects have central nervous system signs or symptoms made evident by diffuse and focal abnormalities of higher cortical, cerebellar, or brainstem activity. Cranial nerve involvement is unusual. Ocular abnormalities secondary to PAN may be present as typical hypertensive changes or as retinal vasculitis manifested by cytoid bodies.

Classic PAN, by definition, does not involve the lung. When lung disease is present, it is usually found in association with granulomatous inflammation in an atopic patient and is included under the category of AAG (Churg-Strauss syndrome) (4), as discussed later. Theses two entities may overlap, a finding that has led to the designation polyangiitis “overlap” syndrome (30).

Skin involvement is reported in 20% to 30% of patients with PAN secondary to small muscular arteritis of the subcutaneous tissue, which may be clinically manifested by tender erythematous nodules from a few millimeters to several centimeters in size (31) or as livedo reticularis. Unfortunately, the term *cutaneous PAN* has been applied to vasculitis of the small arteries of the subcutaneous tissue without systemic involvement (32). Histopathologically, the vessel involvement is indistinguishable from classic PAN; however, the disease is limited to cutaneous vessels. I prefer to consider this a form of cutaneous vasculitis (8), rather than a manifestation of classic PAN.

Although arthralgias are frequently observed in patients with PAN, true arthritis is uncommon. Myalgias, which may herald the onset of the disease (4), are secondary to vasculitis, and they may be found in blind muscle biopsy specimens from symptomatic sites. Testicular epididymal pain may likewise be a presenting manifestation of PAN, and vasculitis of the testes is observed in 30% of postmortem specimens. The clinical manifestations of classic PAN are listed in Table 45.1.

TABLE 45.1. Clinical Profile and Clinical Manifestations in Patients with Classic Polyarteritis Nodosa

No diagnostic laboratory tests are specific for classic PAN. However, various laboratory abnormalities largely reflect the nonspecific findings of any chronic inflammatory disease as well as those resulting from immune complex deposition. Elevations in C-reactive protein may be useful in monitoring disease activity (33). Approximately 30% of patients with PAN are ANCA positive, most of whom are P-ANCA, rather than C-ANCA, positive.

The diagnosis of classic PAN is based on the histologic finding of necrotizing vasculitis of small and medium-sized muscular arteries (Fig. 45.1) in patients with a compatible clinical picture. Biopsy sites are best determined on the basis of accessibility and clinical or laboratory evidence of disease activity. Blind biopsies of asymptomatic sites rarely establish the diagnosis; preferred anatomic locales include skin, muscle, and peripheral nerve.

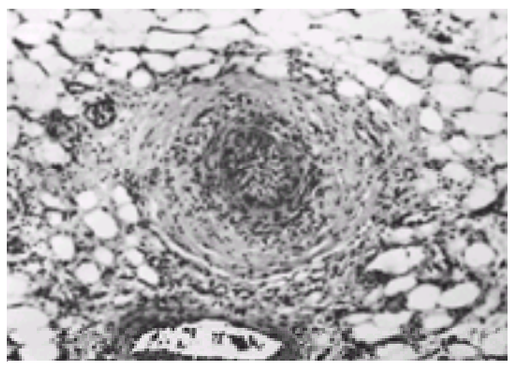

Figure 45.1. Biopsy specimen of a subcutaneous nodule in a patient with classic polyarteritis nodosa. A small muscular artery is involved with an inflammatory process, early fibrinoid necrosis, and obliteration of vessel lumen (hematoxylin and eosin stain, ×200).

The angiographic finding of small, aneurysmal dilatations *(pseudoaneurysms)* in renal, hepatic, and visceral vessels may be helpful in establishing the diagnosis (Fig. 45.2). Although initially thought to be diagnostic of PAN (34), these aneurysms may also be demonstrable in the polyangiitis “overlap” syndrome, systemic lupus erythematosus (35), and fibromuscular dysplasia (36). However, in a patient with a clinical picture compatible with classic PAN but without an easily accessible biopsy site, a positive angiogram substantiates this diagnosis.

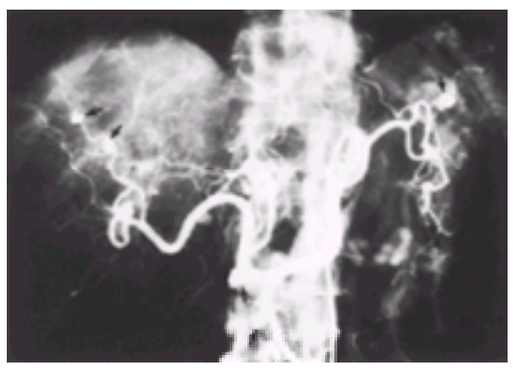

Figure 45.2. Celiac axis angiogram in a 47-year-old man with classic polyarteritis nodosa. The *arrows* indicate typical aneurysm formation in visceral arteries.

Without therapy, the prognosis of classic PAN is extremely poor. In untreated patients, the 5-year survival rate is 13%, and it increases to only 48% with corticosteroid therapy (37). These figures are considerably worse for patients presenting with hypertension or renal disease. Renal failure or a cardiovascular or gastrointestinal event is the usual cause of death. The therapy of classic PAN is described later, in the discussion of treatment of the systemic necrotizing vasculitis group.

Allergic Angiitis and Granulomatosis (Churg-Strauss Syndrome)

AAG is a disease similar to classic PAN in many respects. Unlike classic PAN, however, pulmonary vascular involvement is the *sine qua non* of this syndrome (13,38,39). Additionally, the following features distinguish AAG from classic PAN: (a) vasculitis of blood vessels of different sizes and types (small and medium-sized arteries, veins, and venules), (b) granulomatous inflammation (intravascular and perivascular), (c) peripheral blood and tissue eosinophilia, and (d) a strong association with severe atopic disease (usually asthma). As such, it is probably identical to “PAN with pulmonary involvement” as described by Rose and Spencer (21).

Patients generally present with nonspecific findings including fever, malaise, and weight loss. Pulmonary symptoms are usually the most notable and may include bronchospasm and evanescent pulmonary infiltrates (Table 45.2). There is a high incidence of skin lesions in AAG secondary to small vessel vasculitis. As shown in Table 45.2, the multisystemic nature of this syndrome is similar to that of classic PAN.

Category	Value
General considerations	Results
Age (mean)	46 yr
Sex ratio (male to female)	1.3:1
Duration (mean) of pulmonary symptoms before systemic symptoms	2 yr
Fever	Majority of patients
Organ system involvement	Percentage (%)
Pulmonary	95
Infiltrate on chest radiograph	93
Wheezing	82
Cutaneous	67
Purpura	37
Nodule	35
Peripheral neuropathy	63
Hypertension	54
Gastrointestinal	42
Cardiac	38
Renal	38
Lower urinary tract	10
Arthritis/arthralgia	21

From fit Guppa, Ad Fauci. The vasculitides. Philadelphia: WB Saunders, 1996:145, with permission.

TABLE 45.2. Clinical Profile and Manifestations in Allergic Angiitis and Granulomatosis

Like PAN, AAG has no specific laboratory tests. Most patients have elevated erythrocyte sedimentation rates and white blood cell counts that, in 80% of cases, include absolute eosinophilia (more than 1,000 cells/µL). The presence of peripheral blood eosinophilia excludes the diagnosis of classic PAN but not the polyangiitis “overlap” syndrome. As may be anticipated from the strong allergic history in many patients with AAG, serum IgE levels may be elevated (5).

Histologically, vascular inflammation similar to that of classic PAN is characteristic of AAG. As previously noted, vessels of differing types and sizes may be involved. Unlike in PAN, eosinophils and granulomas may be found in and near involved vessels (21). Biopsy of involved tissues with characteristic pathologic features in concert

with the appropriate clinical setting establishes the diagnosis of AAG.

Without treatment, the 5-year survival rate for AAG is 4%; with corticosteroid treatment, this figure is increased to approximately 60%. The therapeutic recommendations for AAG are the same as for classic PAN.

Polyangiitis "Overlap" Syndrome

In examining large numbers of patients with the characteristics of classic PAN or AAG, it becomes evident that one syndrome of necrotizing vasculitis does not easily fit into these or other clinical categories (8,30). This has been referred to as the *polyangiitis "overlap" syndrome* (4,8). Typically, a patient in this group may have features of both classic PAN and AAG with or without the small vessel cutaneous venulitis characteristic of cutaneous "hypersensitivity vasculitis." Such patients may or may not have pulmonary involvement, an atopic history, eosinophilia, and tissue granulomas. This syndrome is a systemic vasculitis with the potential to progress to end-organ failure and death. Treatment of this subgroup is identical to that of PAN and AAG. Evidence suggests that long-lasting remissions are possible with early diagnosis and prompt institution of therapy (30).

Treatment of the Systemic Necrotizing Vasculitis Group

As noted in the preceding sections, the prognosis of patients with these entities before the advent of corticosteroids was extremely poor (37). Nonetheless, even with these drugs, the overall 5-year survival rate of the group was less than 50% (37). The heterogeneity of patients in this group has contributed to the difficulties in devising successful therapeutic regimens. In this regard, a few patients may do well with only corticosteroid treatment. Conversely, in some patients with rapidly progressive and fulminant disease, combination therapy with corticosteroids and cytotoxic drugs is mandatory to prevent irreversible organ dysfunction and, perhaps, death.

In patients with less severe disease without impending organ failure, a trial of corticosteroids appears justified. Therapy is initiated with prednisone, 1 mg/kg per day in divided doses with consolidation over several weeks to a single daily dose (8). This regimen is continued for 1 to 2 months, followed by an attempt to convert the patient to an alternate-day schedule over the ensuing 1 to 2 months. If this conversion is successful without evidence of relapse, alternate-day corticosteroids are continued for months up to 1 year, with tapering of this dose until the drug is discontinued or until a dosage is reached that is needed to suppress disease activity.

In patients failing to respond to this regimen within a month of its institution or in patients presenting with fulminant disease and end-organ dysfunction, a cytotoxic drug should be begun. Using the protocol originally devised for WG (see later), excellent results have been achieved with cyclophosphamide administered orally at 1 to 2 mg/kg per day. In patients unable to take the drug orally, it can be administered intravenously at the same dose. Prednisone therapy should always be administered together with the cyclophosphamide regimen, particularly early in the course, because cyclophosphamide in the regimen outlined takes 2 weeks to 1 month to achieve a significant immunosuppressive effect. The prednisone regimen should be administered as outlined earlier, with ultimate conversion to an alternate-day regimen after approximately 1 month of daily therapy. The alternate-day regimen should be maintained for at least 3 to 6 months, with gradual tapering until discontinuation or until a dose is reached beneath which disease activity flares. The dose of cyclophosphamide is adjusted to maintain the white blood cell count at approximately 3,000 to 3,500 cells/µL, with an absolute neutrophil count of greater than 1,000 to 1,500 cells/µL (8). At these levels, infection is extremely unlikely. Cyclophosphamide therapy is continued for 1 year after complete remission has been achieved; the dose is then tapered in 25-mg decrements every 2 months until it is discontinued. Using this regimen, we have achieved long-term remissions in most patients with systemic necrotizing vasculitis (40).

Although other cytotoxic drugs have been used in some of these syndromes (e.g., azathioprine, chlorambucil), it does appear that cyclophosphamide is the most efficacious agent for inducing long-term remissions in systemic necrotizing vasculitis (41). As discussed later, cyclophosphamide therapy may result in serious side effects, especially when therapy is prolonged. Enthusiasm for the use of intermittent "pulse" cyclophosphamide, as successfully employed in lupus nephritis, has been dampened by difficulties in inducing and maintaining remissions.

WEGENER GRANULOMATOSIS

WG is characterized by the clinical complex of (a) necrotizing granulomatous vasculitis of the upper and lower respiratory tracts, (b) glomerulonephritis, and (c) varying degrees of disseminated small vessel vasculitis (42,43). As with most of the systemic vasculitides, the cause of WG is unknown; however, it is believed to represent an exaggerated and aberrant hypersensitivity reaction to an unknown antigen that may have entered through the respiratory tract.

A reported series of 158 patients with WG provided a comprehensive review of the clinical, laboratory, and pathologic manifestations of the disease (44). The mean age at onset was 41 years, yet 15% of patients presented before the age of 19 years. No gender differences were noted, and only 3% of affected persons were not white.

The presenting signs and symptoms of WG are listed in Table 45.3. Most patients present with nonspecific complaints (malaise, fatigue) in concert with symptoms referable to the upper airways such as rhinorrhea, sinusitis, nasal mucosal ulcerations, and otitis media. Sinus roentgenograms often appear abnormal, with early signs of mucosal thickening. This may progress to total opacification of air-fluid levels. Nasal septal perforation and loss of supporting nasal structures may lead to the characteristic saddle nose deformity. Most patients with WG develop secondary infection of damaged upper airway structures, almost invariably with *Staphylococcus aureus.* This infection may occur even in patients who are in complete remission from their underlying disease and often represents an increased susceptibility of previously damaged upper airway mucosa to becoming secondarily infected. Such infections may complicate the clinical picture in patients in remission who may initially be misdiagnosed as having a relapse. Clearly, this critical distinction cannot be overemphasized, and in such patients, the appropriate cultures or surgical procedures to establish the diagnosis are recommended.

Sign or Symptom	Percen
Upper or lower airway symptoms	90
Upper airway signs and symptoms	73
Musculoskeletal symptoms	67
Pulmonary infiltrates	45
Fever	23
Cough	19
Ocular inflammation	15
Weight loss	15
Glomerulonephritis	15
Skin rash	13
Hemoptysis	12
Pleuritis or effusion	10
Proptosis	2

Source: Adapted from GS Hoffman et al. Wegener granulomatosis: An analysis of 158 patients. Ann Intern Med 116:488, 1992.

TABLE 45.3. Presenting Signs and Symptoms in 158 Patients with Wegener Granulomatosis

Pulmonary symptoms in the absence of upper respiratory tract signs are unusual. Respiratory complaints such as cough, hemoptysis, and chest pain may herald the onset of WG. Chest radiographs generally reveal multiple, bilateral nodules that may cavitate (Fig. 45.3). Approximately 10% of patients have demonstrable pleural effusions, but hilar adenopathy is rare (45). Pulmonary dysfunction manifested as airflow obstruction, decreased lung volumes, and decreased diffusing capacity is frequently noted.

Figure 45.3. Chest roentgenogram of a 38-year-old woman with Wegener granulomatosis. Note the bilateral nodular cavitary infiltrates.

Patients with WG rarely present with renal failure in the absence of significant disease in other organ systems. Signs of kidney disease may range from minimal urinary sediment abnormalities with normal renal function (e.g., in mild focal and segmental glomerulonephritis) to markedly progressive renal dysfunction with diffuse necrotizing glomerulonephritis including proliferative and crescentic changes. Because of the morbidity and eventual mortality associated with renal disease in WG, and because extrarenal WG usually precedes kidney diseases, it is impernative to establish the diagnosis and to initiate appropriate therapy as early as possible (43).

Ocular disease may occur either early or late in the course of WG. This may range from mild conjunctivitis through episcleritis and scleritis to scleromalacia perforans (46). Proptosis secondary to nasal sinus involvement or retroorbital granulomatous vasculitis may be seen in some patients.

Cutaneous lesions may develop early in the course of WG and are usually secondary to small vessel vasculitis (47). These may be present as ulcerations, papules, vesicles, or subcutaneous nodules.

No diagnostic laboratory studies are available for WG; however, some characteristic abnormalities may be useful. The erythrocyte sedimentation rate is invariably elevated, often greater than 100 mm per hour. This test is perhaps the most sensitive indicator of disease activity in WG and is useful in detecting active disease even in the absence of clinical symptoms. Autoantibodies against extranuclear components of neutrophils may be present in sera from patients with WG (48). As noted earlier, sera from patients with WG frequently contain ANCAs, which diffusely stain the cytoplasm of fixed neutrophils (C-ANCAs) (15,49). These autoantibodies are directed against a 29-kd serine proteinase, termed *proteinase 3.* Additionally, some sera from patients with WG, as well as sera from patients with microscopic PAN, Churg-Strauss syndrome, necrotizing crescentic glomerulonephritis, or Kawasaki syndrome, may stain neutrophils in a perinuclear pattern (P-ANCAs) (49). P-ANCA staining results from autoantibody reactivity with myeloid lysosomal enzymes, most importantly MPO, as well as elastase. Approximately 90% of sera from patients with active WG are C-ANCA positive; with disease remission, this falls to 40% (44). Frequently, low-grade anemia and thrombocytosis are encountered secondary to the inflammatory nature of the disease. Most patients have leukocytosis, often with a left shift. Increased levels of serum IgG and IgA are observed in most patients, a finding reflecting hyperactivity of the immune system (42). Rheumatoid factor and circulating immune complexes can be demonstrated in some patients with WG. We described an increased frequency of HLA-B8 in WG (50).

The pathologic hallmark of WG is necrotizing granulomatous vasculitis (Fig. 45.4). Biopsy of the upper airways, although accessible, may reveal only acute and chronic inflammation. Lung biopsy produces an extremely high diagnostic yield because large tissue specimens can be obtained. In addition to demonstrating the typical granulomatous vasculitis of WG, one can usually rule out other diseases in the differential diagnosis such as pulmonary neoplasms and infectious diseases. The vasculitis primarily involves small arteries and veins. Granulomas may be within, adjacent to, or distant from sites of vascular inflammation. Renal involvement is histologically variable but may range from focal and segmental glomerulonephritis to fulminant crescentic glomerulonephritis (51). Less frequently encountered are vasculitis and granulomas. Histologic evidence of immune complex components can be demonstrated in some, but not all, biopsy specimens.

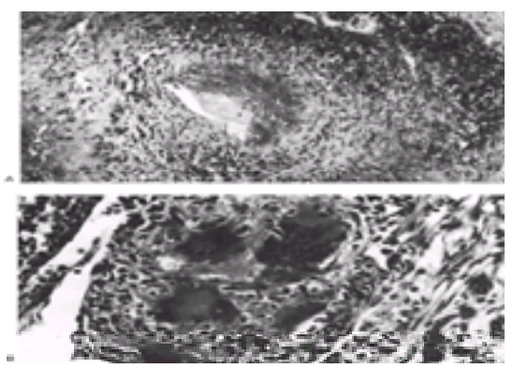

Figure 45.4. Lung biopsy specimen from a patient with Wegener granulomatosis. **A:** Typical pulmonary vasculitis with fibrinoid necrosis of a vessel wall and surrounding inflammatory infiltrate (hematoxylin and eosin stain, ×170). **B:** Multinucleated giant cells within infiltrate in alveolar spaces (hematoxylin and eosin stain, ×270).

Before the advent of cytotoxic therapy, WG was an almost uniformly fatal disease with a particularly rapid and progressive course in the presence of renal disease. In the absence of treatment, the mean survival time was 5 months, with a mortality rate at 1 year of 82%, which rose to more than 90% after 2 years (52). These dismal results were not substantially influenced by corticosteroids. This was the first vasculitis syndrome shown to respond successfully to long-term low-dose cyclophosphamide therapy, because it was demonstrated that this drug, together with alternate-day corticosteroids, could induce long-lasting remissions in most patients (42).

The treatment outcomes of 158 patients with WG were reported (44). Eighty-four percent received standard treatment of daily low-dose cyclophosphamide and corticosteroids, 5% were treated with low-dose cyclophosphamide alone, 6% received only corticosteroids, and 4% received other cytotoxic drugs and corticosteroids. The standard protocol produced marked improvement or partial remission in 91% of recipients; 75% experienced complete remissions, with a median time of 12 months. Fewer than 10% of patients so treated experienced remission as late as 6 years after beginning this protocol. Although almost all patients eventually had disease remissions, nearly one half had relapses. Morbidity, secondary to the disease or its therapy or both, occurred in nearly 90% of patients; most prominent was chronic renal insufficiency, affecting 40%. Hearing loss, nasal deformity, tracheal stenosis, and visual impairment were experienced by up to one third of subjects.

Cyclophosphamide-related morbidity included cystitis (43%), carcinoma of the bladder (3%), and myelodysplasia (2%). Overall, malignant diseases were nearly 2.5 times more frequent in cyclophosphamide-treated patients than in the general population; notably, this was skewed toward bladder cancer (33-fold) and lymphoma (11-fold). Infectious complications, occurring in 46% of the cohort, were observed secondary to immunosuppressive treatment and disease-related tissue injury; one half of these were seen in association with daily corticosteroid treatment. Thirteen percent of the patients died as a direct consequence of WG, its sequelae, or therapy.

Despite the efficacy of low-dose cyclophosphamide-corticosteroid treatment, the considerable toxicity has prompted interest in alternate therapies. Based on the success and relatively low toxicity of high-dose, monthly intravenous cyclophosphamide in systemic lupus erythematosus, this same protocol was employed in 14 patients with WG (53). Patients received cyclophosphamide monthly and tapering doses of corticosteroids for 6 months; if successful, the regimen was continued, but less often. Although initial improvement was observed in more than 90% of patients, nearly 80% had disease exacerbations or were intolerant of this regimen; only 20% had prolonged remissions. Therefore, this protocol is less efficacious than the standard regimen.

Twenty-nine patients with less severe disease were treated with weekly methotrexate and daily or alternate-day corticosteroids, with marked improvement observed in approximately 75% and remission in 70% (54). This protocol has been employed in patients with less than immediately life-threatening illness. Methotrexate is begun at 0.3 mg/kg per week, with a gradual increase in dose as needed and as tolerated up to a dose of 25 mg per week. Seventeen percent displayed continued and accelerating disease activity during the first 6 months of this regimen. Over time, 36% of patients experienced a relapse requiring repeat therapy. Thus, in selected patients, this therapeutic option may be appropriate.

Finally, scattered reports have noted success with trimethoprim-sulfamethoxazole (55). However, these results were not confirmed in a study from the National Institutes of Health. Given the potential role of tumor necrosis factor in WG, studies are under way to look at the possible efficacy of anti–tumor necrosis factor therapy using etanercept and infliximab. These newer therapies certainly hold promise, particularly in view of the limited toxicity of this drug.

LYMPHOMATOID GRANULOMATOSIS

In 1972, Liebow et al. published their landmark description of *LG,* an unusual form of granulomatous vasculitis characterized by an angiodestructive and angioinvasive infiltration of blood vessels with atypical lymphocytoid and plasmacytoid cells (56). Currently, this disease is included with the angiocentric immunoproliferative lesions, as are benign lymphocytic angiitis and granulomatosis and angiocentric lymphoma. The pathogenesis of this syndrome is obscure, and it may differ from other vasculitides in that an immune complex origin may not be apparent. There is a predilection for this disease to involve many of the same organ systems as WG (lung,

skin, kidneys, and central nervous system) (57), and this may cause some confusion between these syndromes. Approximately 50% of patients with LG do not respond to the accepted therapeutic regimen (see later) and go on to develop non-Hodgkin lymphoma (58).

Usually, patients in the fourth or fifth decade present with nonspecific signs including malaise, fever, fatigue, and weight loss (4). More specific symptoms are referable to pulmonary involvement including cough, dyspnea, and chest pain and are manifested by the radiographic finding of bilateral nodular densities. Approximately one fifth of patients with LG have either peripheral or central nervous system signs. Cutaneous disease occurs in up to 40% of patients, as either erythematous macules or indurated plaques. One third of patients with LG have renal involvement secondary to parenchymal infiltrates with atypical mononuclear cells rather than glomerulonephritis. Less frequent manifestations include splenomegaly (18%), hepatomegaly (12%), and lymphadenopathy (8%).

Again, no specific laboratory tests hallmark LG. One half of patients may have leukocytosis, whereas 20% have leukopenia. The erythrocyte sedimentation rate is either normal or minimally elevated, in contrast to the invariable elevations in WG (56).

The unique histologic appearance of LG includes angiocentric and angioinvasive infiltration of arteries and veins with pleomorphic, atypical lymphocytes (primarily T cells) and plasmacytes. Associated atypical B lymphocytes may be infected with Epstein-Barr virus. The granulomas are less frequent and less distinct than in WG. In addition to cellular atypia, mitosis of the infiltrating cells may be observed. Differentiation between LG and frank lymphoma may be difficult; however, the clinical absence of splenic, bone marrow, and lymphatic disease favors the diagnosis of LG. Furthermore, the pathologic appearance of the infiltrating cells is extremely important in establishing the diagnosis.

Without therapy, LG is nearly always fatal (56,59). We described our experience with 15 patients with LG who were treated with the protocol mentioned earlier for systemic necrotizing vasculitis (58). In 50% of patients, long-term remissions were achieved, and therapy was discontinued. The remaining one half who failed to respond evolved into the lymphomatous phase of LG and eventually died. The best indicator of significant long-standing remission and survival is a successfully induced, early and complete remission. In patients with disease limited to the lungs and skin, cyclophosphamide and prednisone may be adequate; however, more extensive and histologically aggressive disease likely deserves more extreme chemotherapy. This situation emphasizes the need for early diagnosis and treatment if remissions are to be realized.

GIANT CELL ARTERITIDES

Within the broad category of *giant cell arteritides* are two distinct disorders: temporal arteritis (or cranial arteritis) and Takayasu arteritis (“pulseless” disease). Although these syndromes are distinct clinically, they are histologically similar and are characterized by vasculitis of medium-sized and large arteries, often accompanied by granuloma or giant cell formation.

Temporal Arteritis

The classic signs and symptoms of *temporal arteritis* include fever, high erythrocyte sedimentation rate, and anemia often accompanied by polymyalgia rheumatica in a person more than 55 years old (60). Although usually involving branches of the carotid artery, temporal arteritis is truly a systemic vasculitis, and virtually any medium-sized or large artery may be involved, including the renal artery and aorta.

Temporal arteritis is a common disorder, with an incidence of 17.4 cases per year per 100,000 people older than 50 years. Rarely, this disease may present in younger adults and even adolescents (61). There appears to be a 2:1 female predominance. Genetic studies have suggested that HLA-DR4 frequency is increased in patients with temporal arteritis or polymyalgia rheumatica.

The presenting symptoms of temporal arteritis are usually nonspecific and include headache (44%), malaise (20%), fatigue (12%), and fever (8%) (60). The only early sign that may be specific is jaw claudication, reported in 12% of patients (60). In one half of patients, physical abnormalities of the temporal artery, such as tenderness, nodules, and decreased pulse, may be found. However, an absence of these findings does not decrease the chances of a positive finding on biopsy because one third of patients have no clinical changes in the area of the temporal artery (62). Approximately 40% of patients with temporal arteritis develop visual impairment (63), although it is unusual for this to be the presenting manifestation with associated blindness. Blindness generally occurs 3 to 4 months after the onset of symptoms, a finding emphasizing the need for early diagnosis and therapy.

Polymyalgia rheumatica, with its characteristic proximal myalgias, periarticular pain, and morning stiffness, is seen in approximately 50% of patients with temporal arteritis (64). Conversely, 40% to 50% of patients with polymyalgia rheumatica have positive temporal artery findings even in the absence of symptoms (64). Clearly, the distinction between these two syndromes may be difficult, but necessary.

Typical laboratory findings in temporal arteritis include mild normochromic, normocytic anemia and a high erythrocyte sedimentation rate. A review of the literature indicated that temporal arteritis may be seen in the absence of elevations in erythrocyte sedimentation rate (65). Headache and jaw claudication were seen less frequently in these patients than in those with elevated values. These findings in an elderly patient with the aforementioned symptoms should alert the clinician to this diagnosis. Elevated acute-phase reactants and serum Igs have been observed in some patients. Frequently, abnormal findings on liver function tests, especially increased alkaline phosphatase levels, are noted (66); less commonly, there may be only slight elevations of serum transaminases. Patients with polymyalgia rheumatic and antiphospholipid antibodies appear to have a greater chance of developing temporal arteritis. Furthermore, levels of anticardiolipin antibodies may drop precipitously after corticosteroid therapy.

Typically, the diagnosis of temporal arteritis is established by the finding of panarteritis of the vessel with mononuclear cell infiltration. Giant cells may or may not be present, and their absence does not exclude the diagnosis. Ig deposits on the internal elastic membrane are found in fewer than 50% of biopsy specimens (67). Infiltrating lymphocytes are primarily T cells of the helper-inducer CD4 subset. Some of these cells are positive for HLA-DR, the integrin receptor very late antigen-1, and IL-2 receptors, indicative of activation (68). Accompanying giant and endothelial cells and macrophages express HLA-DR and LFA-1, LFA-3, and ICAM-1. These same histologic findings are frequently demonstrable in vessels other than the temporal artery, and this situation may result in systemic signs and symptoms.

Investigators have suggested that multiple sections of bilateral temporal artery biopsy specimens should be examined because of the focality and segmental nature of the vasculitis (69). Additionally, it has been proposed that superficial temporal arteriography could assist in identifying high-yield biopsy sites, although the angiographic changes are not diagnostic of this syndrome (70). In general, this is not recommended.

Patients with temporal arteritis generally respond dramatically to corticosteroid therapy. Usually, after several days of 40 to 60 mg of prednisone daily, patients report an improved sense of well-being with a diminution in fever, malaise, and myalgias (71). Concomitant with this is a decrease in vascular symptoms including headache and jaw claudication. Because of the risk of blindness, the diagnosis should be established quickly, and proper therapy should be instituted. The symptoms of polymyalgia rheumatica frequently improve with lower doses of corticosteroids; unfortunately, these are inadequate to prevent the development of visual impairment. Gradually, prednisone can be tapered to 7.5 to 10.0 mg per day, with readjustment should symptoms recur. Therapy should be continued for 1 to 2 years (60). The erythrocyte sedimentation rate may increase during tapering even in the absence of clinically apparent disease. Under these circumstances, tapering can be carefully continued because some symptoms generally reappear before relapse. It appears that many patients can be converted to alternate-day corticosteroid therapy once remission has been induced. This schedule should not be instituted at the onset of therapy because it is unlikely to completely improve symptoms (72). Although nonsteroidal antiinflammatory drugs may relieve some symptoms, they do not prevent blindness. In this regard, visual impairment is generally irreversible, although a few patients may have some restoration of function (72). Alternate corticosteroid regimens have been employed in patients with impending visual loss (73). Resolution of ischemic optic neuropathy with high-dose, “pulse” intravenous methylprednisolone, similar to that administered for optic neuritis, may be useful in these circumstances, although experience is limited. Fortunately, most patients remain in remission even after cessation of an adequate trial of corticosteroid therapy.

A report of nine patients who died of temporal arteritis indicated that myocardial infarction, dissecting aneurysm, and stroke were implicated. No patient in this group was receiving “adequate” corticosteroid treatment (74).

Takayasu Arteritis

Takayasu arteritis (“pulseless” disease), a disease primarily of young women, is characterized by vasculitis and stenosis of intermediate-sized and large arteries with a predilection for the aortic arch and its branches (75,76). Because of the increased incidence in female patients, genetic factors are believed to be important; furthermore, an association with the HLA antigens B5, A10, B52, and DHO has further substantiated this possibility (4). Nonetheless, a study of 21 white North American patients showed no correlation with class I or II antigens (77); DR1 was negatively associated with the disease.

Takayasu arteritis is biphasic. The acute phase is heralded by symptoms of a systemic inflammatory disease; the chronic stage has signs of an occlusive vascular process with insufficiency. The mean time between these phases is 8 years, but it may be as short as several months (78). In 70% of patients, an acute stage can be identifiable historically, whereas in the remainder, ischemic signs and symptoms herald the diagnosis in the absence of prior recognizable systemic inflammatory

disease. In the early period, fever, night sweats, fatigue, myalgias, arthralgias, and weakness are common. Ischemic symptoms dominate the late stage of disease; this most frequently involves the aortic arch and its branches, but any intermediate-sized or large vessel may be involved. In this stage, physical examination may reveal absent pulses or vascular bruits. In addition to vascular insufficiency, claudication or Raynaud phenomenon may be apparent. Renal artery involvement may produce hyperreninemia with resultant hypertension. Other findings may include visual disturbances, syncope, paresthesias, and cerebral infarction. Cardiac signs such as congestive heart failure, angina, aortic insufficiency, myocarditis, and pericarditis may be noted.

Up to 14% of patients with Takayasu arteritis die of the disease, usually secondary to congestive heart failure resulting from myocardial infarction (79). Laboratory studies are nondiagnostic. Most patients have elevations of the erythrocyte sedimentation rate; mild anemia, leukocytosis, and increased serum Igs may likewise be present.

The diagnosis is usually established by the characteristic arteriographic changes ranging from narrowing to occlusion of large arteries with formation of collateral circulation (80). Usual vessels involved are the subclavian artery, descending aorta, renal artery, and carotid artery, although, as noted earlier, any intermediate-sized or large vessel may be involved.

Because biopsy material is usually not available for study, the diagnosis is less frequently made by the histologic finding of panarteritis of large elastic arteries with mononuclear and giant cells in all vessel wall layers (81). Secondary atherosclerotic changes may be observed in previously damaged vessels. Histologically, this vasculitis syndrome suggests a cell-mediated pathogenesis with lesions infiltrated with natural killer and cytotoxic T lymphocytes and gd T cells. Furthermore, adhesion molecule expression in the vessel wall adds additional support to this pathogenic model.

The initial inflammatory stage of Takayasu arteritis can be frequently suppressed by corticosteroids; however, it is not clear that such therapy prolongs life. The effect of these agents on the ultimate outcome of the disease is particularly difficult to interpret in view of the variable course; that is, patients with Takayasu arteritis generally deteriorate gradually, but spontaneous remissions or acute decompensation may occur. Some data suggest that long-term low-dose prednisone therapy (5 to 10 mg per day) may reduce late complications and may prolong life. A report of 20 patients indicated that patients not responding to corticosteroids often benefited by the addition of cyclophosphamide (82). Additionally, methotrexate may be effective in patients in whom corticosteroid therapy fails. In patients with vascular insufficiency secondary to this process, percutaneous angioplasty may be useful for renal, aortic, and coronary vessels.

MISCELLANEOUS SYSTEMIC VASCULITIDES

Mucocutaneous Lymph Node Syndrome (Kawasaki Disease)

MLNS, or Kawasaki disease, is an acute illness of children characterized by the clinical signs of prolonged fever, conjunctivitis, involvement of the oral mucosa and lips, cutaneous changes in the distal extremities, truncal exanthem, and acute cervical lymphadenopathy (27,83). Although initially described in Japan, the disease is now recognized with increased frequency in the United States, where it has been reported in children from 3 months to 13 years old (84).

The cause of MLNS is unknown; initial enthusiastic reports about the possible relationship with *Rickettsia,* Epstein-Barr virus, and streptococci have not been conclusively verified. The disease is seen with greatest frequency in persons of Japanese descent, but HLA associations have not been clearly identified.

Ninety-five percent of patients are febrile during the first week of illness, with gradual defervescence during the ensuing week (83). A common manifestation is marked reddening of the skin of the palms and soles (88% of patients) during the initial 7 days, much like that of scarlet fever. Concomitant with a decline in fever, desquamation of the involved skin occurs, beginning at the distal end of the affected digits. Nonspecific maculopapular dermatitis develops within 3 to 5 days of the onset of fever, with eventual progression to confluence. Patients invariably develop dry erythematous and fissured lips and oral mucous membranes in association with congested conjunctivae and a “strawberry” tongue. Tender, nonsuppurative anterior and posterior cervical lymphadenopathy is present in three fourths of patients.

An infrequent, yet potentially life-threatening, complication of MLNS is cardiac involvement with signs of cardiomyopathy or mitral or aortic valve regurgitation (85). Late cardiac events such as coronary artery vasculitis and aneurysm formation may be responsible for the 1% to 2% case-fatality rate (86). Less common manifestations may include arthralgias or arthritis, liver function abnormalities, diarrhea, and aseptic meningitis (87).

Nondiagnostic laboratory studies such as leukocytosis and increased erythrocyte sedimentation rate are frequently observed in patients with MLNS. Elevations of serum IgE may be present for 1 to 2 months. With myocarditis, electrocardiographic abnormalities may be noted, but these may not necessarily be apparent even in the presence of coronary artery aneurysms. These aneurysms are usually demonstrable angiographically or by echocardiography.

Histologically, panarteritis of the coronary vessels with mononuclear cell infiltration is noted. Aneurysm formation generally occurs at the sites of greatest vasculitic inflammation. Other vessels may be involved less frequently, but usually only endothelial proliferation is observed. Lymph node biopsy generally reveals necrosis, thrombosis, small vessel vasculitis, and immunoblast-like leukocytes. The syndrome originally labeled *infantile (or childhood)* PAN likely represented MLNS with severe coronary arteritis (88).

The diagnosis of MLNS is made by the presence of five of the six criteria described earlier. The striking clinical signs and symptoms of this entity usually make the diagnosis readily apparent.

Although normally MLNS is a self-limited disease without long-term morbidity or mortality, the risk of coronary arteritis and of subsequent aneurysm formation has favored treatment. Initial enthusiasm for corticosteroids or aspirin has not been sustained. Currently, intravenous immune globulin is considered the therapy of choice, particularly with regard to the prevention of late complications (89).

Isolated Central Nervous System Vasculitis

Rarely, vasculitis may be restricted to *vessels of the central nervous system* without evidence of extracranial involvement (90). Although intracranial vasculitis may be observed in patients with certain bacterial infections, syphilis, tuberculosis, fungal infections, and viral illnesses, isolated idiopathic central nervous system vasculitis may occur. Patients usually present with mental confusion, headache, or focal neurologic deficits. Systemic signs and symptoms such as arthralgia and arthritis, myalgias, and fever, which are frequently observed in many systemic vasculitides, are conspicuously absent in isolated central nervous system vasculitis.

Although routine laboratory tests are rarely helpful, cerebrospinal fluid examination may reveal increased opening pressure, mononuclear pleocytosis, and slightly increased protein; however, normal fluid does not exclude the diagnosis. Radiographic examinations, except angiography, are of little value. Cerebral arteriography may demonstrate vessel “beading” with narrowing and dilatation of small blood vessels, vessel cutoff, or aneurysm formation, although these studies may appear entirely normal. Biopsy of involved vessels, usually small arteries and arterioles, reveals mononuclear cell infiltration and occasionally giant cells.

In the absence of therapy, this disease is nearly always fatal (91), with either an abrupt, fulminant course or slow deterioration. Although the precise treatment modality is unproved, corticosteroids alone are the initial drugs of choice. When a corticosteroid regimen fails, or in patients who are intolerant of corticosteroids, the aforementioned regimen of cyclophosphamide plus corticosteroids appears to improve this dismal prognosis significantly.

Behçet Disease

Behçet disease is an unusual clinical syndrome hallmarked by recurrent episodes of oral ulcerations, ocular lesions, genital ulcerations, and skin lesions. Histologically, the basic process is that of venulitis, although virtually any size vessel in any location may be involved. The cause of Behçet disease is unknown. Immune complex deposits in involved tissues (92) and lymphocyte-mediated cytotoxicity against oral epithelial cells (93) have suggested an immune pathogenesis.

The disease is found with increased frequency in eastern Mediterranean and Far Eastern countries; additionally, the incidence of the histocompatibility antigen HLA-B51 is greater than expected in affected patients, especially those with the most severe disease (94). Recognized with higher frequency in people living along the “silk route,” affected patients away from this area do not have the associated HLA-B51 frequency.

The initial manifestation of Behçet disease is usually oral ulcers, noted in 75% of patients. These painful lesions may be multiple and may be found anywhere in the oral cavity; healing is generally spontaneous within 2 weeks of onset. Twenty-five percent of patients present with ocular signs, usually iridocyclitis and hypopyon, which are usually self-limited and without late complications. Posterior eye disease (e.g., choroiditis, retinal vasculitis) may be recurrent and can result in visual loss.

Genital ulcers are clinically similar to oral ulcerations. In male patients, these are most frequently found on the scrotum and less commonly on the penis. Vulvar lesions are more common than vaginal ulcers in female patients. Scar formation after healing is noted. Skin lesions may occur as pyoderma, pustules, papules, vesicles, and

erythema nodosum. Cutaneous nodules may be noted after minor skin trauma such as venipuncture. Superficial thrombophlebitis and deep thrombophlebitis are noted in one third of patients. Heparin may have little effect on this condition and, in some instances, may in fact worsen vessel inflammation.

Although infrequently encountered, neurologic involvement, usually manifested as paralysis, headache, or meningoencephalitis, is associated with increased mortality. These findings occur late in the course of Behçet disease, and recurrences may be noted. Renal involvement manifested as proteinuria or hematuria, cardiac involvement manifested as pericarditis, and gastrointestinal ulcerations are observed in a few patients.

Laboratory tests are of little assistance in establishing the diagnosis. Leukocytosis, increased erythrocyte sedimentation rate, and hypergammaglobulinemia may be present. Mononuclear cell infiltration of small vessels is the typical histologic pattern, with a tendency toward perivascular involvement and thrombosis. The diagnosis, however, is usually made on the basis of clinical findings.

No clearly satisfactory therapy for Behçet disease exists. Chemotherapeutic agents, blood transfusions, colchicine, corticosteroids, and nonsteroidal antiinflammatory drugs have all been tried with varying degrees of success. Without large vessel, gastrointestinal, or central nervous system involvement, the prognosis is good. Posterior ocular involvement, usually refractory to therapy, may improve with cyclosporine. In a 2-year trial of daily azathioprine (2.5 mg/kg of body weight per day), improvement was noted in existing ocular and extraocular disease (95); additionally, the development of new eye disease was substantially less than that in patients receiving placebo.

APPROACH TO THE PATIENT WITH SYSTEMIC VASCULITIS

The systemic vasculitides encompass a wide variety of diverse clinical entities. Many of these are clearly separable, whereas others may overlap in their clinical, pathologic, and immunologic considerations. The clinician must correctly categorize the type of vasculitic syndrome and must delineate the extent and nature of the process. Early diagnosis and proper treatment are necessary if severe and often permanent end-organ failure is to be avoided.

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46 ANTIPHOSPHOLIPID SYNDROME

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The *antiphospholipid syndrome* (APS) is an uncommon condition characterized by thrombosis and miscarriage, in the presence of autoantibodies to specific plasma phospholipid (PL)-binding proteins. The diagnosis of APS requires both clinical and laboratory findings. Patients have a range of clinical findings generally attributable to vascular events in both the arterial and venous circulations, and women are also subject to recurrent miscarriage. The autoantibodies are called antiphospholipid antibodies (aPLs) for historical reasons, but they are actually directed against phospholipid-binding plasma proteins, specifically b_2-glycoprotein I (b_2-GPI) and prothrombin (1,2).

The tests for aPL fall into two groups. *In vitro* coagulation tests may be prolonged by aPL in which case the aPL is called a lupus anticoagulant (LA). The most commonly used enzyme-linked immunosorbent assay (ELISA) for aPL uses cardiolipin-coated wells and buffer containing bovine serum: this is called the anticardiolipin (aCL) assay.

HISTORICAL FEATURES

The appreciation of what is now generally known as APS followed from the intersection of several coincident findings (3). Widespread premarital syphilis screening unveiled a group of young women with biologic false-positive tests for syphilis, many of whom had systemic lupus erythematosus (SLE) (4). These patients frequently had thromboses despite prolonged *in vitro* coagulation tests (5). The coagulation tests generally require the addition of anionic PL, providing a surface to which many coagulation factors must bind to be fully active. Cardiolipin is the antigen detected in the Wassermann reagent (the “reagin” of syphilis tests) and is also an anionic PL. Coating the cardiolipin directly onto a microtiter plate (initially as a radioimmunoassay, now as an ELISA) was found to have greater sensitivity than the syphilis screening tests for detecting the population with thrombosis within SLE (6). Autoantibodies, whether LA or aCL, are therefore known as aPLs, and the syndrome has generally become known as APS. Synonyms include the aCL syndrome, the anti-PL protein syndrome, and the eponymous Hughes syndrome.

Contrary to the name of the syndrome, PL itself is not the target for clinically significant antibodies. Autoantibodies occurring in APS generally do not bind to anionic PL in the absence of human or bovine plasma in the buffer (7). Investigators have shown that most aCLs from patients with APS actually bind b_2-GPI, a plasma protein that binds anionic PL (8,9 and 10). On the contrary, aCLs from patients with infections (not limited to syphilis) without clinical features of APS bind directly to cardiolipin (11). Antibodies with an LA effect generally bind either prothrombin or b_2-GPI (12,13). The two major autoantigens in APS are therefore b_2-GPI and prothrombin, not PLs.

IMMUNOLOGIC HIGHLIGHTS

APS is widely accepted as an autoimmune disease. Support for this concept derives from the following: the presence of autoantibodies, the occasional reports of neonatal thrombosis associated with maternal-fetal transfer of antibodies (14), the results of some experiments in the passive transfer of antibody to mice (15), and reports of T-cell reactivity to b_2-GPI (16,17). The association of APS with SLE indirectly supports an autoimmune basis, as does the development of some features of APS in spontaneous mouse models of SLE.

APS was originally recognized in patients with SLE, but it may also occur without other features of a connective tissue disease. This has led to a dichotomy of secondary APS and primary APS, although in practice a continuum may be seen, and individual patients may move from one state to the other over time (18,19). A patient may also experience deterioration, relapse, or remission; because thrombotic events occur infrequently, assessment of the clinical course should be viewed as stochastic, unlike the more progressive changes in symptoms of a condition such as rheumatoid arthritis.

Although SLE is the most frequent connective tissue disease associated with APS, other disorders in which APS may occur do not exactly parallel those disorders that may overlap with SLE. The American College of Rheumatology (ACR) included a positive routine aCL, LA, or false-positive test for syphilis, but not clinical events such as thrombosis, as classification criteria for SLE (20). We disagree with this inclusion, both for reasons of the test method suggested by the ACR and because it seems illogical to diagnose one condition from the presence of another condition (21).

APS nevertheless remains a curious immunologic disorder. Although autoantibodies are present, the typical pathologic finding is of bland thrombosis in the absence of overt inflammatory change. Paradoxically, although the disease is one of thrombosis, the effect of the autoantibodies is to prolong *in vitro* coagulation assays.

EPIDEMIOLOGY

APS occurs in a population similar to that of other connective tissue diseases; the most commonly affected group comprises women of reproductive age. In secondary APS, the female-to-male gender ratio of 7 to 9:1 mirrors that of SLE, whereas primary APS has a lesser female predominance of about 4:1 (18,22). Cases in the young and old are described. The diagnosis in elderly patients is confounded by the frequent occurrence of aCLs or LA in members of this group without APS (23,24) and the frequency of thrombosis from other causes such as atherosclerosis, cardiac embolism, malignant disease, or immobility.

Several sets of diagnostic criteria for APS have been proposed and are discussed further later in this chapter (19,25). Because the formal diagnostic criteria for APS are not fully accepted and there is no current standard test, it is not possible to establish a true prevalence. Nevertheless, by the criteria of a large single study of patients with SLE in Mexico, up to 23% of patients develop “definite” APS over 15 to 18 years (26). A prevalence within an SLE population of about 25% is consistent with the frequency of thrombosis within SLE in other studies (1,27). Not all patients with SLE and aPL have thrombosis; of patients with SLE, 44% have a positive aCL and 34% have an LA. Robust epidemiologic data on primary APS are lacking, but this variant appears to be less frequent; a particular difficulty is that in an unselected non-SLE population, approximately 80% of aCL and LA detected will be unassociated with thrombosis of any sort (27).

APS may also be a heterogenous disorder: many women may only ever have fetal loss; and in patients with a first arterial or venous thrombosis, any subsequent thrombosis tends to be of the same type (28). Some patients present with or develop fulminant, widespread thromboses described as catastrophic APS (29).

Autosomal dominant inheritance appears to account for a small number of cases of APS (30). Case reports have linked factor V_{Leiden} mutation and APS, but neither this nor any other candidate gene was found consistently to segregate with the disease in a larger study of familial APS (30). Human leukocyte antigen (HLA) studies have identified associations between APS and HLA class II haplotypes, but these associations have not held for interracial evaluations (31).

CLINICAL ASPECTS

The manifestations of APS are largely the consequence of thrombosis in the affected organ (2). The pattern of thromboses in APS is unique; both small and large

vessels are affected in both the venous and arterial circulations. A “full house” case is therefore characteristic and easily recognizable clinically. However, not all aspects are present in every case, especially on presentation, and clinicians prefer not to wait for further thromboses despite their utility in confirming the diagnosis. In addition, the aPL tests are regarded as sensitive but not specific for APS. The careful exclusion of differential diagnoses is therefore paramount even in the presence of positive tests for aPL. Conversely, the diagnosis of APS should only be considered (but not conclusively made) in typical clinical cases in the absence of aPL and anti–b_2-GPI; a caveat is that aPL may transiently disappear at the time of a thrombosis (32).

The ACR proposed criteria for the diagnosis of APS in a general population, with the proviso that “… the classification criteria are not intended to be used outside of the context of clinical and scientific investigations of APS” (25). The ACR criteria regard the diagnosis of “definite antiphospholipid syndrome” to be present if at least one significant aPL is detected on two occasions and one clinical criterion is present; there are no strict exclusion parameters. This is a more generous use of “definite” than in previously proposed criteria for APS within SLE (19), which used “definite” for two or more clinical events plus a moderate-titer or high-titer aCL (see later). We also believe that it is essential to exclude other conditions associated with aPL (drugs, malignant disease, infections) that in the presence of thrombosis may otherwise lead to a fallacious diagnosis of APS.

Signs and Symptoms

The three major clinical features of APS are venous thrombosis, arterial thrombosis, and miscarriage. The signs and symptoms reflect the site of the clinical event and the severity. Because almost any organ may be affected in APS, the range of presentations is protean (2).

The sites particularly commonly affected are as follows: the deep pelvic and leg veins, often causing painful leg swelling with or without the symptoms of pulmonary emboli; the cerebral arteries, causing stroke; and the cutaneous small vessels, causing small painful nodules, ulceration, and, more commonly, an unsightly but generally asymptomatic mottling of the skin termed livido reticularis. Miscarriage may present with bleeding or pelvic pain, or it may be detected ultrasonographically while asymptomatic. Thrombocytopenia is rarely severe enough to cause bleeding and is therefore asymptomatic. Chorea may be frequently unreported and is often noticed informally by the physician.

Neuropsychologic conditions secondary to any chronic disease are frequent in APS and include depression, anxiety, and headache. These conditions should not be dismissed because they may also represent cerebral venous or arterial thrombosis or cerebral lupus.

Clinical Features

The major clinical findings positively correlated with the presence of aCL in patients with SLE from the most comprehensive single study by Alarcón-Segovia et al. are detailed in Table 46.1. The clinical features associated with the presence of aCL in this series have generally been supported by other studies. However, a comparison of primary and secondary APS groups suggests that hemolytic anemia may be a feature of SLE *per se* rather than APS (22). Additionally, the term “transverse myel*itis* ” is presumptive; myelo*pathy* is preferable. Other clinical features suggested to be part of APS are detailed by system in Table 46.2. Some of these features are uncommon enough to escape statistical recognition but are nonetheless characteristic of APS; others have not been borne out by later studies but are included for clarification.

Manifestation	n	Percentage (%)
Livido reticularis	202	30
Thrombocytopenia	132	20
Recurrent fetal loss	45[a]	17
Venous thrombosis	76	11
Hemolytic anemia	59	9
Arterial occlusion	21	3
Leg ulcer	21	3
Pulmonary hypertension	11	2
Transverse myelitis	5	1

[a] Out of 261 patients at risk.
From Alarcon-Segovia D, Perez-Vazquez ME, Villa AR, et al. Preliminary classification criteria for the antiphospholipid syndrome within systemic lupus erythematosus. *Semin Arthritis Rheum* 1992;21:375–386, with permission.

TABLE 46.1. Frequency of Clinical Manifestations Associated with Antiphospholipid Antibodies Applied to 667 Patients with Systemic Lupus Erythematosus

	Manifestation
Central Nervous System (99)	**Chorea**
	Multi-infarct dementia
	Deep white matter lesions, mimicking multiple sclerosis
	Epilepsy
	Sinus thrombosis/intracranial hypertension
	(Migraine (100))
Skin (54)	**Painful skin nodules or macules**
	Acrocyanosis
	Sneddon syndrome
Renal	**Thrombotic microangiopathy**
Respiratory	**Pulmonary hemorrhage**
Cardiac	Vegetations/Libman-Sacks endocarditis
Bone	Avascular osteonecrosis
Obstetric	Infertility/in vitro fertilization failure

In our opinion, features in bold have been generally supported by the literature, features in regular type remain uncertain, and features in parentheses have been discounted by later studies.

TABLE 46.2. Other Clinical Features of Antiphospholipid Syndrome

Venous thrombi are found most commonly in the deep or superficial leg veins, the vena cava, the renal, adrenal, hepatic veins, and the cerebral venous sinuses, but they may occur elsewhere; secondary pulmonary emboli are common. Arterial thrombi are less common and typically affect the cerebral, ophthalmic, coronary, mesenteric, and leg arteries, with the aorta also occasionally involved. Hypertension and other risk factors for atherosclerotic arterial disease may contribute to or account for some arterial thrombi in patients with APS. Most, but not all, studies do not support an association between aPL and stroke or coronary occlusion in unselected populations (33,34). This finding illustrates the rarity of APS such that in unselected populations the rare cases of arterial thrombosis induced by APS are statistically overwhelmed by more common causes.

Miscarriage in APS often occurs in the second trimester, which is unusual for other causes of miscarriage. Some authors suggest that despite the trend to the second trimester, most miscarriages in APS still occur in the first trimester of pregnancy.

Bleeding is unusual in APS despite the prolongation of *in vitro* clotting time. When bleeding is present, it suggests the presence of a high-avidity antiprothrombin antibody with hypoprothrombinemia (35), but it may rarely be caused by thrombocytopenia.

Laboratory Investigations

The diagnosis of APS requires the presence of aCL or LA positivity. A patient with APS may have an abnormality in either the aCL or LA, or both: testing and retesting should therefore be performed for both. Each test has various methods, with varying standardization and performance (36,37): it is therefore reasonable to send samples to a second or reference laboratory if any doubt exists. Syphilis testing offers little extra benefit for the diagnosis of APS, although it may be useful to exclude syphilis with specific serology. The attributes of the specific aCL and LA tests are summarized in Table 46.3.

Test	Antigen Detected	Disorder
aCL	β_2-GPI	APS
"	Phospholipid	Infection, other
anti-β_2-GPI	β_2-GPI	APS
antiprothrombin	Prothrombin	?APS
LA (aPTT,KCT,DRVVT)	Prothrombin (low avidity)	APS
"	Prothrombin (high avidity)	Hemorrhage
"	β_2-GPI	APS

aCL, anticardiolipin assay; APS, antiphospholipid syndrome; aPTT, activated partial thromboplastin time; DRVVT, dilute Russell viper venom time; β_2-GPI, β_2-glycoprotein I; KCT, kaolin clotting time; LA, lupus anticoagulant.

TABLE 46.3. Tests Used in the Antiphospholipid Syndrome

The aCL assay relies on the binding of b_2-GPI to CL, which is, in turn, bound by anti–b_2-GPI autoantibodies. Cardiolipin in organic solvent is evaporated onto the wells of a microtiter plate. Test serum along with the appropriate controls is incubated in the presence of buffer, without calcium ions. Bovine serum is an essential component of the buffer because it contains b_2-GPI, the actual antigen (8). The plate is developed using subclass-specific second antibody and a standard ELISA substrate. The strongest associations with disease are for immunoglobulin G (IgG), and then IgM, aCL, although there is some support for also testing IgA aCL (19,25). The result is often expressed in "GPL" or "MPL" units in which one unit is the activity of 1 µg/mL of standard affinity purified antibody. Positive results are divided into low, medium, and high ranges, representing 2 to 5 standard deviations (SD), 5 to 10 SD, and greater than 10 SD higher than the mean, respectively. An important variation to the aCL is using a serum-free buffer with and without b_2-GPI and assessing the difference; this is termed a b_2-GPI–dependent aCL assay (11). Many variations of the aCL ELISA use other PLs, without clear superiority (37,38).

LAs (previously called "lupus coagulation inhibitor," still the Medline subject title) are antibodies that prolong the *in vitro* coagulation time of platelet-free plasma after the addition of an activating substance plus a limited quantity of PL (39). Another negatively charged surface such as kaolin may be substituted for PL. Studies for LA should be done using at least two different methods because no one method detects all LA. The dilute Russell viper venom time activates the common coagulation pathway directly. The intrinsic clotting cascade may be activated through the contact system by the kaolin clotting time or the activated partial thromboplastin time (Fig. 46. 1). When a test result is prolonged, further checks must be performed. The mixing of LA with normal plasma does not normalize the clotting time, unlike in a clotting factor deficiency. The prolongation of the clotting time by an LA diminishes or normalizes with the addition of excess PL, called the PL (or platelet) neutralization procedure. It is also necessary to exclude inhibitory anticoagulants such as heparin and specific factor inhibitors such as anti-factor VIII (40). The degree of prolongation of the clotting time is usually not considered. Autoantibodies with an LA effect may be shown to bind either b_2-GPI or prothrombin (41).

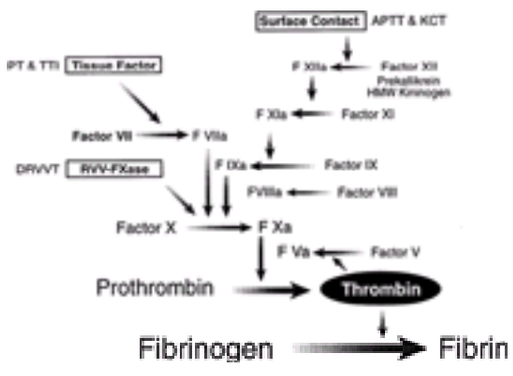

Figure 46.1. The components of the procoagulant pathway as assessed by *in vitro* clotting assays. The prothrombin time (PT) and tissue thromboplastin inhibition test (TTI) assess the tissue factor (extrinsic) pathway; the activated partial thromboplastin time (aPTT) and kaolin clotting time (KCT) assess the contact (intrinsic) pathway; and the dilute Russell viper venom time (DRVVT) assesses the prothrombinase (common) pathway. The DRVVT and the KCT are the most commonly used for the detection of lupus anticoagulant. (Modified with kind permission from T. Exner, Gradipore, Australia.)

A direct anti–b_2-GPI ELISA also has been developed (10). Close homology exists between human and bovine forms of b_2-GPI, and most APS antibodies bind bovine b_2-GPI in the aCL ELISA, but a few bind only to human b_2-GPI (42). Most patient anti–b_2-GPI sera bind only when the b_2-GPI has been coated on irradiated, negatively charged, "high-binding" plates, although the optimal method is not yet clear (43,37). Significant association with APS has been found for IgG, IgM, and IgA isotypes of anti–b_2-GPI, although IgG is the best studied (43,44). Although it is not an ACR accepted test for the diagnosis of APS, the presence of anti–b_2-GPI adds considerably to the confidence of the diagnosis.

The development of an antiprothrombin ELISA for LA-positive samples is proceeding with conflicting evidence of clinical utility to date, although it will probably become a useful secondary test to clarify the antigen in LA-positive plasma (45,46). Antiprothrombin antibodies are not detected by the standard aCL ELISA.

INTERPRETATION OF ANTIPHOSPHOLIPID ANTIBODY ASSAY RESULTS

The quoted predictive power of the aCL, LA, and anti–b_2-GPI tests varies considerably among studies. Because the tests are usually defined in terms of a normal mean +2 to 5 SD, a percentage of a general population will be arbitrarily abnormal. Thrombosis in a general population is also common. Because APS is rarer than either of the foregoing, it follows that some cases of thrombosis associated with aPL are not truly cases of APS. As best as can be determined, the likelihood ratio (pretest-posttest probability of disease after a positive result) of the foregoing tests is around 2–5 (37). In a condition such as SLE in which the pretest probability of APS is 1:3 (25%), these tests will, if positive, raise the probability into the range in which the disease is likely as not or better. However, in many other cases of thrombosis, the likelihood that the cause is APS may be less than 1:10. In this case, a positive result (giving less than 1:1 posttest probability) should not give a clinician confidence in the diagnosis of APS.

The utility of the aCL depends on the cutoff used. Approximately 80% of patients with SLE who have any manifestation of APS, and virtually all with more than three manifestations, have a detectable elevation of aCL (19). However, the false-positive rate is such that SLE patients with aCL in the low positive range have a relative risk for APS little different from that of the baseline SLE population (47). At higher cutoffs, the test is more specific, but approximately 40% of SLE patients with thrombosis have an aCL less than 5 SD higher than the mean (48). Nevertheless, the presence of any elevated aCL titer in a venous thromboembolism study has been shown to predict both recurrence and death (49).

The presence of an LA (by various methods) has been shown in several multivariate analyses of SLE populations to be of greater significance than aCL (50). However, a prospective study of pregnant women found a high proportion of dilute Russell viper venom time results just outside the normal range, without significant consequences (51).

The anti–b_2-GPI assay is less sensitive but has a strong positive predictive value. This test should therefore be used if aPL and clinical features of APS are present but the diagnosis remains equivocal or in the typical clinical case in the absence of aCL or LA (37).

Pathology

The characteristic arterial lesion in APS consists of fibrin thrombus with endothelial changes and intimal proliferation; a variable degree of recanalization may be present (52). Whether intravascular thrombosis or endothelial injury is the primary event is in debate (53). Inflammatory or vasculitic arterial changes are rarely described and should be taken to suggest an alternative diagnosis. Small vessel arterial or arteriolar thrombosis, sometimes with hemorrhage or dermal hemosiderin deposits, is the usual cause of the skin manifestations of APS that, despite their sometimes inflammatory appearance (i.e., painful skin nodules, which look vasculitic),

have an ischemic origin (Fig. 46.2) (54).

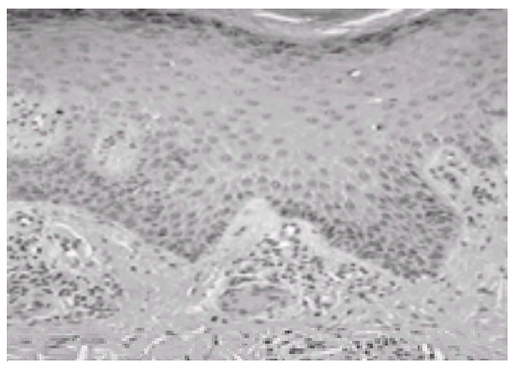

Figure 46.2. Cutaneous small vessel thrombosis in the antiphospholipid syndrome. Skin biopsy from the painful skin nodules of a 33-year-old woman with anticardiolipin, lupus anticoagulant, anti–b_2-glycoprotein I, deep venous thrombosis, and miscarriage. The hematoxylin and eosin–stained section of a skin biopsy shows a small dermal vessel in the center of the lower third of the frame that contains bland fibrin thrombus without inflammation. (Courtesy of Dr. K. Georgeous.) (See Color Figure 46.2.)

The placenta after miscarriage shows uteroplacental vascular disease, with fibrinoid necrosis and sometimes “atherosis” (not atherosclerosis), leading to secondary villous changes including abruption and villous infarcts. Intraplacental vessels may show fibrin thrombi. Less commonly (20%), vasculitis and chronic villitis are present and may occur in the absence of SLE (55). Neither the arterial lesions nor the placental lesions are specific and in particular are similar to those found in malignant or pregnancy-induced hypertension.

Venous thrombi have not been adequately studied histologically despite their frequency in APS. The rare cases of alveolar hemorrhage may be caused by either pulmonary arteriolar thrombosis or pulmonary capillaritis (56).

Therapy

Treatment of APS is with anticoagulation rather than with immunosuppression. Retrospective studies have suggested that patients with APS who have a thrombotic event should receive indefinite anticoagulation with coumarin anticoagulants and that recurrences have occurred at less than an international normalized ratio (INR) of 2.85 to 3 (57,58). With some reagents, the prothrombin time/INR may be falsely prolonged by an LA. This may have the effect of raising the INR of a patient receiving inadequate anticoagulation into the apparently therapeutic range (59). This may have contributed in the foregoing studies to the occurrence of thromboses at INRs of 2 to 3. Anticoagulation to an INR of 3 to 4.5 therefore is not uniformly accepted because of doubts concerning the true degree of anticoagulation and the significant risk of bleeding.

Miscarriage and gestational thrombosis are prevented with the combination of aspirin and low-dose subcutaneous heparin, because coumarin anticoagulants are teratogenic. This regimen has support from randomized clinical trials (60, 61), and it is administered throughout pregnancy after a viable fetus is confirmed by ultrasound until 1 to 2 weeks before term. Low-molecular-weight heparins may be adopted in due course. Consideration should be given to counteracting the osteoporotic effect of heparin. Aspirin is present with regular dosage in breast milk and can affect closure of the *ductus arteriosus;* if required for postnatal prophylaxis of thrombosis, warfarin is minimally present in breast milk (62). Intravenous Ig has not yet been shown to provide significant extra benefit; corticosteroid therapy is not useful and has deleterious effects on the mother.

Immunosuppression does not generally affect outcome in APS. The exceptions are in the treatment of pulmonary hemorrhage, with corticosteroid, and in the rare case of catastrophic APS, when both corticosteroid and plasmapheresis may appear to be of benefit (29). The use of hydroxychloroquine as prophylactic therapy in APS has shown utility in a mouse model and is being tried in human patients (63).

IMMUNOPATHOLOGY

Humoral Response

The major antigens bound by autoantibodies in APS are the plasma proteins b_2-GPI and prothrombin. In APS, b_2-GPI and prothrombin are readily accessible to circulating autoantibody, unlike the nuclear autoantigens generally detected in SLE. Kininogen, annexin V, and protein S have also been cited as autoantigens in isolated cases with features of APS.

Why these proteins should become autoantigens is unclear. The anionic PL phosphatidylserine (PS) moves to the outer layer of the bilaminar cell membrane during apoptosis and necrosis (64). One explanation therefore is that b_2-GPI or prothrombin, bound to PS, may be concentrated on apoptotic blebs with viral proteins from infected cells and may be processed abnormally in individuals with disordered self-tolerance (17,65).

b_2-GLYCOPROTEIN I

b_2-GPI (sometimes apolipoprotein H) is a 326-amino acid glycoprotein present at approximately 200 mg/L in plasma that is able to bind to anionic PL without calcium ions. It is composed of five structurally similar short consensus repeat (SCR, sometimes “sushi”) domains, and this feature characterizes it as a member of the complement control protein family (66,67). Persons without detectable b_2-GPI have been identified who appear clinically well.

The anionic PL binding properties of b_2-GPI are mediated by a cluster of positively charged lysine residues and a hydrophobic loop on domain V (Fig. 46.3) (68,69 and 70). The central portion (domains III and IV) is heavily glycosylated, separating the PL binding site from domains I and II; these domains are of unknown function and project away from the PL surface into the plasma space (69,70). Both domains I and IV have been suggested to contain the major epitopic region for most anti–b_2-GPI autoantibodies (71).

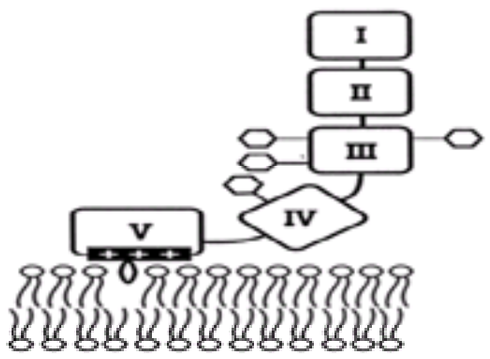

Figure 46.3. Structure of b_2-glycoprotein I (b_2-GPI). Schematic illustration of the structure of b_2-GPI as determined by x-ray crystallography, showing the five domains, the four glycosylation sites, and the positively charged phospholipid binding site with a hydrophobic insertion loop. (Modified from Bouma B, de Groot PG, van den Elsen JMH, et al. Adhesion mechanism of human b_2-glycoprotein I to phospholipids based on its crystal structure. *EMBO J* 1999;18:5166–5174, with permission.)

Autoantibodies are seldom detected when b_2-GPI is coated directly on a standard ELISA plate (Fig. 46.4) (43). This has been interpreted as indicative of a cryptic epitope appearing only when b_2-GPI is bound to PL or the negatively charged surface of an irradiated plate (8,10). Contrary evidence suggests that most antibodies are in the low avidity range and require dimeric binding to b_2-GPI; this concept is supported by higher avidity to a dimer construct of b_2-GPI (72,73 and 74).

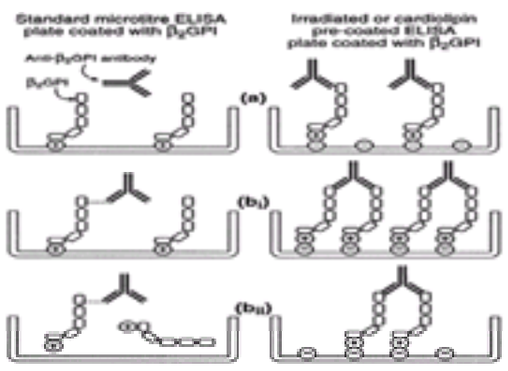

Figure 46.4. Why does anti–b_2-glycoprotein I (b_2-GPI) not bind b_2-GPI on a standard microtiter plate? Anti–b_2-GPI autoantibodies bind b_2-GPI coated on enzyme-linked immunosorbent assay plates only when the plate has been irradiated, inducing a negative charge, or precoated with a negatively charged phospholipid. Theories for this include the following: an unknown conformational change reveals a hidden (cryptic) epitope when b_2-GPI binds to a charged surface *(a);* or the anti–b_2-GPI autoantibodies have a low avidity that requires divalent binding to remain attached to b_2-GPI. On negatively charged plates, an increased antigen density *(b_i)* or uniform orientation of the antigen and its epitope *(b_{ii})* may bring two epitopes sufficiently close that divalent binding occurs.

The physiologic function of b_2-GPI is unknown. Because b_2-GPI may displace other coagulation system proteins from a PL surface, an *in vitro* effect will depend on whether procoagulant or anticoagulant parts of the coagulation system are being evaluated. The plasma level of b_2-GPI is unchanged or increased in APS (75). Anti–b_2-GPI autoantibodies increase the avidity of b_2-GPI for anionic PL (73).

PROTHROMBIN

Prothrombin is well known as the zymogen precursor of thrombin. It is a 579-amino acid glycoprotein present at approximately 100 mg/L in plasma (76). A complete prothrombin deficiency has not been identified in humans and is lethal in mice (77). The amino-terminal region of prothrombin contains a cluster of glutamic acid residues binding PL in a Ca^{++}-dependent manner (gla domain). This region and two kringle domains are cleaved from the thrombin (protease) region by factor Xa in the prothrombinase complex (factors Xa, Va, Ca^{++}, PL^{-}), releasing free thrombin. The major epitopes on prothrombin appear to be on the gla and kringle domains rather than on the thrombin domain (78).

Autoantibodies to prothrombin show wide ranging avidity. Most are low avidity and require similar ELISA conditions (with the addition of calcium) to those required for the detection of anti–b_2-GPI (45,79). Occasional antiprothrombin antibodies are high avidity and result in hypoprothrombinemia, often with bleeding (35). Much of the *in vitro* evidence does not suggest that antiprothrombin directly facilitates the production of thrombin: indeed, it is generally retarded.

Cellular Response

The cellular response in APS has only recently been investigated. T-cell clones reactive to b_2-GPI have been identified by one group in patients with APS but not others: this process may lead to procoagulant activity by expression of monocyte tissue factor (16). Conflicting data have also been published showing T-cell reactivity only to chemically reduced b_2-GPI, but not the protein in native form (17).

Immunopathogenesis

The presence of a physiologic anionic PL surface leads to the production of thrombin and local clot generation in the presence of tissue factor. At the same time, the activation of the protein C pathway and plasmin prevents the clot from spreading away from the exposed surface. Patients with APS have evidence of an ongoing increase in the production of thrombin, despite the inhibition of this *in vitro* by LA (80). One group has reported that purified anti–b_2-GPI may accelerate the *in vitro* prothrombin time (81).

Most groups have shown that the addition of b_2-GPI to purified protein C pathway proteins leads to a slowing in activation of protein C and a diminished effect of activated protein C on factor Va. This effect is potentiated by anti–b_2-GPI (82). However, adding b_2-GPI to a purified procoagulant pathway has a similar slowing effect. One disputed hypothesis is that the anticoagulant protein C pathway may be more inhibited than the procoagulant pathway under certain conditions (83,84).

Annexin V, like b_2-GPI, is an anionic PL binding protein that, presumably by competing with coagulation factors for limited PL, has an *in vitro* anticoagulant effect (85). Previous reports that annexin V is displaced from the placenta in APS by the b_2-GPI/anti–b_2-GPI complex have been debunked (86).

b_2-GPI may also have a direct role in apoptosis by binding to apoptotic cell membrane and functioning as a target protein for phagocytic cells; this may be enhanced by the presence of anti–b_2-GPI (87). It is unclear whether this is the mechanism by which anti–b_2-GPI binds to some endothelial cell preparations or whether there is a specific endothelial cell receptor for b_2-GPI (88). It is debated whether aPL can bind to healthy endothelial cells and, if so, cause either activation or apoptosis, both of which may reasonably be prothrombotic (89).

APS sera reacting to both aCL and oxidized low-density lipoproteins have suggested a link with atherosclerosis, although it has been reported that arterial thromboses correlate with anti–b_2-GPI but not antioxidized low-density lipoproteins (90). The demonstration of the binding of b_2-GPI and the atherothrombotic risk factor lipoprotein (a) is interesting but of uncertain significance (91).

ANIMAL MODELS

Several spontaneous mouse models of SLE may develop aPL and anti–b_2-GPI; they have thrombocytopenia and fetal loss, but not venous thrombosis (92,93). In nonautoimmune mice, anti–b_2-GPI may be induced by immunization with human b_2-GPI, but it is unclear whether they develop any disease (94). Rabbits immunized with human b_2-GPI also develop aPL and anti–b_2-GPI, but they do not develop disease (95).

Passive transfer to mice of purified aPL from women with fetal loss does not routinely lead to murine fetal loss (96). Conversely, passive transfer of purified or monoclonal aPL has been reported to potentiate an induced thrombosis model in mice (15).

A mouse model of APS with fetal loss and thrombocytopenia has been generated by immunization with polyclonal or monoclonal aPL to induce antiantiidiotype antibodies (97). Subsequent studies reported on the treatment of this model. In the SLE version of this model, the findings were not reproducible by an independent group (98).

FUTURE DIRECTIONS

Great progress has been made since the term “antiphospholipid syndrome” was coined in the mid-1980s. It is now a widely recognized syndrome and may even be in danger of being overdiagnosed. There are, however, shortcomings in our ability to deal with this disease. The most important is the requirement for a surrogate marker

of disease activity. This would enable the clinician to titrate the requirement for anticoagulation to the current disease activity. The situation at present in which the clinician and the patient wait for the next thrombosis or hemorrhagic complication before changing therapy is deeply unsatisfactory. The other major shortcoming is the absence of a gold standard test, without which early or less typical cases of APS are difficult to distinguish from other causes of thrombosis or miscarriage in the presence of nonspecific aPL. Current clinical and basic science research is addressing these problems.

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47 SARCOIDOSIS

A. Brent Carter, M.D., and Gary W. Hunninghake, M.D.

Sarcoidosis is a systemic granulomatous disorder that can affect essentially any tissue in the body, but it primarily affects the lung and lymphatic systems. The presence of epithelioid granulomas in more than one organ system is necessary to make the diagnosis of sarcoidosis. In addition, other causes of granulomas must be excluded to make the diagnosis. Although the disease was first described in 1877, the cause remains unknown. The immunologic and pathologic features, however, are well understood and are emphasized in this discussion.

PATHOLOGY

The pathologic hallmark of sarcoidosis is a noncaseating epithelioid cell granuloma (Fig. 47.1). The central portion is composed primarily of $CD4^+$ lymphocytes, whereas the peripheral portion consists of $CD8^+$ lymphocytes, macrophages, and fibroblasts (1,2). The mononuclear phagocytes may also form giant cells in which cytoplasmic asteroid bodies, Hamazaki-Wesenberg bodies, and Schaumann bodies may be found (1,2,3,4,5 and 6).

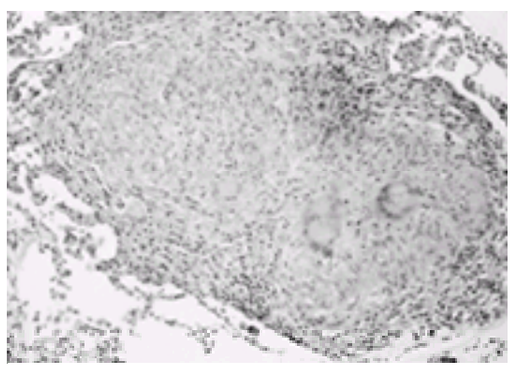

Figure 47.1. Histologic section of a transbronchial biopsy revealing a typical well-formed noncaseating granuloma with multinucleated giant cells and perigranulomatous fibrotic changes (hematoxylin-eosin stain, original ×100).

The presence of cytoplasmic inclusion bodies may be seen in other granulomatous disorders and thus is not specific for sarcoidosis (2). Asteroid bodies are stellate inclusions composed of a collection of cellular organelles in giant cells that have formed by fusion (7). Hamazaki-Wesenberg bodies appear as yellow-brown spirals, which represent extracellular lysosomes or residual bodies (3). Schaumann bodies are a mixture of mucopolysaccharides, calcium salts, and iron, and they appear as concentrically lamellated bodies (8).

Epithelioid granulomas occasionally contain areas of focal necrosis. This may represent a variant of the disease (1,9). Investigators have estimated that slightly less than half the granulomas in sarcoidosis may contain some focal necrosis (10,11). Because infectious diseases are important in the differential diagnosis of sarcoidosis, special microbiologic stains and cultures are necessary, especially when macroscopic necrosis is present. Other causes, however, also need to be considered, such as a tumor-related granulomatous lesion (12,13,14 and 15) and granulomatous lesions of unknown significance (GLUS syndrome) (13). Table 47.1 summarizes the differential diagnosis of sarcoidosis based on specific organ involvement.

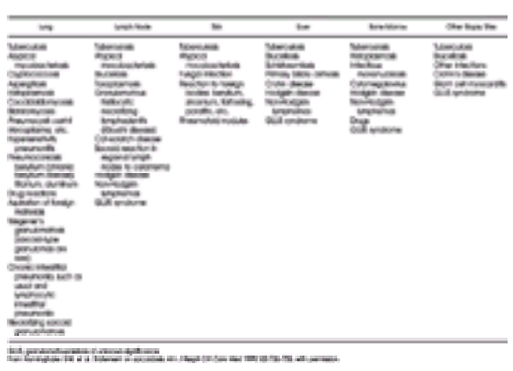

TABLE 47.1. Major Pathologic Considerations in the Differential Diagnosis of Sarcoidosis at Biopsy and Surgical Pathology

The most common tissues that contain granulomas in sarcoidosis are the lung, lymph nodes, skin, liver, and spleen (1,2,4,6,16,17). In the lung, granulomas are typically localized in areas of lymphatic drainage, such as in bronchovascular bundles, perilobular spaces, or subpleural spaces (Fig. 47.2).

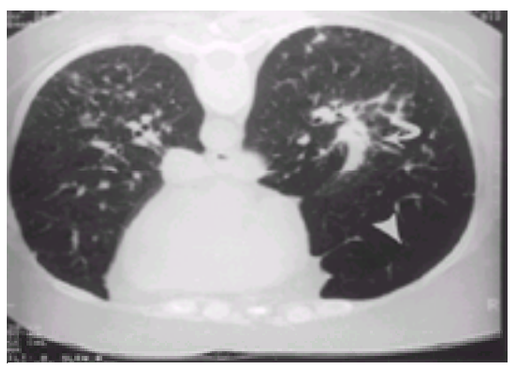

Figure 47.2. Disease activity is intracompartmentally localized in sarcoidosis. High-resolution computed tomography scan of the lung of a patient with sarcoidosis. The disease is localized predominantly around the central bronchovascular bundles *(curved arrow)* while sparing the peripheral gas-exchanging parenchyma *(arrowhead).* This intracompartmentally localization of inflammation suggests that sarcoidosis has a patchy distribution, and in the lung, the lymphatics are found in the bronchovascular bundles.

The course of granulomas is either complete resolution or the development of fibrosis. Most patients have complete resolution of granulomas, but those whose lesions undergo fibrosis may eventually develop honeycombing of the lung. At this time, the factors that initiate the development of fibrosis are unknown (1,2).

EPIDEMIOLOGY

Sarcoidosis occurs worldwide, but it has a predilection for certain areas, especially those with temperate climates (18). The prevalence of sarcoidosis in Sweden, for example, is 60 per 100,000. Africa and Southeast Asia, on the other hand, have an extremely low prevalence (18). The disease has a peak incidence in adults between 20 and 29 years of age (19). In Scandinavia and Japan, however, a second peak incidence occurs in women older than 50 years of age (20,21 and 22). In the United States, the prevalence is highest in the Southeast and averages 10 per 100,000 (18). There is a slightly higher incidence of sarcoidosis in women, and in a population-based incidence study in the United States, the rates were 5.9 per 100,000 person-years for men and 6.3 per 100,000 for women (23). The highest prevalence rates are found in Sweden, in Denmark, and in African Americans (24). In comparison with the white population of the United States, African Americans have an age-adjusted annual incidence rate 3.3 times higher and a lifetime risk of developing sarcoidosis that is 2.8 times higher (23,25,26).

Familial clustering of sarcoidosis has been reported in the United States, Ireland, and Japan (27,28 and 29). In the United States, familial clustering is found more commonly in African-American families (27). Genetic studies in familial clustering of sarcoidosis suggest that the most common genotype inherited in these families include human leukocyte antigen (HLA) class I HLA-A1 and HLA-B8 and class II HLA-DR3 (30,31 and 32).

Although the specific etiologic factor that is responsible for the development of sarcoidosis is unknown, several studies suggest that the disease occurs in persons with a genetic susceptibility who are exposed to certain environmental agents. Some of these studies have suggested the possibility of person-to-person transmission or simultaneous exposure to a specific agent. For example, a study of residents of the Isle of Man reported that 40% of cases occurred in persons with prior interaction with a resident known to have the disease (33,34). These interactions were noted to occur in the same household, at work, and between friends who did not live in the same household. The development of disease within the same household, especially between spouses, suggests the possibility of a mutual infectious or environmental exposure.

Environmental exposure, whether infectious, inorganic, or organic, has long been suggested to be involved in the origin of sarcoidosis. Several of the agents considered to be etiologic factors in sarcoidosis are listed in Table 47.2. Some of the early studies relied on the increased prevalence of sarcoidosis in the southeastern United States. These studies evaluated exposure to pine, lumberyards and mills, soil, pets and farm animals, and water supply (35,36,37,38,39,40,41,42,43,44 and 45). Studies evaluating occupational exposure were subsequently developed; however, no direct association with the development of sarcoidosis was made in these studies (46,47,48 and 49). Unlike with many other pulmonary diseases, nonsmokers have a higher incidence of sarcoidosis or severity of the inflammatory response than smokers (50,51 and 52). The reason may be the decreased number of alveolar macrophages or the increased number of $CD8^+$ lymphocytes recovered from the bronchoalveolar lavage fluid of smokers (51).

Infectious agents
Viruses (herpes, Epstein-Barr, retrovirus, Coxsackie B virus, cytomegalovirus)
Borrelia burgdorferi
Propionibacterium acnes
Mycobacterium tuberculosis and other mycobacteria
Mycoplasma
Inorganic agents
Aluminum
Zirconium
Talc
Organic agents
Pine tree pollen
Clay

From Hunninghake GW, et al. Statement on sarcoidosis. *Am J Respir Crit Care Med* 1999; 160:736–755, with permission.

TABLE 47.2. Examples of Agents Suggested to be Involved in the Etiology of Sarcoidosis

The infectious agents currently proposed to be involved in the development of sarcoidosis include several viruses, *Borrelia burgdorferi, Propionibacterium acnes, Mycoplasma,* and both tuberculous and nontuberculous mycobacteria. Mycobacteria are the most heavily studied among all of these infectious organisms because they are known to cause granulomatous inflammation during infection (53). In fact, two different studies measured antibodies to mycobacteria and found these antibodies in 50% to 80% of patients with sarcoidosis (54,55). Other antigenic components of mycobacteria, such as tuberculostearic acid, muramyl dipeptide, and portions of cell wall–deficient forms, have been measured in patients with sarcoidosis (56,57 and 58). As an aggregate, these studies did not provide evidence that an infectious organism is the etiologic agent in sarcoidosis. Specifically, mycobacteria, although often present in granulomatous inflammation in sarcoidosis, were not shown to represent the antigen that causes development of the disease.

IMMUNOLOGY AND PATHOGENESIS

Although the cause of sarcoidosis is unknown, much of the immunology of this disease has been studied in detail. The initial discernment of the immunology and pathogenesis occurred in 1941, when Ansgar Kveim found that the intradermal injection of lymph node tissue from patients with sarcoidosis resulted in a papular eruption in 12 of 13 patients with sarcoidosis (59). Louis Siltzbach organized an international study after he found similar results using a suspension from spleen cells (60). The Kveim-Siltzbach test, which recognizes these investigators' contributions to understanding the pathogenesis, is positive in up to 80% of patients with sarcoidosis (61,62). A positive reaction requires the presence of epithelioid granulomas 4 to 6 weeks after the inoculation.

Granuloma formation depends on the accumulation of activated $CD4^+$ cells and macrophages (Table 47.3). The increase in activated $CD4^+$ cells in areas of inflammation occurs secondary to both proliferation at the site of inflammation and accumulation of cells derived from the peripheral blood (63,64). Interleukin-2 (IL-2) mediates proliferation of $CD4^+$ cells at the site of inflammation. T lymphocytes obtained from patients with sarcoidosis release increased amounts of IL-2 and express IL-2 receptors (65,66 and 67). In fact, IL-2 has been shown to enhance T-lymphocyte proliferation in areas of inflammation in patients with sarcoidosis (68,69). Not only does this mechanism increase the proliferation of $CD4^+$ cells, but the release of IL-2, in addition to interferon-g, also results in the differentiation of Th0 T-helper cells into a Th1 phenotype (70).

Intraalveolar and interstitial accumulation of $CD4^+$ cells with helper-inducer activity and release of IL-2
Expansion of T cells bearing a restricted TCR repertoire in involved tissues; this pattern is consistent with a TCR oligoclonality; expansion of the lung α/β TCR cell pool in a subset of patients
Increased *in situ* production of Th1 cell–derived cytokines (IL-2 and IFN-γ) during granuloma formation
Increased expression of members of TNF ligand and TNF receptor superfamilies by sarcoid T cells
B-cell hyperactivity and spontaneous *in situ* production of immunoglobulins
Increased spontaneous rate of proliferation of lung immunocompetent cells
Accumulation of monocyte-macrophages with antigen presenting cell capacity and expressing increased levels of activation markers (HLA-DR, HLA-DQ, CD71) and adhesion molecules (CD49c, CD64, CD102)
Increased release of macrophage-derived cytokines (IL-1, IL-6, IL-8, IL-15, TNF-α, IFN-γ, GM-CSF) and chemokines (IP-10, RANTES, MIP-1α, IL-16); most of these cytokines favor granuloma formation and lung damage
Increased production of macrophage-derived fibrogenetic cytokines (TGF-β and related cytokines, PDGF, and IGF-1), favoring evolution toward fibrosis

GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated on activation normal T cell expressed and secreted; TCR, T-cell receptor; TGF, transforming growth factor; TNF, tumor necrosis factor.

TABLE 47.3. Immunologic Abnormalities Observed in Patients with Sarcoidosis

The accumulation of cells derived from the peripheral blood at sites of inflammation is induced by cytokines and chemokines, such as IL-8, IL-15, and RANTES (regulated on activation normal T cell expressed and secreted), released from macrophages (65,71 and 72). The bronchoalveolar lavage fluid from patients with sarcoidosis has elevated levels of IL-8 and has been correlated with the degree of granulomatous inflammation (73,74). In addition, alveolar macrophages from patients with sarcoidosis and a $CD4^+$ cell alveolitis express high levels of IL-15 (75). RANTES protein levels are increased in bronchoalveolar lavage fluid from patients

with sarcoidosis when compared with control subjects (76,77), and RANTES mRNA levels are increased in alveolar macrophages obtained from patients with sarcoidosis (78,79). Furthermore, increased RANTES expression has been correlated with $CD4^+$ redistribution into the lung. This interaction of activated $CD4^+$ lymphocytes and macrophages is summarized schematically in Fig. 47.3.

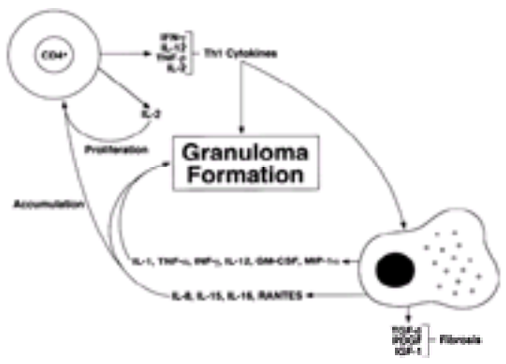

Figure 47.3. Schematic diagram of the classic Th1 immune response.

Most $CD4^+$ and $CD8^+$ lymphocytes use the T-cell receptor (TCR) to identify specific antigens, and exposure of cells to these antigens results in the expansion of T lymphocytes that bear a restricted TCR. More specifically, $CD4^+$ cells use the a/b-chain variable region of the TCR to recognize specific antigens, and the expansion of this restricted variable portion of the TCR results in the oligoclonality of $CD4^+$ cells in areas of inflammation. Several studies involving patients with sarcoidosis have shown the presence of $CD4^+$ cells with a restricted TCR gene expression (80,81,82,83,84,85 and 86). Investigators have demonstrated in patients with sarcoidosis that the oligoclonality of the restricted TCR in $CD4^+$ cells disappears when the granulomatous inflammation resolves (86). These studies showing restricted TCR usage during the period of inflammation suggest that a specific antigen triggers sarcoidosis.

Other nonspecific immunologic abnormalities that can be found in sarcoidosis include polyclonal hypergammaglobulinemia and immune complex formation with elevated levels of rheumatoid factor and antinuclear antibodies (87,88,89,90 and 91). Similar cell-cell interactions that result in granuloma formation drive the increase in antibody production in sarcoidosis (92). Activated T lymphocytes release B-cell growth factors and B-cell differentiation factors, which induce immunoglobulin production by B lymphocytes (93). It is unclear how frequently hypergammaglobulinemia occurs in sarcoidosis, but investigators have shown that T lymphocytes from the lungs of patients with active sarcoidosis can spontaneously activate B lymphocytes to secrete immunoglobulins, and a correlation exists between the number of activated T lymphocytes and the number of immunoglobulin-secreting B lymphocytes in sarcoidosis (88).

Although not clinically relevant, immune complex formation in patients with sarcoidosis has been reported to be as high as 50% (89,94). Elevation of rheumatoid factor and antinuclear antibodies has also been seen in patients with sarcoidosis (89,94), whereas other studies have demonstrated no elevation in these serum factors (90). One other study measured serum factors in patients with inactive sarcoidosis and found that their levels were similar to those of control subjects (95). Thus, the increase in serum factors, such as rheumatoid factor, antinuclear antibodies, and erythrocyte sedimentation rate, is a nonspecific finding and does not appear to be clinically important.

Although many studies propose that a specific antigen is necessary for the development of sarcoidosis, the genetic susceptibility of those who develop the disease is an important factor as well. The basis of evaluating the role of genetic factors in the development of sarcoidosis is that cases of sarcoidosis have occurred in families, and certain races appear to be predisposed to develop the disease (26,43). As discussed previously, studies of familial clustering of sarcoidosis have revealed a high prevalence of certain HLA genotypes in family members who develop the disease (30,31 and 32). These genes may be important in regulation of antigen presentation and signal transduction in the cells that process the antigen and initiate the inflammatory response. Other studies, however, suggest that genetic factors are important in determining the prognostic features—presentation and progression—of sarcoidosis. One study compared 126 cases of sarcoidosis from the Czech Republic with 107 cases from Italy (96). These investigators found a positive association with disease in both populations with HLA-A1, HLA-B8, and HLA-DR3 and a negative association with HLA-B12 and HLA-DR4. Early onset of sarcoidosis occurred in patients with HLA-B13 and HLA-B35, and late onset was found in those with HLA-A30, HLA-B8, HLA-DR3, and HLA-DR4. The primary difference between these ethnic groups was that in the Czech Republic, HLA-B13 was found in patients with extrapulmonary localization of disease. In Italy, conversely, systemic disease was associated with HLA-B22. These findings support the notion of clinical heterogeneity in sarcoidosis (96).

Another study that determined genotypes in Scandinavia, which has a relatively homogeneous population, found that patients with HLA-DR17 had a better prognosis than those with HLA-DR14 or HLA-DR15 (97). HLA-DR17 was present in a high percentage of Scandinavian patients, but it was also shown to be associated with a favorable outcome in other studies (31,96). As an aggregate, these genetic studies indicate that certain genotypes may be related to the development of sarcoidosis and others may be related to the clinical outcome of the disease. In addition, the effect of specific genotypes varies, depending on the ethnic population.

CLINICAL PRESENTATION

Sarcoidosis can affect any organ of the body, so physicians from a variety of specialties may make the initial diagnosis or may be involved in the care of a patient with sarcoidosis. As discussed earlier, the ethnic and genetic background of a patient has a significant influence on the disease manifestation. For example, persons of Swedish, Puerto Rican, or Irish descent often present with Löfgren syndrome, which is characterized by erythema nodosum, polyarthritis, iritis, and fever (98). Another study compared patients of West Indian descent with white patients who lived in the same area (99). These investigators found that the patients from the West Indies had more disseminated disease, and respiratory symptoms were common at presentation. Whites, on the other hand, presented more commonly with erythema nodosum. The effect of various genotypes—specifically, HLA antigens—is discussed earlier and has been associated with variability in disease presentation and overall prognosis.

Constitutional symptoms are common at presentation in sarcoidosis, and they occur more frequently in African Americans and Asian Indians than in whites. Fever is typically low grade and is occasionally associated with night sweats. Sarcoidosis has been known to present as a fever of unknown origin (100). Weight loss, although significant, is usually limited to less than 6 kg. Fatigue and malaise do not occur as frequently as other constitutional symptoms, but they can be incapacitating when present initially.

The lungs are the organs most frequently involved in sarcoidosis; at least 90% of patients have evidence of granulomatous inflammation, even if they are asymptomatic. If symptoms are present, they usually include dyspnea, nonproductive cough, and chest pain (101). Radiographic staging of pulmonary sarcoidosis is an internationally accepted means of classifying the disease. Stage 0 is normal without evidence of pulmonary disease. Stage I is bilateral hilar lymphadenopathy. Stage II is bilateral hilar lymphadenopathy accompanied by diffuse parenchymal infiltrates. Stage III is diffuse parenchymal infiltrates without hilar adenopathy. Stage IV is advanced parenchymal disease with evidence of end-stage fibrosis with honeycombing (18,102). Sarcoidosis involving the airway, which includes the larynx, trachea, and bronchi, is less common than parenchymal disease.

Pulmonary function tests in patients with sarcoidosis show primarily restrictive physiology. Abnormal gas exchange with resultant hypoxemia may be seen in patients with stage IV radiographic disease (102). Sarcoidosis involving the airway often gives a different pattern on pulmonary function tests. These patients may have obstructive physiology and airway hyperreactivity (103,104).

The lymphatic system is the second most common organ system involved in sarcoidosis. Besides the hilar and mediastinal lymph nodes, the most frequently involved are lymph nodes in the cervical, axillary, epitrochlear, and inguinal regions. Lymph nodes in sarcoidosis are nontender and easily movable. Pancytopenia may be present if spleen involvement is significant (105).

The liver is frequently involved in sarcoidosis. Although these lesions are usually insignificant clinically, studies have estimated that between 50% and 80% of patients have granulomas in the liver (106,107 and 108). Treatment is usually not required for mild hepatic dysfunction, but patients with severe abnormalities and evidence of hepatic failure may benefit from corticosteroids or cytotoxic therapy (107,109,110).

Cardiac involvement in sarcoidosis is rare, but if present, the risk of mortality is substantial. Granulomas in the heart can result in both benign and lethal arrhythmias and cardiomyopathy if myocardial involvement is significant. Cardiac sarcoidosis may be detected by several different tests, but thallium-201 imaging has been shown to be the preferred study to detect segmental contractile dysfunction (111,112). If thallium-201 imaging shows contraction abnormalities, coronary artery catheterization

should be performed to exclude coronary artery disease. Endomyocardial biopsies are typically not helpful because of patchy involvement of the myocardium with granulomas. Electrocardiograms may be normal in patients with arrhythmias, but Holter monitoring may be used to detect arrhythmias in patients suspected of having conduction abnormalities.

Ocular sarcoidosis is often asymptomatic, but all patients with sarcoidosis should have an ophthalmologic examination because ocular involvement can result in blindness (113). Although any part of the eye or orbit may be involved, the most commonly affected structure is the anterior uveal tract. Anterior uveitis is the primary disorder in ocular sarcoidosis, and it can be diagnosed with slit-lamp examination. It is usually treated adequately with corticosteroid drops applied locally. When the posterior chamber or the retina is affected, more invasive testing is necessary for detection (114).

The two most important cutaneous manifestations of sarcoidosis are erythema nodosum and lupus pernio. Skin involvement is reported to occur in about 25% of patients with sarcoidosis (115). Erythema nodosum, which is one of the clinical findings in Löfgren syndrome, appears as raised, red, tender nodules on the anterior surface of the lower legs. Joints near the affected skin may also be painful. Erythema nodosum is typically self-limiting and resolves in 6 to 8 weeks. This cutaneous manifestation is primarily seen in European, Puerto Rican, and Mexican patients.

In contrast to erythema nodosum, lupus pernio is a chronic manifestation of cutaneous sarcoidosis, and resolution is extremely rare. It is most commonly seen in African Americans. Lupus pernio appears clinically as indurated plaques associated with discoloration of the nose, cheeks, lips, and ears. It is also associated with bone cysts and pulmonary fibrosis.

Sarcoidosis can involve any portion of the nervous system in the form of diffuse inflammation or a mass lesion (116), but this manifestation occurs in fewer than 10% of patients (117,118). Neurosarcoidosis is difficult to diagnose clinically, and, therefore, histologic samples are often required. Laboratory testing is nonspecific, but both computed tomography and magnetic resonance imaging can be used to support the diagnosis. The presentation is also variable and may include cranial nerve palsies, polyneuritis and polyneuropathy, meningitis, mass lesions, pituitary and hypothalamic syndromes, and memory loss (119).

Hypercalcemia is seen in fewer than 10% of patients, whereas hypercalcuria is seen in as many as 50% (120,121). The mechanism for both hypercalcemia and hypercalcuria is increased production of 1,25-dihydroxycholecalciferol (calcitriol) by granulomas (120,121). Persistent hypercalcuria can result in nephrolithiasis, nephrocalcinosis, and renal failure (122).

THERAPY

The therapeutic regimen used to treat patients with sarcoidosis needs to be individualized because of the variability in the prognosis. In fact, sarcoidosis is often a self-limited disease, so frequent observation, rather than specific drug therapy, is often the best approach (123). In patients with mild skin, ocular, or airway disease, topical therapy, including inhaled corticosteroids, may be sufficient. Systemic therapy is indicated in patients with progressive disease and in those with hypercalcemia and cardiac, neurologic, or ocular sarcoidosis.

Corticosteroids, whether topical or systemic, are the first-line drugs used for treatment of sarcoidosis. Topical therapy may be used in patients with skin involvement, uveitis, nasal polyps, and airway disease (124,125,126,127,128 and 129). Many studies have shown that topical therapy is beneficial (124,125 and 126,129,130 and 131), but there has been some disagreement in the efficacy of this type of therapy (128).

Systemic corticosteroids have been shown in clinical trials to be beneficial in patients with pulmonary sarcoidosis, as measured by symptoms, radiographic studies, and pulmonary function studies (132,133,134,135,136,137,138,139 and 140). Some reports, however, have noted that symptoms and radiographic abnormalities return with cessation of therapy (135,141). The duration of therapy needs to be individualized, but patients should be expected to have some response after 3 months. Patients who respond to corticosteroids should gradually have the dosage tapered over a minimum of 12 months. If relapses occur, patients with sarcoidosis may require long-term, low-dose corticosteroids (135,141).

Because of the many side effects of systemic corticosteroids, many studies have been undertaken to evaluate the efficacy of steroid-sparing agents. These agents primarily fall in the category of cytotoxic agents. These drugs, specifically methotrexate and azathioprine, are useful in some patients with sarcoidosis, but often no clear indication exists regarding when to initiate therapy with these agents. Methotrexate has been used to treat chronic forms of the disease. One study found a response in 66% of patients taking methotrexate alone, and another 18% responded when low-dose corticosteroids were added to the regimen (142,143). Relapse of sarcoidosis has been shown to occur when this agent is discontinued (143). Like methotrexate, azathioprine has been used in the treatment of chronic disease. In fact, two studies found a response to therapy in chronic sarcoidosis when azathioprine was used in the treatment regimen (144,145).

SUMMARY

Sarcoidosis is a systemic disorder that is characterized by a classic Th1 immune response. In fact, sarcoidosis was the first human disease in which the Th1 response was clearly delineated. This immune response appears to be modified by genetic factors, and it likely occurs in response to exposure to an environmental agent. Therapy of the disease is directed at the immune response. Further advances in the understanding and therapy of this disease are likely to depend on defining the cause of the disorder.

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48 DIABETES MELLITUS

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CLASSIFICATION

In the broadest sense, *diabetes mellitus* can be defined as a group of disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The long-term sequelae of the chronic hyperglycemia associated with diabetes mellitus include damage to the small blood vessels (microangiopathy) of various organs, especially the eyes, kidneys, and nerves, and damage to large arteries (macroangiopathy) (1). These complications of diabetes mellitus are among the leading causes of blindness, lower extremity amputation, and end-stage renal disease and are a major factor contributing to premature death (2). Classifications of diabetes mellitus have been based on the requirement for insulin therapy (e.g., insulin-dependent or IDDM, non–insulin-dependent or NIDDM), the age of onset (adult onset, juvenile onset), and the pathogenesis (e.g., malnutrition related, “other types,” gestational). IDDM signified a form of diabetes in which, in the absence of insulin therapy, a patient died. It was realized, however, that insulin dependence could not be tested; many patients early in the course of type 1 diabetes had significant endogenous insulin secretion, and most patients with type 2 diabetes eventually are treated with insulin. A newer classification of diabetes mellitus, put forth by the American Diabetes Association, serves to encompass the increasing knowledge of the etiology and natural history of diabetes (1). This classification system is based on our current understanding of etiology, and the terms IDDM and NIDDM are no longer used. The four major categories for diabetes are as follows: *type 1 diabetes,* characterized by b-cell destruction that often progresses to absolute insulin deficiency); *type 2 diabetes,* which ranges from primarily insulin resistance to insulin secretory defect with insulin resistance; *other specific types of diabetes,* a group that includes mutations such as those causing maturity-onset diabetes of the young (MODY) (MODY2-glucokinase, MODY3 hepatocyte nuclear factor (HNF)-1a mutation), pancreatitis, endocrinopathies, drug-induced diabetes, diabetes associated with “stiff man” syndrome or antiinsulin receptor antibodies, and chromosome abnormalities associated with diabetes (e.g., trisomy 21); and *gestational diabetes mellitus,* defined as diabetes with onset or first recognition during pregnancy.

Type 1 diabetes is divided into type 1A (immune-mediated) and type 1B diabetes (idiopathic loss of insulin secretion), depending primarily on the presence or absence of antiislet autoantibodies. In type 1A diabetes, the rate of b-cell destruction is variable, rapid in some persons (mainly infants and children) and slow in others (mainly adults). With current autoantibody assays, adults with immune-mediated but slowly progressive diabetes, termed latent autoimmune diabetes of adults (LADA) (3) or slowly progressive IDDM (4), can be classified as having type 1A diabetes (1). A report from Japan suggests that a form of type 1B diabetes can be extremely rapid in onset and is associated with pancreatic exocrine inflammation (5). This chapter concentrates on what has been termed type 1A diabetes or the form of diabetes resulting from immune-mediated destruction of the cells that produce insulin.

HISTORICAL FEATURES OF TYPE 1A(IMMUNE-MEDIATED) DIABETES

Recognition of distinct forms of diabetes and appreciation of the immunologic basis for a subset of the disease are relatively recent. Without the separation of diabetes into type 1 and type 2 forms, lymphocytic invasion of the islets (which is associated only with type 1 diabetes) was rarely observed (6,7,8,9 and 10).

A major advance was the recognition of genetically distinct forms of diabetes. Studies of monozygotic twins (MZTs) with diabetes revealed different patterns of disease expression (earlier age of onset for type 1 diabetes) and lower concordance for childhood onset diabetes compared with adult-onset diabetes. Specific HLA alleles were found to be associated with the insulin-dependent, usually childhood-onset, form of diabetes (what we now term type 1A diabetes) (11,12). It was proposed that type 1A diabetes was predominantly a disorder of acute viral origin, whereas for some patients, it was of autoimmune origin. In particular, patients with other autoimmune diseases such as Addison disease were thought to have the autoimmune form of diabetes (13,14). In 1974, cytoplasmic islet cell autoantibodies were found in the sera of patients with diabetes who had polyendocrine autoimmunity (e.g., Addison disease) (15). Islet cell autoantibodies were then found to be present before the development of diabetes for most patients with insulin-dependent diabetes. Studies of MZTs of patients with type 1A diabetes led to the observation that years before the development of type 1A diabetes, associated with the presence of islet autoantibodies, insulin secretion was progressively lost (16,17 and 18).

IMMUNOLOGIC HIGHLIGHTS

Over the past decade, type 1A diabetes has become one of the best studied autoimmune disorders. Prospective studies of large populations beginning at birth now track disease development. Evaluation of antiislet autoantibodies in these studies reveal that they often appear before 1 year of age. Depending on the spectrum of autoantibodies present, hyperglycemia develops within several years.

Although the detection of antiislet antibodies is useful for disease prediction, the consensus among investigators is that b-cell destruction is mediated by T cells, and not by autoantibodies (19,20 and 21). With the discovery of two spontaneous animal models of type 1A diabetes, the NOD mouse and the BB rat, studies of animal T cells have contributed to our understanding of the immunopathogenesis of this disease (21,22). Islet-specific T-cell clones have been isolated from the spleen, lymph node, and islets of Langerhans of NOD mice, and such islet-specific T cells are capable of inducing diabetes after adoptive transfer into young NOD mice (23). Evidence of disease “transfer” in human patients is also available with the recurrence of islet b-cell destruction in pancreas transplanted from a nondiabetic identical twin into a twin with long-term diabetes (24). The development of diabetes in the transplanted pancreas was remarkable in that diabetes recurred within a matter of weeks after organ transplantation. This finding suggests that the immune process that leads to type 1A diabetes remains intact for years after the development of diabetes. In contrast, the development of diabetes usually occurs over years. One hypothesis, which we favor, is that the development of type 1A diabetes is a chronic process with long-term immunologic and progressive metabolic abnormalities. This hypothesis allows the development of diabetes to be divided into a series of stages, beginning with genetic susceptibility and ending with essentially complete b-cell destruction, as shown in Fig. 48.1. In human patients, not only is there progressive loss of insulin secretion after intravenous glucose before the diagnosis of diabetes, but also, after the diagnosis of diabetes, C-peptide secretion is progressively lost (Fig. 48.2).

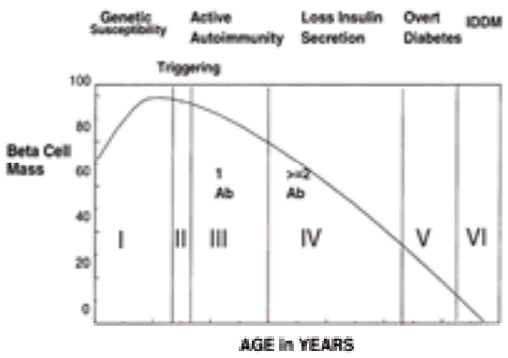

Figure 48.1. Stages in the development of type 1A diabetes. (Adapted from Eisenbarth GS. Type I diabetes mellitus: a chronic autoimmune disease. *N Engl J Med* 1986;314:1360–1368, with permission.)

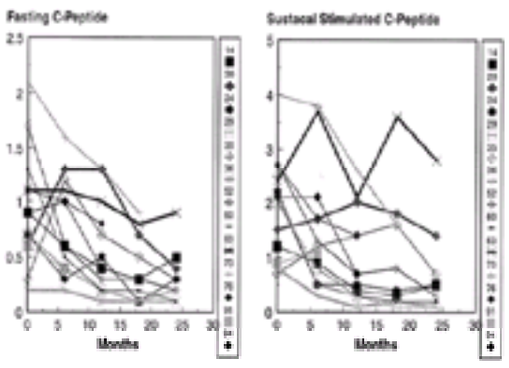

Figure 48.2. Example of progressive loss of C-peptide secretion in children after the diagnosis of type 1A diabetes. Younger children often lose C-peptide secretion even more rapidly.

Investigators have suggested that b-cell destruction in NOD mice occurs just before the onset of overt diabetes. This suggestion is based on the observation that the splenocytes change from a predominantly Th2-cell (T-helper) phenotype to a predominantly Th1-cell phenotype just before the development of hyperglycemia (25), as well as on evidence of accelerated islet graft rejection 2 weeks before the onset of diabetes (26). However, a study of b-cell mass and rate of b-cell proliferation in NOD mice by Sreenan and co-workers found that b-cell proliferation (which could be a compensatory response to ongoing autoimmune b-cell destruction) was observed in mice at 8 weeks of age. Despite the compensatory b-cell proliferative response, b-cell mass was significantly reduced by 13 weeks of age; only 11% of NOD mice at this age were overtly diabetic, and most mice did not become diabetic until after 16 weeks of age (27). These data suggest that b-cell destruction occurs gradually, eventually progressing to overt diabetes in NOD mice. We reported that insulin autoantibody (IAA) expression at 8 weeks in NOD mice can predict the early development of diabetes at 16 to 18 weeks (28). Identifying a cohort of NOD mice expressing IAA at 8 weeks should facilitate the study of heterogeneity of the timing of b-cell destruction.

EPIDEMIOLOGY

Genetics

Similar to most autoimmune disorders, type 1A diabetes develops in persons with genetic susceptibility (29,30,31 and 32). In the general population, the risk of type 1A diabetes by the age of 15 years is approximately 1 in 400 for children (33). This is in marked contrast to the risk to a child of a father with type 1A diabetes (1 in 40) or the risk of a child of a mother with type 1A diabetes (1 in 66) (34). For siblings of a patient with type 1A diabetes, the risk of diabetes is 1 in 20. Although the risk of diabetes is greatly increased for first-degree relatives of patients with type 1A diabetes, 85% to 90% of persons who develop diabetes do not have a relative with the disorder.

The concordance among MZTs of patients with type 1A diabetes (50%) differs markedly from the concordance of dizygotic twins (DZTs) (5%), in whom the risk is similar to that of other siblings (35). Although the probability that a discordant MZT will develop diabetes decreases with the duration of discordance, twins can become concordant more than 40 years after the development of diabetes in their twin (Fig. 48.3). This difference in concordance between MZTs and DZTs reflects a significant role for inherited factors. However, the finding that the concordance among MZTs is not closer to 100% suggests that environmental factors, somatic mutation, or stochastic events may also play a role in pathogenesis. Expression of antiislet autoantibodies is similarly much greater for the discordant MZT as compared with the DZT mate of patients with type 1A diabetes. Most MZTs of patients with type 1A diabetes expressing antiislet autoantibodies develop diabetes (36).

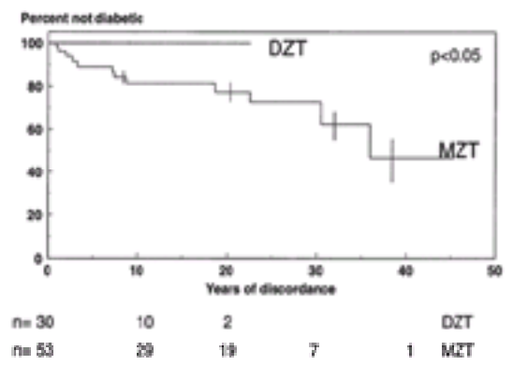

Figure 48.3. Progression to diabetes of initially discordant monozygotic twins by life-table analysis. *X-axis:* years of discordance. (Adapted from Redondo MJ, Rewers M, Yu L, Garg S, Pilcher CC, Elliott RB, et al. Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study. *BMJ* 1999;318:698–702, with permission.)

One study suggested that either serum interleukin-4 (IL-4) levels or IL-4 production by natural killer (NK) T cells could aid in the definition of risk of diabetes among twins (37). The serum IL-4 assay used in this work artifactually measured heterophile antibodies directed against the murine monoclonal anti–IL-4 antibodies used in the IL-4 assay (38,39). The presence of such heterophile antibodies are correlated with diabetes-protective human leukocyte antigen (HLA) alleles (e.g., DQB1*0602) and thus correlated with lack of disease progression. Prospective study of NK T cells will be necessary to determine whether their lack of IL-4 secretion is causally related or is a result of the development of type 1A diabetes.

HUMAN LEUKOCYTE ANTIGEN GENES (IDDM 1)

The association between autoimmune diseases and genes of the major histocompatibility complex (MHC) has been well documented. The establishment of large repositories with DNA (e.g., Human Biologic Disease Interchange, British Diabetes Association Warram Repository) from hundreds of complete families with type 1A diabetes and developments in genetic analysis have led to large studies of HLA alleles and other genetic loci (40,41,42 and 43).

Class II molecules, in particular, DQ alleles, are most strongly associated with diabetes risk, but DR, DP, and class I alleles can influence diabetes risk and age of diabetes onset. DQ and DR alleles are in linkage dysequilibrium. For example, the DR3 allele DRB1*0301 is almost always associated with DQA1*0501, DQB1*0201,

whereas the DR4 allele DRB1*0401 is commonly associated with DQA1*0301 and one of three DQB1 alleles, DQB1*0301, DQB1*0302, DQB1*0303. The highest risk for type 1A diabetes is associated with persons expressing both DQA1*0501-DQB1*0201 (DQ2) and DQA1*0301-DQB1*0302 (DQ8) (DR3/4 or DQ8/DQ2 heterozygotes).

Using a rapid method for screening HLA alleles from umbilical cord blood samples, we have typed more than 20,000 newborns in Denver, Colorado. The high-risk genotype (DRB1*03/DRB1*04, DQB1*0302) is present in 2.4% of newborns (44). Both relatives of patients with type 1A diabetes and infants from the general population are studied. As many as 40% of siblings of patients with type 1A diabetes with DR3/4 (DQB1*0302) develop antiislet autoantibodies by the age of 3 years, in contrast to fewer than 5% of general-population children with DR3/4 (DQB1*0302).

In addition to these high-risk HLA haplotypes, less common HLA haplotypes are associated with diabetes risk. From our analysis of the transmission of DQ haplotypes to affected children of the Human Biological Data Interchange family collection, DQA1*0501-DQB1*0201 and DQA1*0301-DQB1*0302, when present in a parent, were transmitted to more than 80% of affected children, as expected. The DQA1*0401-DQB1*0402 haplotype was transmitted to 82% of diabetic children when a parent had this haplotype (without DR3 and DR4 as the alternative parental haplotype). This haplotype is uncommon in the general population, although it is important to a person or family in which this haplotype occurs (Table 48.1) (45). DQB1*0402 contains an aspartic acid residue at position 57 of the DQ b chain that has been reported to be associated with protection from type 1A diabetes (46,47). In Korean and Japanese patients with type 1A diabetes, an allele related to DQB1*0402 also with Asp57 is commonly present, a finding suggesting that similar HLA alleles contribute to type 1A diabetes risk in all populations, but specific alleles differ in frequency for different ethnic groups (48).

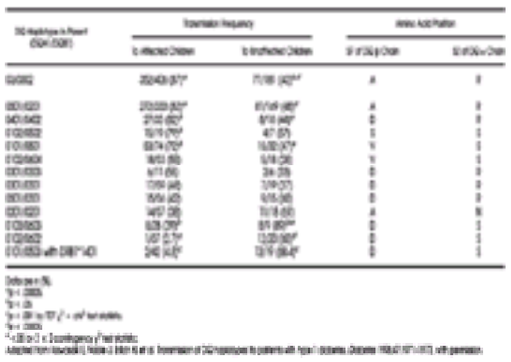

TABLE 48.1. Transmission Frequencies DQ Haplotypes to Affected and Unaffected Children from Heterozygous Parents

The HLA haplotype DQA1*0102-DQB1*0602 is present in 20% of the general population and provides dominant protection from type 1A diabetes (49). DQA1*0102-DQB1*0602 was transmitted to only 1 of 37 (2.7%) children with diabetes (Table 48.1). In contrast, 60% of unaffected children received this haplotype (45). Further analysis indicates that rare alleles such as DRB1*1401 and DQA1*0201, DQB1*0303 may be as protective as the protective DQ molecule DQA1*0102, DQB1*0602 associated with DR2. It appears that in humans, as in the NOD mouse, both DQ and DR alleles (mouse I-A and I-E, respectively) can protect from type 1A diabetes. We have evidence that DQB1*0602 does not prevent the expression of antiislet autoantibodies, although most autoantibody-positive persons with DQB1*0602 express only a single autoantibody.

At present, it is not possible to encapsulate the protection and susceptibility associated with the foregoing alleles by reference to a single polymorphic amino acid residue. For example, the hypothesis that aspartic acid at position 57 of DQb alleles is associated with diabetes risk (47) fails to account for the high-risk allele DQB1*0402 or to differentiate a series of alleles with dramatically different “protection” (e.g., DQB1*0602 versus DQB1*0301, both having Asp 57) or the influence of HLA DR alleles (50). The “most” diabetogenic HLA DQ allele, DQ8 (DQA1*0301, DQB1*0302), when associated with DRB1*0403 does not form a high-risk haplotype. For the haplotype DRB1*1401, DQA1*0101, DQB1*0503, it is the DRB1 allele rather than DQ that is protective (e.g., when DQA1*0101, DQB1*0503 is present without DRB1*1401, it is transmitted to children with diabetes approximately 50% of the time).

HLA-DR and DQ alleles in type 1A diabetes have been well studied; however, the role of the DP allele or class I allele is less well documented. Erlich and co-workers reported an association of type 1A diabetes with the DPB1 allele 0301 in a population of Mexican Americans by an association study between patients and control subjects (51). An increased frequency of DPB1*0301 among patients was observed in the group excluding DRB1*0405 subjects (which is one of the susceptibility alleles in Mexican Americans), a finding suggesting that polymorphisms at the DPB1 locus may also influence diabetes risk.

DQA1*0301 is associated with susceptibility to type 1A diabetes in Japanese patients (52,53). Nakanishi and co-workers have reported the association of the HLA class I allele, A24, with less residual b-cell function (54). They studied the association of the HLA-A24 with type 1A diabetes in Japanese persons who did not have DQA1*0301. All patients with type 1A diabetes without DQA1*0301 had HLA-A24, whereas 60% of normal control subjects or 58% of patients with type 1A diabetes with DQA1*0301 had HLA-A24 (55). This finding suggests that HLA class I may contribute to susceptibility to type 1A diabetes independent of HLA class II alleles.

A major unanswered question is how HLA alleles contribute to the development of diabetes and autoimmunity. Although this question has no definitive answer, several hypotheses and data are provided by studies in humans, by spontaneous animal models of type 1 diabetes, and by transgenic animal models. One hypothesis is that high-risk class II molecules may be unstable, and protective molecules may be more stable. The I-A^{g7} molecule (NOD murine homolog of DQ) is reported to be unstable (56,57), and the protective DQA1*0102/DQB1*0602 is reported to be more stable than other HLA-DQ molecules (58). It is proposed that such a lack of stability may allow autoreactive T lymphocytes to escape negative selection in the thymus. The observation that the haplotype DRB1*1501, DQA1*0102/DQB1*0602 allele is associated with a high risk of multiple sclerosis but protection from type 1A diabetes argues against a global association of DQ allele stability with protection from autoimmunity.

Schmidt and co-workers studied an animal model with diabetes that developed subsequent to introduction of a transgenic T-cell receptor (TCR) (59). These animals are protected from diabetes by several different class II HLA molecules, all of which induce thymic deletion of this T-cell clonotype. Thus, mechanisms of central tolerance may play an important role in preventing autoimmunity and diabetes. Wen and co-workers described a fascinating murine model in which the high-risk human DQA1*0301/DQB1*0302 allele was introduced into the mice (60). When this DQ8 transgene was combined with islet expression of a costimulatory molecule (B7-1), spontaneous diabetes occurred. This finding suggests that the DQ8 allele directly influences the development of type 1A diabetes. Such an influence on diabetes susceptibility may result from presentation of a critical islet peptide to T cells or the shaping of the TCR repertoire.

INSULIN GENE (IDDM 2)

Studies of the genetics of type 1A diabetes may provide an indication of how expression of a target autoantigen, namely, insulin, can affect tolerance induction and susceptibility to autoimmune disease. The insulin gene on chromosome 11 (IDDM 2) contributes to diabetes susceptibility. The promoter region of this gene has a variable nucleotide tandem repeat *(VNTR)*, which is associated with risk of type 1A diabetes (61,62). Such repetitive sequences often undergo expansion or contraction. Given its location in the 5' regulatory region of the insulin gene, such length polymorphisms have been reported to affect and to correlate with intrathymic expression of insulin messenger RNA (63,64). The insulin gene polymorphism with a larger repeat region results in greater thymic expression of insulin mRNA (presumably favoring more efficient negative selection and tolerance) and is associated with protection from diabetes. This finding is in contrast to the smaller repeat region of susceptible persons, in which there is a lowered intrathymic expression of the insulin gene (63,64). Thus, a lack of central tolerance to insulin as a result of altered thymic expression may underlie part of the pathogenesis of type 1A diabetes.

Thymic expression of peripheral and sequestered antigens (e.g., insulin, myelin basic protein) has been determined to be a normal event (65), and it is believed to be critical for the establishment of central tolerance (66,67). These data are further supported by the work of Egwuagu and colleagues, who studied the differences in susceptibility to experimental autoimmune uveoretinitis (EAU), an animal model for human intraocular inflammation (uveitis). These investigators reported that thymic (mRNA and protein) expression of ocular autoantigens in the thymus correlated with resistance to autoimmune disease (EAU) (68). These data (together with the data on the insulin gene) suggest that thymic malfunction, in particular, alterations of thymic antigen expression, may be necessary but not sufficient for the development of autoimmunity.

OTHER NON–MAJOR HISTOCOMPATIBILITY COMPLEX GENES

Investigators believe that the combined effects of haplotype sharing for the HLA and insulin (INS) regions account for, at most, 60% to 70% of the familial aggregation of type 1A diabetes. However, in some populations, the combined effects of HLA and INS contribute less than 50% of the familial risk, a finding suggesting that other

non-MHC loci contribute to the genetic risk. To identify regions that may contain susceptibility genes, several genomewide linkage studies have been undertaken. There has been great difficulty in reproducibly identifying specific genes underlying diabetes susceptibility outside of MHC alleles and the insulin gene. Most of genetic studies have implicated only large chromosomal regions in diabetes susceptibility, and for most of these putative loci, confirmation by multiple groups is lacking. Currently, three major modes of inheritance may account for the majority of type 1A diabetes: polygenic, oligogenic, and monogenic inheritance. (If the disorder is oligogenic or monogenic, there must be heterogeneity.) The existence of all three modes of inheritance in different families, we believe, is likely because of animal models with different modes of inheritance.

Evidence for Polygenic Inheritance

More than 20 candidate regions associated with type 1A diabetes have been reported in studies evaluating pairs of siblings with diabetes (69,70,71,72,73,74,75,76,77,78 and 79) (Table 48.2). Most of the known or suspected susceptibility loci have been designated IDDMn, where n is a numeric identifier. The associations reported to date are relatively weak and have proved difficult to confirm (79,80 and 81). Although the non-MHC loci described to date have a weak influence on diabetes risk in a mixture of families, extremely potent and important regions may confer a diabetes risk in specific families.

Locus	Region	λ-s	Candidate gene or closely linked marker
IDDM1	6p21.31	1.7–4.2	HLA class II
IDDM2	11p15.5	1.6	5′ VNTR insulin gene
IDDM3	15q26	<1.5	D15S107
IDDM4	11q13	1.0–1.5	FGF3
IDDM5	6q25	1.0–3.0	ESR
IDDM6	18q21	1.0–1.5	D18S64
IDDM7	2q31–33	1.0–1.6	D2S152
IDDM8	6q27	1.0–2.1	D6S446/D6S264
IDDM9	3q21	1.0–1.7	D3S1300
IDDM10	10p11–q11	1.1–2.2	D10S193
IDDM11	14q24.3–14q31	<1.5	
IDDM12	2q33	<1.5	CTLA-4/CD28
IDDM13	2q34	<1.5	D2S164
IDDM15	6p21	<1.5	
IDDM17	10q25.1		D10S1681

TABLE 48.2. Insulin-Dependent Diabetes Mellitus (IDDM) Candidate Regions Identified by Linkage/Association Studies

Evidence for Oligogenic Inheritance (IDDM17)

We performed linkage and haplotype analysis in a large Bedouin Arab family with 20 members with type 1A diabetes (Fig. 48.4) (82). In this family, homozygosity (suggesting autosomal recessive inheritance) at a locus on chromosome 10 (now termed IDDM17) and HLA alleles DR3 or DR4 convey a risk of diabetes of approximately 40% (nonparametric linkage score greater than 4). When the gene underlying this association is identified, based on the genetic heterogeneity model, we predict that this gene contributes to the development of diabetes in a subset of families from other populations.

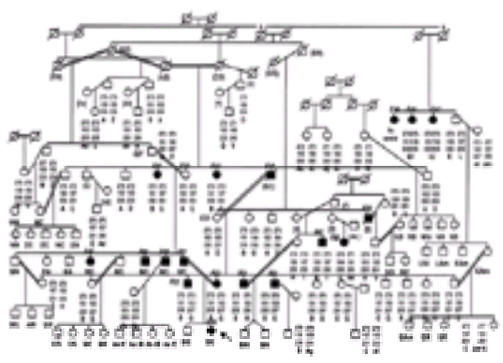

Figure 48.4. Family tree in which 18 members have developed type 1 diabetes, and diabetes appears to be inherited as an autosomal recessive mutation at a locus on chromosome 10 plus typical type 1 HLA alleles. The B haplotype on chromosome 10 occurs in most patients with diabetes (*numbers* refer to chromosome 10q25.1 microsatellite alleles), and patients with diabetes have homologous B-like 10q 25.1 regions for other diabetic haplotypes (e.g., haplotypes, Ae, O, I, J, C, T, G, and H).

Evidence for Monogenic Inheritance: Autoimmune Polyendocrine Syndrome Type I: A Mutation of the Autoimmune Regulator Gene

The two distinct autoimmune polyendocrine syndromes are termed autoimmune polyendocrine syndrome type I (APS-I) and type II (APS-II) (13,14 and 15,83,84,85,86 and 87). APS-II is strongly associated with type 1A diabetes and Addison disease as well as autoimmune thyroid disease. HLA alleles, in particular DR3 and DR4 haplotypes, contribute to disease, but given the familial aggregation of the syndrome, other genes contributing to susceptibility are likely. In contrast, the APS-I results from a recessive mutation of a gene on chromosome 21 (21q22.3), termed the autoimmune regulator gene (AIRE) (88,89). APS-I is a distinctive syndrome, with the major disorders being mucocutaneous candidiasis, hypoparathyroidism, and Addison disease and, less frequently, autoimmune hepatitis. Other endocrine and nonendocrine disorders may also develop in these patients and include type 1A diabetes, gonadal failure, alopecia, vitiligo, pernicious anemia, nail and dental enamel dystrophy, and keratopathy. Approximately 18% of patients with APS-I develop type 1A diabetes. More patients express glutamic acid decarboxylase 65 (GAD65) autoantibodies (almost 50%) than develop diabetes (90,91). Both type 1A diabetes and Addison disease are strongly associated with the HLA-DR3 and DR4 alleles, but patients with APS-I show no susceptibility to class II HLA association. This finding suggests that the mutation that results in APS-I is able to bypass the influence of HLA alleles on disease risk. The syndrome is so distinctive that essentially all families with the clinical syndrome, when studied, have mutations of the AIRE gene (92).

The manner by which mutations of the AIRE gene influence autoimmunity is unknown. Analysis of the AIRE gene revealed that it harbors several domains (two zinc fingers, a nuclear targeting signal, four nuclear receptor binding motifs, and a putative DNA binding domain), a finding that suggests a role in transcriptional regulation (93). Bjorses and co-workers demonstrated that AIRE activated transcription of a reporter gene in a eukaryotic transactivation assay (94). Which cells express the AIRE protein remains controversial. mRNA for the AIRE gene has been detected in the thymus, lymph node, and fetal liver (92,95). By immunohistochemical analysis, the AIRE protein was detected in the nuclei of several immunologically relevant tissues, namely, thymus, spleen, lymph node, and peripheral leukocytes (93,95). Given its putative role in regulating transcription and its restricted cell and tissue expression, it is likely that the AIRE protein regulates genes important for the development of immunologic tolerance.

Environment

The incidence of type 1A diabetes varies worldwide, with some populations having remarkably high incidence rates, such as in Scandinavia, which has a rate of approximately 45 in 100,000 (96), whereas others, particularly Asian countries such as Japan, have a low incidence of approximately 1.8 in 100,000 (97). In the United States, the incidence in children is approximately 15 in 100,000. The incidence of type 1A diabetes also varies among members of different racial backgrounds living in the same geographic region, although they are not of the same magnitude as the geographic differences. In the United States, for example, non-Hispanic whites are approximately one and a half times more likely to develop type 1A diabetes than other racial groups.

Geoethnic variation in type 1A diabetes risk probably reflects differences in the frequencies of modifier genes (susceptibility or protective) or environmental factors (causative or protective) or some combination thereof. The precise role that these factors have on the variation in the incidence of type 1A diabetes has been difficult to establish. Several studies suggest that most of the intercountry variation in diabetes risk may relate to genetic differences and not environmental factors. Investigators found that MZTs and first-degree relatives of patients with type 1A diabetes in Japan had a risk of diabetes similar to that of twins and relatives of patients in the United States (98). The island of Sardinia has one of the highest incidences of type 1A diabetes in the world. On migration from Sardinia to mainland Italy (a lower-incidence region), the risk of diabetes is unchanged (99). In contrast, data indicate that Yemenite Jews who came to Israel now have the highest incidence of type 1A diabetes in Israel. In Yemen, childhood diabetes is reportedly rare (100).

The incidence of type 1A diabetes has been increasing in many countries over the past 40 years, with the greatest differences occurring in the youngest children

developing diabetes. Some studies reported an early peak in diabetes incidence around age 5 years (33,34,101,102). The risk of developing diabetes in children increases until puberty (reaching a peak onset at 12 to 13 years). The incidence peak at this time may be related to physiologic changes (increases in sex hormones) associated with insulin resistance or lifestyle changes and the associated change in the pattern of infection (34). Type 1A diabetes can occur at any age, even in the eighth and ninth decades of life. Only a few studies have examined the incidence or prevalence of type 1A diabetes in older groups, mainly because of the difficulty of distinguishing slowly progressive type 1A diabetes from insulin-requiring type 2 diabetes in older persons. Many patients, particularly adults, presenting to their physician with what appears to be non–insulin-requiring diabetes have an early stage of type 1A (immune-mediated) diabetes (103). The diagnosis of diabetes in such adult patients is now possible because of the availability of autoantibody assays with high sensitivity and specificity (48). Asian adult patients are likely to have a lower prevalence of adult-onset type 1A diabetes compared with the reports from Finland (3,48,104).

Several European countries such as Great Britain and Finland have experienced more than a doubling in incidence of type 1A diabetes over the past three decades (105,106). This increase in the incidence of type 1A diabetes is almost certainly the result of environmental factors. What may be changing in the environment is unknown, but one hypothesis is that sanitation and vaccination create a “cleaner” environment that results in reduced exposure to microbial antigens postnatally. Such postnatal infection may protect from autoimmunity by shaping the immunologic repertoire. BB rats and NOD mice raised in a germ-free environment develop more diabetes than those raised in standard animal rooms (107). It is not yet clear whether type 1A diabetes is actually increasing in incidence or whether the age of onset of the disorder is decreasing. An alternative hypothesis to that of a decrease in environmental factors that protect from diabetes is that a diabetogenic factor is increasing. Many investigators believe that viral infections contribute to diabetes development.

A common observation is that the incidence of diabetes increases in the fall and winter. With the long prodrome preceding type 1A diabetes, many investigators now believe that this seasonal variation results from stress associated with infections precipitating metabolic decompensation and diagnosis. Thus, more recent studies have searched for viruses that may initiate the development of diabetes rather than merely precipitate hyperglycemia (34,108). Previous studies linked the risk of type 1A diabetes to congenital rubella infection, but intriguingly, noncongenital infection does not appear to convey this risk (109,110). Approximately one fourth of children with congenital rubella will develop diabetes. As many or more of these children also develop autoimmune thyroiditis, a finding suggesting that the virus has altered the immune system and has thereby increased the risk of developing a series of autoimmune disorders (109,111). No other viral infection is as clearly linked to diabetes.

One manner in which viral infection may influence diabetes risk is thorough molecular mimicry. In this model, a peptide of the virus with homology to an islet antigen can induce the proliferation of T cells that cross-react with the islet antigen, thus leading to antiislet autoimmunity. Coxsackieviruses have been of particular interest because of a molecular homology involving six amino acids (PEVKEK) with the antigen GAD65 (112,113 and 114). A series of studies related anticoxsackie antibodies to type 1A diabetes, either at onset or during the development of diabetes, but both negative and positive results were reported (115,116,117,118,119 and 120). Viral infection of the mother may also contribute to the development of diabetes (116,121). A possible explanation for the lack of definitive association may derive from the finding that viral infections are ubiquitous and are not unique to persons with diabetes.

Microbial superantigens that act by bridging between class II MHC and the Vb portion of specific subsets of TCRs have been implicated in the pathogenesis of autoimmune disease (122). One study reported the isolation of an endogenous retrovirus from patients with type 1A diabetes and proposed that this superantigen may initiate b-cell destruction (123). However, other groups could not confirm the association between the viral gene and type 1A diabetes (124,125).

A potential environmental trigger of type 1A diabetes is ingestion of dietary proteins. This is analogous to celiac disease in which the ingestion of the wheat protein gliadin is essential for antiintestinal autoimmunity, and in particular the presence of antitransglutaminase autoantibodies. Celiac disease is frequently found in patients with type 1A diabetes (approximately 1 in 20). Of patients with diabetes who have the HLA genotype DR3/3, almost one third express antitransglutaminase autoantibodies, and one half of these patients have celiac disease on biopsy (126). For most such persons, the disease is asymptomatic and is detected only with autoantibody screening.

Milk proteins have garnered most of the attention of investigators studying the role of dietary proteins in the development of type 1A diabetes. Both antibodies and T-cell responses to milk proteins have been reported to be increased among children with type 1A diabetes. In one study, investigators suggested that certain forms of milk casein vary in their protein sequence and can be converted to peptides with immunomodulatory properties (127). Investigators also suggested that milk albumin introduced in a neonate's diet may increase the risk of type 1A diabetes. The link between milk albumin and type 1A diabetes has been suggested to result from the homology between a milk albumin peptide and the islet autoantigen, ICA69 (128,129 and 130).

Insulitis and diabetes in BB rats can be triggered by exposure to wheat and cow's milk protein at the time of weaning (131). Borch-Johnsen and co-workers reported that a nationwide decrease in breast-feeding correlated with an increase in incidence of the type 1A diabetes (132), and retrospective studies have implicated early milk ingestion in the development of diabetes (133,134 and 135). The increased risk in most of these studies was small, and prospective studies following healthy children until the expression of autoimmunity (Baby-Diab study of Munich and DAISY study of Denver) have failed to find an association between milk ingestion and antiislet autoimmunity (136).

Given the high penetrance of the disorder for MZTs and relatives of patients with type 1A diabetes with HLA DR3/4, and a similar risk of diabetes and autoantibody expression for DZTs and other siblings (36), if a triggering environmental factor is important, it is likely to be ubiquitous. This would be similar to celiac disease, in which most persons are exposed to wheat gliadin, but only a few of those exposed develop celiac disease. Current studies indicate that each TCR can be triggered by as many as 1 million different peptides. Many peptides able to trigger the same receptor have little or no linear sequence in common (137). Thus, the number of peptides that may be mimotopes of an important islet autoantigen and may potentially contribute to disease could be enormous. As the target molecules and immune pathogenesis are better defined, it should become easier to identify relevant environmental factors.

CLINICAL CONSIDERATIONS

Signs and Symptoms

The classic signs of type 1A diabetes are polyuria, polydipsia, and weight loss. At diagnosis, most patients who present with type 1A diabetes have had overt hyperglycemia for many months, some for more than a year, as evidenced by the presence of an elevated level of hemoglobin A_{1c} (HbA_{1c}). Despite the classic signs and symptoms of diabetes, the diagnosis is often missed by the first health care provider to see the child. Such children are at increased risk of developing ketoacidosis. Even with institution of insulin therapy, some of these children develop cerebral edema, which has a high mortality.

Diagnostic Criteria

DIAGNOSIS OF DIABETES

The American Diabetes Association 1997 revised guidelines for the diagnosis of diabetes include a fasting plasma glucose level of more than 126 mg/dL (7 mmol/L) or a plasma glucose level of more than 200 mg/dL (11.1 mmol/L) taken randomly (with symptoms of diabetes) or 2 hours after an oral glucose load (1.75 g glucose/kg up to a maximum of 75 g). Confirmation of such abnormalities on more than one occasion is recommended. “Impaired” fasting glucose values are from 110 to 125 mg/dL (6.1 to 6.9 mmol/L), and impaired 2-hour values are from 140 to 200 mg/dL (7.8 to 11.1 mmol/L). Transient hyperglycemia, though uncommon, can occur particularly with severe illness. Children with such transient hyperglycemia do not usually have antiislet autoantibodies and usually have a normal HbA_{1c}.

DIAGNOSIS OF TYPE 1A DIABETES

At present, the best indicator of type 1A diabetes in a patient with hyperglycemia is the presence of antiislet autoantibodies (see later). In addition, HLA genotypes can suggest the presence or absence of type 1A diabetes. A child, for instance, without antiislet autoantibodies and with the protective HLA allele DQA1*0102, DQB1*0602 is unlikely to have type 1A diabetes. More often, such children have type 2 diabetes. Approximately one half of Hispanic and African-American children presenting with diabetes have type 2 and not type 1A diabetes (138). Some of these children, after initial treatment with insulin, can be treated with oral hypoglycemic agents.

Laboratory Tests

The laboratory tests used for the prediction and diagnosis of type 1A diabetes relate to hyperglycemia, insulin secretion by islet b cells, autoimmunity and genetic polymorphisms. Standard laboratory tests of blood glucose and HbA_{1c} are readily available. The HbA_{1c} test provides a determination of average blood glucose for approximately 3 months. Because type 1A diabetes develops slowly, it is unusual for a child to present with hyperglycemia with a normal HbA_{1c}. This situation can occur with fulminant type 1B diabetes (5), children presenting with transient hyperglycemia associated with stress (139), and children followed to the development of diabetes such that diabetes is diagnosed soon after the earliest abnormalities of glucose metabolism.

In a nondiabetic person expressing antiislet autoantibodies, the best predictor of type 1A diabetes and approximate time of onset of diabetes is loss of insulin secretion

after a bolus of intravenous glucose. The intravenous glucose tolerance test has been standardized, and insulin levels are usually measured at 1 and 3 minutes after the glucose bolus. Loss of rapid secretion of insulin precedes loss of the ability of the pancreas to secrete insulin after a meal. Once diabetes is diagnosed, patients are treated with insulin. Thus, after diagnosis, C-peptide, rather than insulin, is the measurement of choice to define remaining b-cell function. C-peptide (connecting peptide) of the proinsulin molecule is stored in insulin secretory granules after cleavage from the proinsulin molecule. It is then secreted by pancreatic b cells in amounts equal to that of insulin. Within 3 years of the onset of type 1A diabetes, most children have a severe impairment of insulin secretion such that fasting C-peptide is no longer detectable. The maintenance of C-peptide secretion is also used as a measure of effective immunotherapy in clinical trials.

The initial assay for antiislet autoantibodies (detection of cytoplasmic islet cell antibodies) consisted of indirect immunofluorescent detection of antibodies binding to frozen sections of human pancreas (15,140). Assays for autoantibodies reacting with the surface of islet cells were also studied but lacked disease specificity and are rarely performed today (141). The ICA or islet cytoplasmic autoantibody assay can provide important diagnostic and prognostic information. Unfortunately, the assay is difficult to standardize, and dramatic variation in the sensitivity and specificity among laboratories has been observed. The development of “biochemical” fluid-phase radioassays to detect a series of autoantibodies reacting with islet antigens replaced the immunofluorescence assay. The ICA assay detects a subset of autoantibodies reacting with glutamic acid decarboxylase, the molecule ICA512 (also termed IA-2), and additional poorly characterized islet antigens, but it does not detect antiinsulin autoantibodies. Assays for autoantibodies reacting with insulin, glutamic acid decarboxylase, or ICA512 (IA-2), when performed with fluid-phase assays (not enzyme-linked immunosorbent assays), can be set with high specificity such that less than 1 in 100 nondiabetic persons have positive results. These autoantibody assays are now usually performed in 96-well filtration plates. Labeled antigens are either purchased or obtained through *in vitro* transcription and translation of cDNAs of the respective molecules (28,142,143,144 and 145). Antibody-bound labeled antigen can be detected with b counting directly in the 96-well plate making the assay easier to perform and more cost-effective. With such semiautomated assays, the assay costs can be reduced to less than $10 per sample. One or another of the three autoantibodies is present in approximately 95% of children with recent-onset type 1A diabetes.

Pathology

The hallmark of type 1A diabetes is the presence of insulitis (lymphocytic invasion of islets) and the selective loss of islet b cells. Islets comprise approximately 1% of the pancreas; exocrine pancreatic cells comprise most of the pancreas. Islets contain multiple endocrine cells, including cells secreting insulin (b cells), glucagon (a cells), somatostatin (d cell), and pancreatic polypeptide. Type 1A diabetes leads only to the complete loss of b cells. This selective b-cell loss occurs despite the presence of two islet autoantigens in human a cells, namely, GAD and ICA512. Pancreatic tissues from a large series of patients who died shortly after the development of type 1A diabetes were analyzed by Foulis and Clark (10). These investigators described three types of islets: (a) insulin-deficient islets with no insulitis, which make up the largest fraction (approximately 70%), (b) insulin-containing islets with a chronic inflammatory cell infiltrate, and (c) insulin-containing islets that appear normal. The diversity of islet types likely represents chronic progressive invasion and destruction of b cells of single islets. This type of autoimmune-mediated destruction may be analogous to the “spotty” lesions of vitiligo, whereby patches of skin melanocytes are destroyed over time. Once all the b cells within an islet have been destroyed, the insulitis disappears. The study of biopsy specimens from the pancreas of patients with newly diagnosed type 1A diabetes revealed that the infiltrating mononuclear cells consisted of T cells ($CD4^+$ and $CD8^+$), B lymphocytes, and macrophages. Among them, $CD8^+$ T lymphocytes were the predominant cell type, whereas macrophages were the next most prevalent (146). However, $CD4^+$ T cells made up the predominant cell type in adult type 1A diabetes patients with residual b-cell function (147). These data suggest that the subtype of the islet infiltrating cells may be variable, depending on the phenotype or the stage of diabetes. Interferon-g (IFN-g) mRNA transcripts have been shown to be highly expressed in the pancreas of the patients, whereas IL-4 mRNA was deficient (148). Huang and co-workers emphasized the presence of IFN-a (149).

Treatment and Prognosis

The discovery of insulin in 1921 was one of the most important medical discoveries of our time. Insulin therapy has saved millions of lives, but it remains an inadequate form of therapy, primarily because of the limited therapeutic window. A major risk with insulin therapy of type 1A diabetes is severe hypoglycemia, which can result in coma, seizure, or death. Therapies are, however, improving with genetically engineered (rapid-acting and long-acting) insulins. In addition, devices capable of measuring cutaneous glucose continuously have been approved by the United States Food and Drug Administration and are likely to have a major beneficial impact on improving glycemic control.

Hypoglycemia becomes more of a problem as blood glucose is lowered toward normal. Normal blood glucose, associated with a lower HbA_{1c}, is essential for prevention of renal failure and proliferative retinopathy (150). Approximately 30% to 40% of persons with type 1A diabetes eventually develop renal failure or loss of vision. Both complications can be decreased if patients are monitored for early eye disease (annual ophthalmologic exam) or early nephropathy (annual determination of microalbuminuria).

IMMUNOPATHOLOGY OF TYPE 1A DIABETES

Humoral Response

Studies of antiislet autoantibodies have filled an important gap in our knowledge concerning the development of type 1A diabetes. Children have been followed from birth until the development of type 1A diabetes (28,44,151,152 and 153). From these studies, it is apparent that antiislet autoantibodies in genetically susceptible persons (e.g., HLA DR3/4 heterozygous persons) can develop in the first 6 to 9 months of life. Autoantibodies often develop sequentially over 6 months to 1 year, with antiinsulin autoantibodies appearing first. GAD65 autoantibodies also occur early, and ICA512 (IA-2) autoantibodies often develop after GAD65 and IAAs (151,153,154).

Many biochemically defined autoantigens have been found to be targets of the autoimmune process that precedes type 1A diabetes (155). The association of autoantibodies to GAD65 with the stiff man syndrome, a rare neuromuscular disease characterized by muscle spasms, contributed to the identification of GAD65 as the elusive islet 64-kd autoantigen (156). The antiislet autoantigen ICA512 was identified by screening an islet cDNA library with sera from patients with type 1A diabetes (157,158,159 and 160). Autoantibodies reacting with 40- and 37-kd tryptic fragments of labeled islet proteins were identified by Christie (161). It was later realized that the 37-kd protein is ICA512 and the 40-kd molecule is a fragment of a molecule termed phogrin by Hutton and co-workers, IA-2b by Notkins and co-workers, and IAR by other investigators (162). ICA512 and IA-2b are both molecules associated with secretory granules. Both ICA512 and I-A2b have tyrosine phosphatase-like domains and are homologous in their C-terminal intracytoplasmic domains. Essentially all the autoantibodies in patients with type 1A diabetes are directed to this intracytoplasmic domain. Most of the anti–IA-2b reactivity can be absorbed with synthetic ICA512 antigen, a finding suggesting that most autoantibodies directed against IA-2b cross-react with ICA512. Approximately 10% of children with diabetes express antibodies that react with ICA512 but fail to react with phogrin (163). We have found that only 1% of children with diabetes who have anti–IA-2b autoantibodies are negative for ICA512 autoantibodies.

Although insulin was the first autoantigen biochemically characterized (164), the assay for antiinsulin autoantibodies has been much less convenient than assays for GAD65 and ICA512. The conventional IAA assay uses 600 µL of serum per determination and is performed in centrifuge tubes. International workshop comparisons of the conventional assay indicated that IAA assays that used less than 600 µL were significantly less sensitive in detecting relevant antiinsulin autoantibodies (165). Williams and co-workers described an IAA radioassay that uses protein A rather than polyethylene glycol and requires much less sera (20 µL) than the conventional assay (153,166,167). Modifications of this assay by our laboratory now allow IAAs to be determined with the same throughput as for GAD and ICA512 autoantibody assays (28). In addition, several laboratories have reported that the polyethylene glycol precipitation of the conventional assay produces false-positive results for hemolyzed sera. Such false-positive results are abrogated with the protein A assay.

COMBINATORIAL AUTOANTIBODY DETERMINATION

The detection of autoantibodies, when combined with the measurement of insulin secretion, is important for the accurate prediction of diabetes (168). For three of the islet-cell autoantibodies (anti-GAD65, antiinsulin, and anti–ICA512/IA-2), the frequency of positivity is high (approximately 95% of children with recent-onset type 1A diabetes express one or more of the three autoantibodies). The assays are in a form that allows sensitive and specific testing of thousands of samples (Table 48.3). Expression of multiple autoantibodies confers a high risk for progression to type 1A diabetes (Fig. 48.5).

Antigen	Sensitivity (Specificity)	Comment
Insulin	40–95% (99%)	Prevalence inversely correlated with age of onset
GAD	70% (99%)	Prevalence increases with age
ICA512 (IA-2)	60% (99%)	Islet tyrosine phosphatase homolog
IA2β (Phogrin)	55% (99%)	These autoantibodies predominantly a subset of ICA512/IA-2 autoantibodies

TABLE 48.3. Biochemical Antiislet Autoantibody Assays

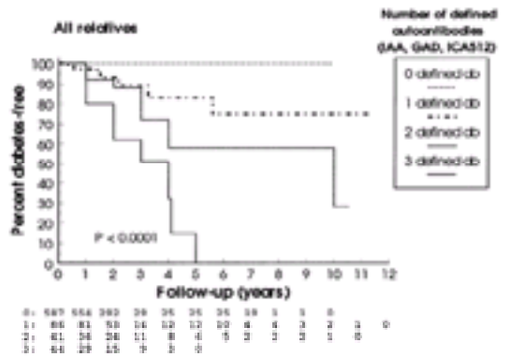

Figure 48.5. Life-table analysis of the risk of type 1 diabetes in first-degree relatives according to the number of autoantibodies to insulin, GAD65, and ICA512/IA-2. (Adapted from Verge CF, Gianani R, Kawasaki E, et al. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 1996;45:926–933, with permission.)

A study examining the risk of type 1A diabetes in first-degree relatives according to the number of autoantibodies found that, in relatives not expressing 1 of the autoantibodies, only 1 relative out of approximately 600 developed diabetes. Fewer than 20% of relatives expressing only a single autoantibody (usually GAD65 or IAAs) developed diabetes. In contrast, expression of 2 or 3 autoantibodies was associated with high risk. Most persons expressing only a single autoantibody may not be destroying b cells. For example, an adult patient with expression of only GAD65 autoantibodies at autopsy was found to have a normal pancreas. Many of the relatives at autopsy with a single autoantibody at initial testing in Fig. 48.5 and who went on to develop diabetes eventually expressed multiple autoantibodies before the onset of diabetes (151). Autoantibody reactivity “spreading” to multiple autoantigens and to multiple epitopes of a given autoantigen can be readily observed in children less than the age of 5 years (169). Expression of multiple autoantibodies appears to be associated with a high diabetes risk for even the general population. Less than 1 in 300 persons in the general population expresses more than a single autoantibody. This is similar to the risk for type 1A diabetes in the United States. In contrast, 80% of children at the time of type 1A diabetes onset express two or more autoantibodies.

INSULIN AUTOIMMUNE SYNDROME

Nearly all patients with diabetes develop antibodies to insulin after subcutaneous insulin therapy. These “induced” IAAs are distinct from those produced spontaneously in that they have a lower affinity for insulin. These induced IAAs usually do not interfere with insulin therapy. In 1970, Hirata and co-workers identified the first patients with antibodies to insulin who had not been treated with insulin. These patients had spontaneous hypoglycemia associated with the production of IAAs with the designation, insulin autoimmune syndrome or Hirata disease (170). Insulin autoimmune syndrome is characterized by a combination of fasting hypoglycemia and polyclonal (rare monoclonal not HLA associated) antibodies to insulin and is associated with HLA-DR4 (DRB1*0406) (171). Since 1970, at least 226 cases in Japanese patients and 26 cases in whites have occurred.

The IAAs in these patients appeared spontaneously or after administration of drugs such as methimazole, penicillamine, or a-mercaptopropionylglycine (172,173). The mechanism of induction of antiinsulin antibodies by these drugs is not well understood, but all these drugs contain a sulfhydryl (S-H) group. Investigators have suggested that the treatment with reducing agents may cleave disulfide bonds of the insulin molecule *in vivo* and may expose insulin-derived peptides to the DRB1*0406 allele (173).

Cellular Response

Although islet autoantibodies predict the development of type 1A diabetes, no evidence indicates that these antibodies themselves are pathogenic (174). Rather, the disease appears to result from cell-mediated immunity. Cell-mediated autoimmunity has not been easy to study in humans because the autoimmune process is well advanced by the time of diagnosis and because diseased human islets are difficult to obtain. Several studies in human patients have reported abnormalities in peripheral T-cell function (175,176,177,178,179,180 and 181). Early studies suggested that differences in the ratios of T-cell subsets, CD4/CD8, could be detected between patients and control subjects. A common abnormality noted across many of these studies is an increase in activated T cells. The data on T-cell reactivity to various candidate autoantigens have been inconsistent (182), and international workshops with “blinded” antigens have highlighted the difficulties. Many explanations have been offered for the reason that this reactivity has been difficult to detect reproducibly. Explanations include a low frequency of circulating autoreactive T cells (183), peripheral immune regulation of autoreactivity (184), assay systems with a low signal-to-noise ratio (elevated background reactivity), or undue reliance on proliferation of T cells as the sole determinant of reactivity.

Effector Mechanisms for b-Cell Destruction

Pathogenic CD4 and CD8 T cells in type 1A diabetes are believed to express a Th1 phenotype. A substantial amount of evidence in the NOD mouse supports the hypothesis that protection from diabetes is associated with nondestructive inflammation and a shift in the cytokine pattern to one exhibiting Th2 cytokines (IL-4, IL-5, IL-10, and transforming growth factor-b) (185,186,187,188,189 and 190).

Diabetogenic CD4+ or CD8+ T-cell clones are capable of mediating b-cell destruction independently in certain experimental situations. However, in human and in rodent models, evidence indicates a complex interaction between antigen-presenting cells (dendritic cells, macrophages, and B cells) and T lymphocytes in the destruction of b cells. For example, depletion of B cells (191) or macrophages (192) in the NOD model blocks development of diabetes. Numerous studies have determined that certain proinflammatory cytokines, including TNF-a, IL-1, IL-6, and IFN-g, play an important role, as well as Fas-FasL interactions, perforin, and granzymes and reactive oxygen intermediates.

Programmed cell death or apoptosis has a role in the normal regulation of the immune response (193,194). Investigators have been examining apoptosis to determine both how b cells die and whether abnormalities in apoptosis may be found in the pathogenic lymphocytes that cause b-cell destruction (195,196). NOD mice express Fas on the surface of b cells during the development of insulitis and diabetes. b cells can die by programmed cell death through the engagement of Fas by Fas-ligand expressed on lymphocytes. In human type 1A diabetes, data are conflicting regarding b-cell–specific Fas expression. One group noted Fas expression on b cells only, a finding suggesting a direct role for Fas in b-cell apoptosis. In contrast, two groups using confocal microscopy found Fas expression on all islet cells including a and b cells. When intracellular mRNA message for apoptotic pathway molecules was assessed, it appeared that critical caspases needed for signaling through the Fas pathway were reduced in diabetic islets, but not in control islets. If this observation is verified, then it would suggest that other pathways of apoptosis, such as mediated by TNF receptor, may be more important for b-cell death.

Loss of Insulin Secretion

First-phase insulin secretion after the administration of intravenous glucose is a parameter that aids the prediction of the time to development of clinically overt diabetes

(197). One to 3 years before the onset of type 1A diabetes, most persons have lost first-phase secretion.

The unique association of the levels of IAAs with the age of development of type 1A diabetes and rate of progression to diabetes is not found for GAD65 autoantibodies and ICA512 autoantibodies. For example, infants less than 5 years of age usually have remarkably elevated levels of IAAs (thousands of nanounits per milliliter), whereas children presenting with diabetes after the age of 15 years have levels in the hundreds, and most are negative for IAAs with current assays. This association of IAAs with a young age of onset may relate to a faster progression to diabetes with high levels of IAAs. We model the approximate time of onset of type 1A diabetes with a dual-parameter model using first-phase insulin secretion on intravenous glucose tolerance testing and levels of IAAs. The model was used to analyze two independent data sets from Boston and Melbourne patients. As illustrated in Fig. 48.6, for both groups, ICA-positive relatives with a predicted time to diabetes of less than 2.5 years (by this formula) had a much more rapid and greater progression to diabetes, in contrast to relatives with a predicted time of greater than 2.5 years (198).

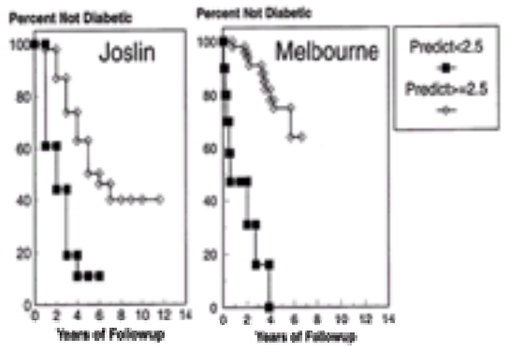

Figure 48.6. Dual-parameter model for prediction of diabetes onset. Levels of insulin autoantibodies and loss of first-phase insulin secretion on intravenous glucose tolerance testing correlate with progression to diabetes. (Adapted from Eisenbarth GS, Gianani R, Yu L, et al. Dual parameter model for prediction of type 1 diabetes mellitus. *Proc Assoc Am Physicians* 1998;110:126–135, with permission.)

Investigators have estimated that at the time of diagnosis of type 1A diabetes, 80% to 90% of the b cells have already been destroyed. Examination of pancreas of patients who died shortly after the onset of type 1A diabetes revealed that insulitis is observed only within the islets that still contain b cells.

In some patients newly diagnosed with type 1A diabetes, initial insulin therapy may be accompanied by a brief metabolic remission, termed the “honeymoon phase.” During this time, low doses of insulin are sufficient to achieve glycemic control. This brief metabolic remission usually lasts for less than 1 year, and it probably reflects decreased insulin resistance after treatment of severe hyperglycemia. The ability to induce this temporary metabolic remission makes the search for an agent that can maintain long-term remission an important goal.

ANIMAL MODELS

Spontaneous Rodent Models

Our understanding of the pathogenesis of type 1A diabetes has been greatly facilitated by the availability of rodent models. Although most animal models of autoimmune disease are experimentally induced (e.g., through chemical toxins, autoantigens, infections, immunotherapy), the NOD mouse and the BB rat both spontaneously develop diabetes. Because the study of diabetes in humans is hampered by the limited access to the target organ, the use of animal models allows investigators to study immunopathologic features at various stages of the disease process. Furthermore, the relative ease of genetically manipulating and breeding mice has allowed better characterization of the role of specific immune mediators and has facilitated the study of disease prevention.

NOD MOUSE

A spontaneous model of diabetes, the NOD (nonobese diabetic) mouse, was discovered in Japan by Makino and colleagues (22). Disease in the NOD mouse is similar to the process in humans in that b-cell destruction is autoimmune mediated. Cellular and humoral immune responses specific for b cells are present, antiislet autoantibodies precede overt diabetes, and a strong association with MHC genes exists. Lymphocytic infiltration of the islets, termed insulitis, can be detected in the NOD mouse at 4 to 5 weeks by light microscopy.

Genetic studies in the NOD mouse reveal that diabetes in this model is characterized by multiple genetic loci determining disease susceptibility. The MHC (termed idd-1) plays a key role but is necessary and not sufficient for disease. The class II region in NOD mice encodes the I-A^{g7} molecule, the homolog of human diabetes-associated HLA-DQ molecules. The I-A^{g7} molecule is unusual in that it has a histidine at position 56 and a serine (instead of an aspartic acid) at position 57 of the b chain, which differs from I-A molecules of most other mouse strains. The a chain of I-A^{g7}, however, is identical to the a chain expressed by nondiabetes-prone mice (e.g., BALB/c). Susceptibility to diabetes in the NOD mouse is strongly influenced by this unique I-A^{b} chain because transgenic NOD mice expressing a “normal” I-A or with amino acid substitutions of position 56 and 57 of the NOD I-A^{g7} are protected from disease. The NOD mouse does not express I-E (the murine homolog of HLA-DR) because of a mutation in the Ea promoter. Transgenic replacement of a normal I-Ea gene blocks diabetes development (198).

NOD mice housed and maintained in standard environmental conditions display a high frequency of diabetes, which predominantly affects female mice (approximately 80% of females and 30% of males at 30 weeks of age). Despite being genetically identical, only certain mice develop diabetes.

With the development of a micro-IAA assay, prospective evaluation of IAAs in NOD mice has become possible. Our studies of IAAs in NOD mice revealed heterogenous expression of IAAs. IAAs appeared between 8 and 24 weeks and often disappeared before the onset of diabetes. Early appearance of IAAs at 8 weeks of age was strongly associated with early development of diabetes, which occurred at 16 to 18 weeks of age (Fig. 48.7). These data suggest that a high risk of early development of diabetes is often determined by 8 weeks of age, and the program for developing diabetes of NOD mice is “fixed” relatively early in life (28).

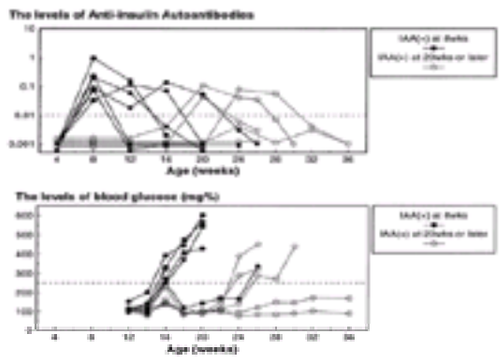

Figure 48.7. Insulin autoantibodies in NOD mice. Insulin antibodies and blood glucose for the NOD mice expressing insulin autoantibodies (IAAs) at 8 weeks of age and at 20 weeks or later followed from 4 weeks of age until diabetes or 36 weeks. Positive index of IAAs > .0. (Adapted from Yu L, Robles DT, Abiru N, et al. Early expression of anti-insulin autoantibodies of man and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc Natl Acad Sci USA* 2000;97:1701–1706, with permission.)

In the NOD mouse, multiple investigators have isolated T-cell clones reactive to islet antigens (199). Islet cell–reactive T-cell clones are able to transfer diabetes (200). The TCR of the clone BDC2.5 (autoantigen unknown), when introduced transgenically into SCID mice, is associated with accelerated development of diabetes (201,202). Of the many CD4+ T-cell clones isolated from NOD islets, we concentrate on *insulin*-reactive T-cell clones that have been well characterized with regard to

the peptide recognized. Wegmann and co-workers isolated T cells from islets of prediabetic NOD mice and created T-cell lines using irradiated whole islets as antigen. These lines were later discovered to react with insulin (203). More than 95% of the insulin-reactive clones recognize a dominant peptide of insulin that spans the amino acid residues 9 to 23 of the insulin B chain (B:9-23 peptide) (204). These clones accelerate disease in young NOD mice, and one was able to transfer disease into NOD/*scid* mice (204). The B:9-23 peptide of insulin can be administered subcutaneously or intranasally to prevent diabetes (205). Molecular characterization of the known B:9-23 reactive CD4+ T-cell clones derived from the NOD islet reveals that the TCR a chain is highly restricted to AV13S3 and Ja 45 or Ja 34. This TCR motif is expressed by approximately 60% of insulin-reactive T-cell clones (206). Despite use of the same Va chain (AV13S3), B:9-23 reactive T-cell clones can recognize two different peptides (B:9-16 and B:13-23) sharing only four amino acids B:13-16 EALY (207).

NOD CD8 T cells reacting with islet antigens have also been cloned (208,209). Schmidt and Verdaguer and their colleagues produced a panel of CD8 T-cell clones that accelerated the development of diabetes when transferred to NOD mice but not to NOD SCID mice (59,210). A “mimotope” of the unknown antigen for these T cells has been identified. The T cells are restricted by K^d. A tetramer with the mimotope peptide is apparently able to detect T cells in early insulitic lesions. Wong and co-workers developed a CD8 T-cell clone with transgenic islets that express the B7-1 costimulatory molecule (208). The clone reacted with a B-chain peptide of insulin, B:15-23. The T cells were restricted by the K^d molecule, and a K^d tetramer with the insulin peptide reacted with a proportion of CD8 T cells in young NOD mice (211). These T-cell clones are able to transfer diabetes to NOD/SCID mice (208).

The relative importance of $CD8^+$ versus CD4 T^+ cells is controversial (212). Data from Christianson et al. indicated a greater contribution of $CD4^+$ than of $CD8^+$ T cells for the adoptive transfer of diabetes into NOD/SCID mice (213). The $CD8^+$ T-cell clone developed by Wong et al. can induce disease in NOD/SCID recipients. Wang and co-workers found that anti-CD8 antibody treatment is only effective in inhibiting diabetes when administered between 2 and 5 weeks of age, a finding suggesting that CD8 T cells are required in the early phase of disease (214). b_2-Microglubulin–null NOD mice, which lack $CD8^+$ T cells, do not develop insulitis or diabetes (215). In adoptive transfer to the NOD/SCID-b2m (null) mice, the disease can be transferred by spleen cells from older, but not young, NOD mice (216). Class I antigen recognition by $CD8^+$ T cells is likely to be required for early activation of islet-reactive CD4 T cells.

There has been considerable emphasis on GAD as a potential primary autoantigen (217,218 and 219). Suppression of GAD expression in b cells of NOD islets by antisense GAD constructs prevented diabetes in two of five strains (219). However, deleting the GAD65 gene failed to protect NOD mice from diabetes, and apparently with further breeding of the antisense transgene constructs onto NOD mice, diabetes occurs in “protected strains” (220). Expression of proinsulin transgenically in NOD mice, driven by a class II promoter, prevents diabetes (221). Similar studies with widespread GAD expression did not protect NOD mice (222).

BB RAT

The BB rat, discovered at the Bio-Breeding laboratory in Canada, is another spontaneous diabetes model. Diabetes in BB rats develops with equal frequency among male and female animals, with 90% of both sexes developing insulitis and then overt diabetes between 50 and 90 days of age. Selective breeding of BB rats resulted in the production of a diabetes-prone (DP) and a diabetes-resistant (DR) BB rat. The BB rat differs from the NOD mouse in that only two to four genetic loci contribute to susceptibility for most crosses. The DP-BB rats are T-cell lymphopenic, a characteristic not shared by NOD mice or humans with type 1A diabetes.

Genetic susceptibility for type 1A diabetes in the BB rat is also determined by MHC genes. In the BB rat, diabetes is independent of class I alleles but requires the MHC class II haplotype $RT\text{-}1^U$ (iddm2). At least one $RT\text{-}1^U$ allele must be present for diabetes development. A second locus *lyp* (lymphopenia), also termed iddm1, is inherited in an autosomal recessive manner and is responsible for the T-cell lymphopenia. Thus, spontaneous disease requires homozygosity at this locus. The lymphopenia in these animals involves the loss of $CD8^+$ T cells, NK T cells, and $RT6^+$ T cells (223). RT6 is a T-cell glycosylphosphatidylinositol-anchored alloantigen with both enzymatic and signal transducing capabilities (224). The RT6 surface antigen marks a subset of T cells capable of immunoregulation.

The DR-BB rats are immunocompetent and nonlymphopenic (225), but they do have autoreactive cells. Adoptive transfer of DR-BB splenocytes (226) or thymocytes (227) to histocompatible athymic rats can induce diabetes. DR-BB rats express normal numbers of $RT6^+$ T lymphocytes. Depletion of $RT6^+$ lymphocytes with anti-RT6 monoclonal antibodies can induce diabetes in DR-BB rats (228). In addition, infection with the Kilham rat virus can induce diabetes in these mice. The Kilham rat virus does not infect the b cells but is believed to alter immune regulation.

LONG-EVANS TOKUSHIMA LEAN (LETL) RAT

The Long-Evans Tokushima Lean (LETL) rat, an inbred spontaneous diabetes rat model, was established from an outbred colony of Long-Evans rats at the Otsuka Pharmaceutical Laboratory in Japan. These animals develop diabetes between 90 and 120 days, with no gender bias. As in the DP-BB rat model, destruction of b cells is autoimmune mediated; however, diabetes in the LETL rat differs from the DP-BB rat model in that T-cell lymphopenia is not present. Genetic analysis of these animals indicates that at least two recessive genes are involved in the pathogenesis of insulitis, one of which is closely linked with the rat MHC $RT1^U$ allele (229). Yokoi and Komeda and co-workers isolated a diabetes substrain of the LETL rat, the Komeda diabetes-prone (KDP) rat. The KDP rat showed a 100% development of insulitis, with 82% of animals progressing to diabetes within 220 days of age. A genome-wide scan for non-MHC type 1 diabetes susceptibility genes in the KDP rat revealed that a non-MHC gene, termed *iddm/kdp1,* on rat chromosome 11 is essential for the development of insulitis and diabetes (230).

LYMPHOPENIA-INDUCED PVG.$RT1^U$ RAT

Fowell and Mason developed a lymphopenia-induced model of diabetes in a nonautoimmune rat that shares the same MHC genotype ($RT1^U$) as the BB rat. They used a protocol of adult thymectomy and sublethal g irradiation (231). Neonatal tolerization with insulin B-chain peptides protected the rat from diabetes. The epitope recognized by the insulin-reactive T-cell line was mapped to residues 1 to 18 of the insulin B chain. As observed in the NOD mouse, diabetes can be adoptively transferred to syngeneic recipients by T-cell lines specific for insulin B:1-18 peptide (232).

Transgenic and Gene Targeting Models

Transgenic and knock-out technologies have been used to study the role of cytokines in the development of diabetes. Local pancreatic expression of proinflammatory cytokines using transgenes under the control of the rat insulin promoter have been well studied. NOD mice expressing IFN-g specifically in the islets (under the control of the rat insulin promoter) developed a massive inflammatory infiltrate with complete destruction of islets b cells (Table 48.4) (233).

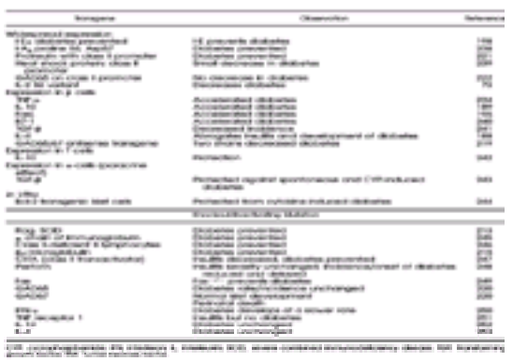

TABLE 48.4. Summary of Transgenic and Knock-Out Studies on the NOD Background

The role of TNF-a provides an example of how the progression to diabetes is determined in part by the temporal regulation of proinflammatory cytokines. TNF-a is not expressed in the neonatal period in TNF-a transgenic NOD mice. Systemic administration of TNF-a in neonatal NOD mice or islet-specific expression of TNF-a (234) accelerates the disease process, whereas neutralization of TNF-a at this stage (235) or expression later, at 7 weeks (236), protects against diabetes.

Studies of the TCR transgenic mouse (BDC2.5) developed by Katz and colleagues shed light on the progression of diabetes in the NOD mouse (201). The BDC2.5 transgenic T cells are CD4+, bear a TCR that is reactive to an unknown antigen, and are diabetogenic. Insulitis develops in the BDC2.5 transgenic NOD mouse at 3 weeks and rapidly progresses to a massive infiltrate. Despite the development of a massive lymphocytic infiltrate, diabetes onset occurs at a relatively normal period (10 to 25 weeks). Based on these observations, Andre and Mathis and colleagues proposed a hypothesis that suggests that the development of diabetes occurs through two critical checkpoints (237). Checkpoint 1, which marks the appearance of insulitis, occurs at 3 weeks of age. The time leading up to checkpoint 1 is believed

to be important for the recruitment and activation of self-reactive T cells. Checkpoint 2 involves the conversion from insulitis to overt diabetes. At this time, the regulatory factors responsible for keeping the autoreactive T cells in check (e.g., inhibitory cytokines, regulatory T cells, inhibition of costimulatory signals) are no longer in place, and the result is destruction of b cells.

Several studies provide evidence for the importance of B lymphocytes in the pathogenesis of type 1A diabetes in NOD mice. NOD mice genetically deficient in B lymphocytes are resistant to diabetes (245,246,247,248,249,250,251,252,253 and 254). Noorchashm and co-workers developed NOD mice with a MHC class II ($I\text{-}A^{g7}$) deficiency confined to the B-cell compartment. This NOD mouse with $I\text{-}A^{g7}$–deficient B cells were resistant to development of diabetes, a finding suggesting that B cells are critical antigen-presenting cells (246).

The idea that a Th1-dominant response is destructive, whereas a Th2-dominant response is protective to islet b cells, provides the basis for efforts to skew the T-cell response toward the Th2 phenotype. In support of this hypothesis, administration of IL-4 (255) or IL-10 (256) is associated with protection from diabetes in NOD mice, whereas systemic administration of Th1-type cytokines, IL-12 (257) and TNF-a (235), accelerates disease progression. However, studies of transgenic and knock-out NOD models involving these cytokines provided conflicting results and suggest that the Th1-Th2 paradigm for the development of type 1A diabetes is oversimplified. For example, NOD mice expressing an IL-4 transgene specifically in islet b cells are protected from diabetes, whereas IL-4 knock-out NOD mice do not develop diabetes at an accelerated rate. Treatment of NOD mice with anti–IFN-g prevents diabetes development in NOD mice (258), but NOD mice made genetically deficient in IFN-g continue to develop diabetes, albeit at a reduced rate (250). Furthermore, whereas NOD mice administered IL-10 are protected from diabetes (256), mice expressing an IL-10 transgene specifically in the islet b cells develop accelerated diabetes (189). Although the reasons for these differences are not yet clear, the foregoing observations suggest that the ability to dissect the role of individual cytokines may be difficult. There is significant redundancy in cytokine action, individual cytokines can affect the expression of other cytokines, and significant temporal differences may exist in the cytokine influence on the disease process.

FUTURE DIRECTIONS

Prediction

With current autoantibody assays, genetic typing and metabolic testing, it is relatively easy to identify persons with a risk of type 1A diabetes exceeding 90% in a given period. For example, relatives of a patient with type 1A diabetes expressing more than two of GAD65, ICA512, or antiinsulin autoantibodies and with first-phase insulin release below the tenth percentile and lacking DQB1*0602 have a high risk (144). Identifying a high-risk group of persons (e.g., a risk of 90% within 5 years) does not imply that the remaining 10% will not develop diabetes. It is much more likely that a significant proportion of the remaining persons will develop diabetes within 10 years. Similarly, a moderate-risk group (e.g., risk of approximately 50% within 5 years: more than autoantibodies, preserved first-phase insulin secretion, lacking DQB1*0602) does not mean that the remaining 50% will not develop diabetes. It is likely that the 50% of patients in this group who do not develop diabetes within 5 years and whose first-phase insulin secretion declines to less than the tenth percentile have a 90% risk of diabetes within the next 5 years.

Prevention

With accurate prediction, studies aimed at preventing or at least delaying the onset of diabetes are now being conducted worldwide. A few investigators are not participating in current trials (259).

IMMUNOSUPPRESSIVE THERAPY

The first major trial of general immunosuppressive therapy for newly diagnosed subjects evaluated cyclosporine (260,261). Cyclosporine suppressed antiinsulin autoantibodies but not cytoplasmic ICA and preserved C-peptide secretion. After discontinuation of cyclosporine, the preservation of C-peptide secretion was rapidly lost. Hyperglycemia recurred in patients who could discontinue insulin therapy even though cyclosporine therapy continued. The recurrence of hyperglycemia appeared to be metabolically mediated in a setting in which most b cells had been destroyed. The failure of immunosuppression to “cure” diabetes and to preserve C-peptide secretion after discontinuing the drug led to relatively little interest in generalized immunosuppression. The most efficacious drug, cyclosporine, required large doses, and it can be associated with both renal toxicity (apparently no permanent toxicity from follow-up of patients in these trials) and a significant risk of malignant disease with long-term therapy.

ADJUVANT THERAPY

Studies in the NOD mouse and BB rat models suggested that injection of either complete Freund's adjuvant or bacille Calmette-Guérin (BCG) prevents diabetes (262). The success of such therapy in the rodent models, however, has not been matched in human subjects. Several randomized trials of BCG treatment of newly diagnosed patients indicate that BCG is not effective in preserving C-peptide secretion or inducing remission of type 1A diabetes (263,264).

NICOTINAMIDE

Treatment with nicotinamide, a free radical scavenger, reduces the development of diabetes in NOD mice but not in BB rats. Its mechanism of action may relate to reduction of free radical-induced islet destruction or blockade of lymphokine-mediated islet destruction. A pilot trial of nicotinamide treatment in human patients was associated with a reported 50% delay in the development of diabetes (262). In the first population-based trial involving nicotinamide intervention, a large cohort of New Zealand schoolchildren were screened for expression of cytoplasmic islet cell antibodies, whereas a matched group of schoolchildren were not screened. ICA-positive children were then treated with nicotinamide and were compared with the nonscreened and nontreated children. Results from this trial suggested a 50% delay in progression to diabetes after an average 7-year follow-up. These nonrandomized trials were followed by two randomized trials of diabetes prevention among ICA-positive relatives. In the smaller “DENIS” trial in Germany, nicotinamide did not have any protective effect in a high-risk group of relatives, and the trial was terminated (approximately 25% of the treated and placebo group developed diabetes) (265). The larger European Nicotinamide Trial (ENDIT) is under way, with outcome data expected in the next few years.

ANTIGEN-SPECIFIC THERAPIES

Insulin is currently being used for antigen-specific therapy in human patients (266). Trial design and implementation followed the reports that insulin, given subcutaneously, intranasally, or orally could prevent diabetes of NOD mice. The mechanisms by which insulin-based immunotherapy protects are not fully defined. Hypotheses include downregulation of islet antigens by the metabolic activity of intact insulin, alteration of lymphocyte function by insulin binding to insulin receptors on lymphocytes, and induction of “regulatory” T cells capable of secreting “antiinflammatory” cytokines (IL-4, IL-10, transforming growth factor-b). Metabolically inactive insulin analogs or insulin B chain can also prevent diabetes (267,268). This indicates that the metabolic activity is not essential for prevention. Zhang and co-workers reported that oral administration of insulin delays the onset and reduces the incidence of diabetes in NOD mice (269). Bergerot et al. studied the mechanisms of protection by oral insulin treatment and reported that insulin-reactive $CD4^+$ T cells induced by oral treatment protect from diabetes by blocking Th1 cytokine secretion and migration of diabetogenic effector T cells to the pancreas (270). Small pilot trials of insulin therapy for diabetes prevention have been reported (271). In a trial from the Joslin and Barbara Davis Center, all eight nontreated persons developed diabetes within 3 years of follow-up. In contrast, three of nine relatives treated with intravenous and subcutaneous insulin remain nondiabetic, and six of nine remained nondiabetic for longer than 4 years, with one nondiabetic relative now followed for 10 years. In the group treated with subcutaneous insulin alone, six of eight persons remain nondiabetic, with the longest follow-up now at 6.6 years and six of eight followed for longer than 4 years.

The National Institutes of Health initiated a randomized trial of parenteral insulin therapy and a placebo controlled trial of oral insulin (the Diabetes Prevention Trial-1 or DPT-1) in 1994. The goal of DPT-1 is to determine whether insulin therapy can prevent or delay the development of disease in relatives at risk for type 1A diabetes. Entry criteria for DPT-1 include the expression of cytoplasmic ICA in relatives of patients, the absence of DQB1*0602, the loss of first-phase insulin secretion, and the expression of IAAs. More than 90,000 relatives throughout the United States have been screened for participation in the trial; 339 have been entered into the parenteral portion of the trial, and more than 223 (target 490) entered into the oral portion of the trial. First-degree relatives can be screened for the DPT-1 trial at centers throughout the United States and Canada (call 1-800-HALT-DM1).

Many adults have slowly progressive type 1A diabetes (3). A study from Japan suggests that insulin therapy may be of help in preserving C-peptide secretion in autoantibody-positive patients presenting with non–insulin-dependent diabetes (272).

It is likely that insulin peptides without the risk of hypoglycemia will be preferable to insulin for diabetes prevention. Muir and co-workers studied insulin B chain, and Daniel and Wegmann found that a single subcutaneous injection of the insulin B:9-23 peptide prevented 90% of diabetes in NOD mice (205). The company Neurocrine has developed an altered peptide ligand of B:9-23 peptide with alanines at position 16 and 19. Administration of this peptide prevents diabetes in NOD mice, and phase I clinical trials are under way. Additional phase I trials for prevention of diabetes with peptides or full-length protein are under way or are planned, such as heat shock

protein (Peptor) and glutamic acid decarboxylase (Diamyd).

CONCLUSION

Type 1A diabetes is highly predictable, has multiple animal models in which disease is preventable, and has reached the stage at which multiple trials of prevention in human patients are under way. Despite this progress, many fundamental questions remain unanswered. It is hoped that as preventive therapies are developed, an interplay between advances in fundamental knowledge and clinical trials will lead to effective therapies.

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49 Autoimmune Thyroid Disease, Addison Disease, and Autoimmune Polyglandular Syndromes

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Autoimmune endocrine diseases represent the prototypes of organ-specific autoimmune disease. This chapter reviews these endocrinopathies, which comprise autoimmune thyroid disease (ATD), Addison disease, premature ovarian failure, and autoimmune pituitary disease; often, these conditions are associated in autoimmune polyglandular syndromes. The predisposing factors, clinical presentation, and treatment are discussed briefly, whereas special attention is given to the immunopathology of these disorders. The key features of animal models of ATD and Addison disease are summarized.

AUTOIMMUNE HYPOTHYROIDISM

In 1912, Hashimoto identified a condition in which goiter developed in four women as a result of lymphoid infiltration (1). More than 40 years later, thyroid antibodies were identified in patients with a similar clinical presentation (2), and this goitrous form of chronic autoimmune thyroiditis is now known as *Hashimoto thyroiditis*. Some patients are euthyroid at presentation because their concerns resulted from the goiter. An atrophic form of ATD also exists and is referred to as *atrophic thyroiditis* or *primary myxedema*. These patients are identified because of hypothyroid features.

Epidemiology

Autoimmune hypothyroidism is a common disease that affects 1% of women but no more than 0.1% of men, generally between 40 and 60 years of age (3). As with other autoimmune diseases, susceptibility to autoimmune hypothyroidism is thought to be influenced by an interaction of genes and environment in addition to endogenous factors such as age and pregnancy.

GENETICS

Human leukocyte antigen (HLA) alleles have been studied extensively in autoimmune hypothyroidism (4), and an association of HLA-DR3, -DR4 and -DR5 with autoimmune hypothyroidism has been recognized in most studies of white subjects. Negative associations (HLA-DQB1), offering protection from the disease, also have been reported (4); however, variability between different ethnic groups has been noted, and in all cases, these associations have been rather weak with inconsistent linkage results, suggesting that HLA genes play only a minor role in the susceptibility to autoimmune hypothyroidism.

Non-HLA loci also have been studied, including polymorphisms of immunoglobulin and T-cell receptor (TCR) genes. Studies of immunoglobulin allotypes in patients with autoimmune hypothyroidism have produced conflicting results (5,6), and a recent comprehensive family study excluded any linkage between immunoglobulin (IgG) heavy chains, TCR polymorphism, and autoimmune hypothyroidism (7,8), and therefore these genes are unlikely to play a major role in the pathogenesis of the disease.

Currently, attention is focused on genes controlling other components of the immune system. Cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4) is found on T-cell surface and binds the B7 family of receptors, thereby regulating T-cell activation. Although an association between polymorphisms of the CTLA-4 gene and autoimmune hypothyroidism in white patients has been documented (9), linkage was excluded recently (8), again indicating that CTLA-4 is not a major determinant of genetic susceptibility to autoimmune hypothyroidism.

Recent linkage studies suggested new susceptibility loci, in particular one locus on chromosome 13 (HT1; 96cM) was linked to Hashimoto thyroiditis with a maximum logarithm of odds (LOD) score (MLS) of 2.1. Another locus (HT-2, 97cM) showed linkage to Hashimoto thyroiditis in a subgroup of families with an MLS of 3.8 (10). Interestingly, only one locus (AITD-1) was found to confer susceptibility to both Graves disease and Hashimoto thyroiditis (MLS of 2.2), indicating that these two diseases are genetically heterogeneous.

ENVIRONMENT

Age probably plays an important role in susceptibility through increasing exposure to environmental agents, in addition to the changes in immunoregulation associated with aging (11). Furthermore, the possibility of cryptic antigen release, secondary to tissue damage (for example, from infection or ischemia), increases with aging and may be a factor in the development of autoimmune hypothyroidism.

Sex hormones are highly likely to be involved in the etiology of ATD in general, and hormone changes also may be responsible for the fact that pregnancy ameliorates the disease, with exacerbation of thyroiditis (or even *de novo* appearance of disease) occurring in the postpartum period (12). Moreover, estrogens exacerbate experimental autoimmune thyroiditis, whereas testosterone ameliorates it (13). In addition to sex hormones, prolactin may play a role because autoimmune thyroiditis is more common in women with hyperprolactinemia (14).

The involvement of infectious agents in the etiology of autoimmune diseases has always been an attractive concept, but their role in autoimmune hypothyroidism remains debatable (15). Epidemiologic data point to iodide as an important factor in enhancing thyroid autoimmunity (16). For example, a study demonstrated a correlation between high iodine intake and the development of autoimmune hypothyroidism (17). Furthermore, a high iodine diet exacerbates thyroiditis in animal models of autoimmune hypothyroidism, whereas a low iodine diet results in amelioration of the disease (18). The mechanism of these effects is unclear, but high concentrations of iodide are directly toxic to thyroid follicular cells (19), possibly leading to release of autoantigens. This toxicity may be mediated by the formation of highly reactive compounds with oxygen metabolites (20). Another possible explanation is the increased iodination of one of the major thyroid autoantigens,

thyroglobulin (TG), which may enhance its immunogenicity (21,22). In addition, iodide forms highly toxic species with oxygen metabolites (22), which could precipitate autoimmunity in a genetically predisposed person.

Exposure to sublethal radiation predisposes to thyroid autoimmunity (23), and lithium exacerbates autoimmune thyroiditis (24). Both probably mediate these effects by altering the function of regulatory T cells. Treatment with amiodarone can result in thyroid dysfunction, particularly in the presence of preexisting thyroid abnormalities. Thyroid dysfunction is due mainly to metabolic abnormalities arising from a disturbance of iodide organification or secondary to the high iodine load; however, direct toxic effects of the drug on thyroid follicular cells have been shown repeatedly but with only inconsistent evidence of autoimmune involvement (25).

Clinical Presentation

Symptoms and Signs

Table 49.1 summarizes the symptoms and signs of autoimmune hypothyroidism. The changes are usually subtle and insidious in onset and may be rather nonspecific, such as complaints of tiredness, lassitude, and weight gain. Although often the most severe symptoms occur in patients with the most severe hypothyroidism, the progression of thyroid failure over several years allows patients to adapt to their altered hormone status, leading to a paucity of complaints in some subjects who have obvious hypothyroidism. Patients are forgetful and may have psychological problems, ranging from mild depression to frank psychosis (*myxedema madness*). In severe cases, patients may present with myxedema coma, a life-threatening complication of hypothyroidism that necessitates urgent treatment.

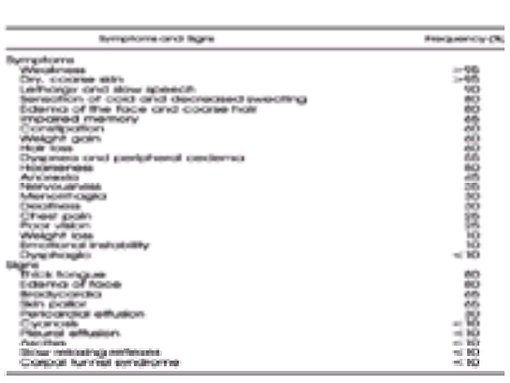

TABLE 49.1. Main Symptoms and Signs in Autoimmune Hypothyrodism

PHYSICAL FINDINGS

In obvious cases, the facial appearance (dull expression, facial puffiness, and periorbital swelling) together with a hoarse voice and slow speech will suggest the diagnosis. Yellowish skin resulting from hypercarotenemia, particularly notable on palms and soles, is not unusual, and slow relaxing reflexes may be present. Fluid accumulation in the middle ear may cause deafness, and there may be fluid accumulation in serous cavities.

LABORATORY TESTS

The diagnosis is usually established easily by finding a low thyroxine (T_4) and elevated thyroid stimulating hormone (TSH) levels. An elevated TSH with normal T_4 suggests subclinical hypothyroidism (Table 49.2), in which there is early evidence of thyroid damage. About 2% of such patients progress to overt thyroid failure annually, but the rate is 5% if the elevated TSH is accompanied by detectable thyroid antibodies in the serum. The autoimmune nature of hypothyroidism is confirmed by the presence of thyroid antibodies against TG or thyroid peroxidase (TPO) (Table 49.3). TSH-receptor (TSH-R) blocking antibodies can be detected in up to 20% of patients, but assay for these is not routinely performed (see later). Antibodies against the Na^+/I^- symporter (NIS) have been described in a minority of patients with autoimmune hypothyroidism (see later) but have no diagnostic value.

	Serum TSH	Serum free T4	Symptoms
Subclinical hypothyroidism	Elevated	Normal	Usually absent
Grade 1	Upper limit of reference range—10 mU/L	Normal	
Grade 2	10.1–20 mU/L	Normal	
Grade 3	>20 mU/L	Normal	
Clinical or overt hypothyroidism	Elevated	Low	Usually present

T4, thyroxine; TSH, thyroid-stimulating hormone.

TABLE 49.2. Definition of Hypothyroidism

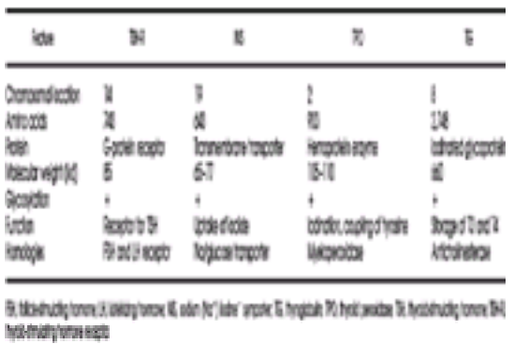

TABLE 49.3. Key Features of the Thyroid Autoantigens

A number of nonspecific laboratory abnormalities can be found in patients with autoimmune hypothyroidism, including raised cholesterol, creatinine phosphokinase, and lactate dehydrogenase levels. A normochromic, normocytic anemia is often present and a macrocytic anemia also can be found, which in some but not all cases is due to the association of pernicious anemia with autoimmune hypothyroidism.

TREATMENT

This is generally straightforward with replacement T_4. Synthetic preparations of thyroxine (L-thyroxine) provide optimal treatment. Triiodothyronine (T_3, liothyronine) has too short a half-life to provide replacement, even though it is the active metabolite of T_4. The place of combined T_3 and T_4 replacement is being reevaluated but is not generally necessary. The goal of T_4 replacement is to return TSH levels to normal. Myxedema coma is a medical emergency treated with intravenous T_4 or T_3 and

intensive supportive therapy, including ventilation.

PATHOLOGY

Hashimoto thyroiditis is characterized by a lymphocytic infiltrate with many plasma cells and the formation of germinal centers (Fig. 49.1); $CD4^+$ T cells predominate over $CD8^+$ T cells (26) The thyroid follicles are small and contain some macrophages, plasma cells, and occasional multinucleated giant cells. The epithelial cells are enlarged with a distinctive eosinophilic cytoplasm resulting from an increased number of mitochondria (Fig. 49.2). A variable amount of fibrosis can be identified. The primary myxedema variant shows extensive replacement by fibrous tissue and atrophy of the gland. Scattered islands of lymphoid cells and enlarged epithelial cells can be identified between areas of fibrosis. It is unclear whether this simply represents an end stage of Hashimoto thyroiditis, a variant of the fibrosis seen in Hashimoto thyroiditis, or a distinct pathologic entity.

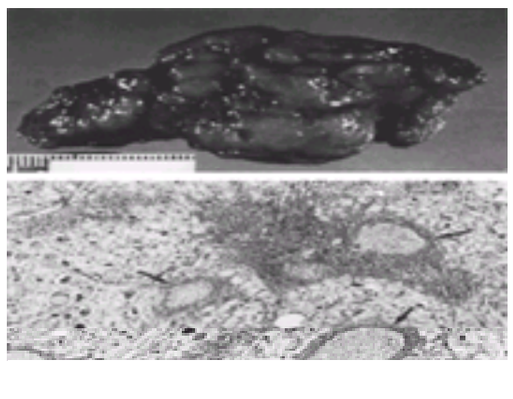

Figure 49.1. Pathology of Hashimoto thyroiditis. **Top:** Gross appearance of thyroid gland. The lobe of the gland is enlarged and has lobulated, freshly appearance. **Bottom:** Low-magnification photomicrograph showing prominent lymphoid infiltrate with multiple reactive germinal centers *(arrows)*. Many of the follicles are atrophic, and there is scanty colloid. (From Mendelsohn GF. Pathology of thyroid disease. In: Mendelsohn GF, ed. *Diagnosis and pathology of endocrine diseases.* Philadelphia: JB Lippincott, 1988:56, with permission.)

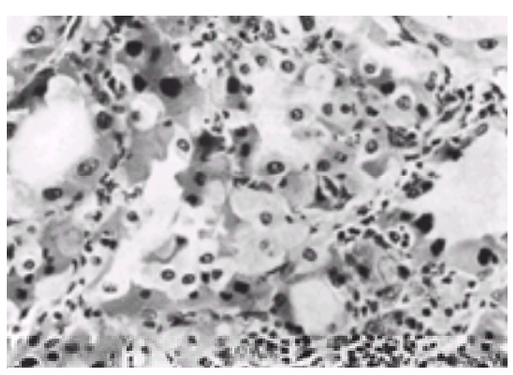

Figure 49.2. Typical Hürthle or Askanazy cells in Hashimoto thyroiditis. These are mitochondrion-rich, altered follicular cells that give an oxyphil staining pattern and indicate thyroid damage. These cells show a moderate degree of pleomorphism and cytologic atypia. A lymphoplasmacytic infiltrate can be seen in the lower right corner. (From Mendelsohn GF. Pathology of thyroid disease. In: Mendelsohn GF, ed. *Diagnosis and pathology of endocrine diseases.* Philadelphia: JB Lippincott, 1988:58, with permission.)

Immunopathology

The three major autoantigens in ATD are TSH-R, TPO, and TG (Table 49.3). Accumulating evidence indicates that the NIS is an autoantigen. TSH-R is a member of the G-protein-coupled receptor family (27). It is 764 amino acids long and comprises three extracellular loops that contain the amino terminus, seven transmembrane segments, and three intracellular loops, ending with a carboxyl terminus. The extracellular domain contains six potential glycosylation sites that are important for the correct function of the receptor. Stimulation of the receptor by TSH causes generation of cyclic adenosine monophosphate (cAMP), stimulating both growth and function of thyroid follicular cells. High concentrations of TSH also can stimulate the phosphatidyl-inositol (PI) cascade, but this pathway seems to have only a minor role in signal transduction in the thyroid (28).

Because iodine is an essential component of T_3 and T_4, its accumulation by the thyroid gland is of utmost importance. Although the thyroid proteins involved in hormonogenesis are now well characterized, relatively little is known about the NIS. The NIS gene in humans encodes a protein of 643 amino acids arranged in a structure with 12 probable transmembrane domains (29,30).

Previously known as the microsomal antigen, TPO is the primary enzyme involved in iodination of tyrosine residues and, in their coupling, to form thyroid hormones (31). It belongs to the family of mammalian peroxidases and has a high sequence homology with myeloperoxidase. In man, TPO is a membrane-associated protein of 933 amino acids with five potential glycosylation sites. An alternatively spliced variant of TPO mRNA giving a 57-residue smaller protein (876 amino acids) has been described and termed TPO-2. TPO-2 usually coexists in the thyroid with the full-length TPO of 933 amino acids (TPO-1). TPO-2 lacks enzymatic activity and is believed to have a different conformation to TPO-1 and hence different antibody reactivity (32). The importance of this observation in relation to ATD is unclear at present. TPO-2 may be expressed earlier than TPO-1 during the embryonic stage of thyroid development, and therefore immunologic tolerance could occur to TPO-2, but not TPO-1, in some persons (33).

Thyroglobulin serves as the precursor and storage form of thyroid hormones and is the major intrathyroidal store of organified iodide. It consists of two monomers of 2,750 amino acids extensively disulfide-linked to form a globular homodimer. TG is secreted primarily into the thyroid follicles, although small amounts gain access to the circulation. In response to demand, TG is endocytosed by the thyroid follicular cells and hydrolyzed, liberating T_3 and T_4 into the circulation. Although some studies suggested an abnormal TG structure in patients with autoimmune hypothyroidism, others failed to confirm these findings (34,35), and in animal models, altered TG structure is not responsible for the induction of autoimmune thyroiditis (36), making a role for TG mutations in the pathogenesis of autoimmune hypothyroidism unlikely. The degree of iodination of TG correlates with its immunogenicity in experimental models of ATD (37), and antibody epitope recognition is highly dependent on the iodination state of the molecule (20,21).

HUMORAL RESPONSES

Patients with autoimmune hypothyroidism almost always have serum antibodies (Ab) to TG and TPO (Fig. 49.3) and less commonly to NIS and TSH-R. Low titers of TG Ab can be found in normal individuals (especially in elderly patients or following viral infections) (3,11), and these antibodies are usually of the immunoglobulin M (IgM) or IgG class. In contrast, patients with autoimmune hypothyroidism have high titers of TG Ab with high affinity, and they are mainly of the IgG class (13). TG Abs are predominantly IgG_1 and IgG_4, although all four subclasses are typically present, with IgG_2 showing the highest functional activity (38). TG Abs are relatively restricted to two major and one minor epitope on each subunit, but many minor epitopes are recognized by TG Abs as titers rise (39). In some patients, there is restricted heavy-chain variable (V) region gene use, with only a moderate restriction of light chains (40).

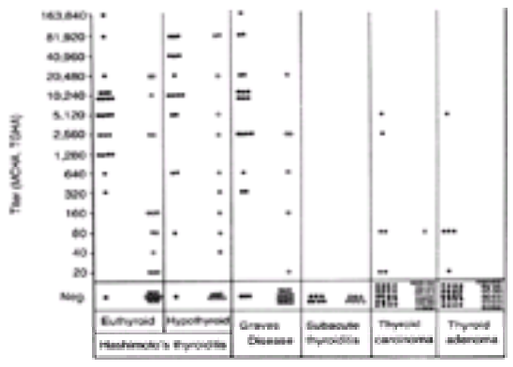

Figure 49.3. Titers of thyroid microsomal hemagglutination antibodies (MCHA, *closed circles*) and thyroglobulin hemagglutination antibodies (TGHA, *open circles*) in patients with various thyroid diseases. (Modified from Amino N. Measurement of circulating thyroid microsomal antibodies by the tanned RBC hemagglutination technique: its usefulness in the diagnosis of autoimmune thyroid disease. *Clin Endocrinol* 1976;5:115, with permission.)

The detection of TG Ab in apparently normal individuals, together with the inability of TG Ab to fix complement (probably because of wide separation of epitopes on the TG molecule preventing IgG cross-linking) casts some doubt on their importance in ATD. TGAb can mediate antibody-dependent cell-mediated cytotoxicity (ADCC) *in vitro* (41), however, and a catalytic role for TG Ab also has been described (42), which could be important in liberating cryptic epitopes for T-cell recognition.

In about 5% to 10% of clinically normal persons, TPO Abs also are detected; the exact figure depends on assay sensitivity. As with TG Ab, IgG_1 and IgG_4 subclasses predominate in TPO Ab (38); although a clear restriction in light chains is not evident, a predominance of light-chain use has been observed in most patients (43). TPO Abs from patients with Graves disease and Hashimoto thyroiditis exhibit similarity in sequences and reactivities to native TPO, indicating a similar antibody response in both types of ATD (44). Conformational TPO Abs in ATD sera are restricted mainly to two closely spaced overlapping epitopes on TPO (45,46). Antibodies in ATD sera occasionally also react with linear TPO epitopes (47), but their significance in ATD pathogenesis is difficult to ascertain. The assumption is that these antibodies arise at a late stage in disease. TPO Abs are able to mediate ADCC, and unlike TG Ab they can also fix complement. The ability of TPO Ab to cause enzymatic inhibition *in vitro* indicates a potential direct role for these antibodies, but it seems most unlikely that TPO inhibition by antibodies occurs at any significant extent *in vivo* (48).

Bispecific Abs interacting with both TG and TPO have been termed TGPO Abs. These Abs are present in sera with high TGAb and TPOAb titers, and they are more frequently detected in patients with Hashimoto thyroiditis than in patients with other ATD (49). Their role in the pathogenesis remains to be resolved, but their existence may help to explain why TG and TPO antibodies frequently coexist.

Several types of Abs to TSH-R have been identified. Thyroid-stimulating antibodies (TS Abs) are dominant in patients with Graves disease, whereas thyroid-blocking antibodies (TB Abs), which prevent TSH-mediated signaling, are found mainly in patients with Hashimoto thyroiditis or primary myxedema. A minority of patients with autoimmune hypothyroidism and patients with Graves disease have reactivity to TS Abs and TB Abs, respectively. Whereas assays for TS Ab and TB Ab measure functional activity, usually in terms of cAMP production by thyroid cells *in vitro*, assays for TSH-binding inhibiting immunoglobulins (TBIIs) measure binding to the receptor (via displacement of radiolabeled TSH) and hence detect the broad range of TSH-R antibodies. TBIIs are present in both Graves disease and autoimmune hypothyroidism, but levels of TBII do not correlate with TS Ab or TB Ab activities; the latter two activities can be determined only by bioassay (27). It is worth noting that Graves disease can develop in patients who have hypothyroidism (and vice versa), and this appears to be related to the balance between TB Abs and TS Abs. The existence of TB Ab was first demonstrated by the development of transient neonatal hypothyroidism secondary to transplacental transfer of these antibodies (50), but subsequent studies showed the association of TB Abs with autoimmune hypothyroidism in Japanese, Korean, and white patients (36). The existence of autoantibodies that increase or inhibit thyroid follicular cell growth independently of the TSH-R (51), potentially resulting in goiter formation or thyroid atrophy respectively, remains debatable.

Numerous studies recently demonstrated the presence of antibodies in Hashimoto thyroiditis patient sera that bind to the rat NIS peptides and recombinant protein (52,53), and antibodies that bind the human symporter have been detected in 24% of autoimmune hypothyroidism sera (54). These antibodies inhibit the function of the rat and human NIS (54,55) and this may have important clinical implications. NIS-inhibitory antibodies could contribute to hypothyroidism in the initial phases of the disease before widespread tissue destruction takes place.

CELLULAR IMMUNITY

T cells play a critical role in the development of autoimmune hypothyroidism. These cells migrate from the periphery to the thyroid gland, where they interact with thyroid follicular cells and extracellular matrix. Many studies found a reduction in the proportion of circulating $CD8^+$ T cells in autoimmune hypothyroidism, and the concept of an antigen-specific T-suppressor cell defect as a factor in the development of autoimmune hypothyroidism has attracted considerable debate (56). Defects in the putative T-suppressor cell response to TPO and TG (but not to irrelevant antigens) have been demonstrated in autoimmune hypothyroidism, but these assays have not been reproducible or physiologic (57), and therefore the role of suppressor cells in the pathogenesis of autoimmune hypothyroidism remains unclear.

Restricted use of TCR by intrathyroidal T cells has been the focus of several studies. If T-cell autoreactivity is clonally limited, this would permit the application of novel therapeutic approaches based on inactivation of selected subpopulations of T cells. Marked restriction in TCR Va gene use by intrathyroidal T cells in ATD (58) has not been confirmed even after analyzing interleukin-2 (IL-2) receptor positive T cells (59,60). Using cell-fractionation studies, Va restriction in the intrathyroidal $CD8^+$ T cells from three Hashimoto thyroiditis patients has been observed, suggesting a role for clonally restricted T-cell-mediated cytotoxicity in Hashimoto thyroiditis (61).

T cells may destroy thyroid follicular cells in Hashimoto thyroiditis and primary myxedema by direct cytotoxicity or indirectly via cytokine secretion. The presence of perforin in Hashimoto thyroiditis-derived $CD8^+$ T cells indicates that these cells are activated *in situ* (62). Furthermore, $CD8^+$ cytotoxic T-cell clones, capable of killing autologous thyroid follicular cells in an HLA class I-restricted fashion, have been raised from Hashimoto thyroiditis patients, but T-cell stimulation in culture by IL-2 favors the expansion of nonspecific natural killer (NK) cells, rendering the study of thyroid-specific $CD8^+$ cells difficult and often inconclusive (63,64).

The involvement of apoptosis in causing tissue destruction in autoimmune hypothyroidism has been suggested by the high frequency of apoptotic thyroid follicular cells in thyroid tissue from patients with Hashimoto thyroiditis (65). An increased expression of Fas by thyroid follicular cells from Hashimoto thyroiditis tissue has been shown (66,67), and this may increase the chance of Fas interacting with its ligand (FasL), for example, on T cells, to trigger thyroid cell apoptosis. *In vitro* studies have shown that Fas expression by thyroid follicular cells is upregulated by cytokines and inhibited by TSH (67). This may explain why apoptosis is uncommon in Graves disease as a result of the protective effects of TS Abs (mimicking TSH action) on Fas expression. FasL expression also has been detected on thyroid cells from Hashimoto thyroiditis thyroid (67), which may provide a protective mechanism against apoptosis, by eliminating Fas^+ autoaggressive T cells. On the other hand, the coexpression of Fas and FasL by thyroid cells may result in these cells killing each another or even committing suicide (67). The massive apoptosis that would occur in such a case is not seen *in vivo*, casting doubts about the relevance of this hypothesis, and there is considerable debate over the specificity of thyroid cell staining for FasL (67). Iodide excess can also induce thyroid cell apoptosis through a p53-independent pathway (68).

Cytokines produced by the infiltrating T cells in Hashimoto thyroiditis have diverse effects on thyroid follicular cells. Cytokines may modulate thyroid cell growth and function and can alter the immunologic properties of these cells, for instance, by inducing HLA class II and adhesion molecule expression (69). *In vitro* studies showed that Hashimoto thyroiditis-derived T cells have a high potential for tumor necrosis factor-a (TNF-a) and interferon-(IFNg) but not IL-4 production (69), consistent with a T-helper cell (Th1) pattern of cytokine production in this condition, capable of mediating delayed-type hypersensitivity and cytotoxicity. Because thyroid antibodies can be detected in almost all patients with autoimmune hypothyroidism, a Th2 response is clearly also important in disease pathogenesis, and some Hashimoto thyroiditis-derived $CD4^+$ T clones produce a mixed Th1/Th2 cytokine pattern (70).

The reverse transcription-polymerase chain reaction (RT-PCR) technique demonstrated IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IFNg, and TNF-a mRNA in Hashimoto thyroiditis thyroid tissue samples (71,72), but a quantitative RT-PCR showed the predominance of a Th1 response (73). By applying cell fractionation techniques to Hashimoto thyroiditis-derived thyroidal lymphocytes, mRNA for all these cytokines has been detected in infiltrating $CD4^+$ and $CD8^+$ T cells, except for IL-1 (72). Besides the infiltrating inflammatory cells, thyroid follicular cells have been shown to produce a number of cytokines *in vitro*, including IL-1, IL-6, IL-8, IL-12, IL-13, IL-15, and TNF-b and transforming growth factor-b (TGF-b), suggesting an interplay between cytokine production by these cells and infiltrating lymphocytes in the autoimmune process (69) (Fig. 49.4).

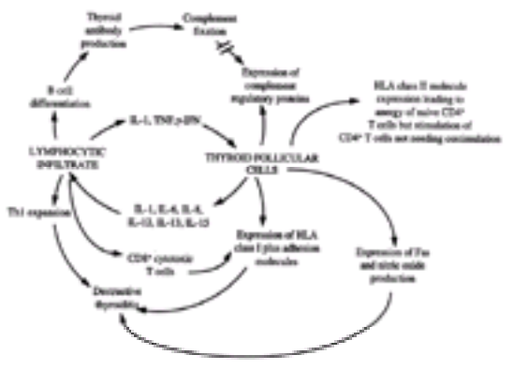

Figure 49.4. Interaction of cytokines and other factors with thyroid cells in the pathogenesis of autoimmune thyroid disease. (From Weetman AP, Ajjan R, Watson PF. Cytokines and Graves' disease. *Baillieres Clin Endocrinol Metab* 1997;11:418, with permission.)

Further evidence for a critical role of cytokines in ATD comes from observations following experimental or therapeutic administration of cytokines. Animals with experimental autoimmune thyroiditis (EAT) induced by immunization with thyroid antigen plus adjuvant develop a disorder similar to Hashimoto thyroiditis in humans, characterized by lymphocytic infiltration of the thyroid gland (see later). IL-1b administration accelerates the onset of thyroiditis in this model of EAT and blocks the induction of tolerance by TG treatment (74,75). A mild thyroiditis, but not hypothyroidism, can be induced by IFN-g administration to normal mice (76), whereas anti-IFN-g antibody treatment reduces disease severity in EAT (77). In humans, IFN-a treatment for malignancy or chronic hepatitis is associated with impairment of thyroid function, ranging from the appearance of thyroid autoantibodies alone to the development of frank thyroid dysfunction (78). The frequency of thyroid dysfunction after IFN-a treatment ranges from 2.5% to 40%, with most patients developing hypothyroidism and a minority hyperthyroidism. A study on euthyroid patients treated with IL-2 alone documented the development of thyroid dysfunction, including both hypothyroidism and hyperthyroidism, in 41% of patients (79,80). It should be noted that these studies were conducted on patients already suffering from serious diseases, and the effects of exogenous cytokine administration on thyroid function in the normal population remains unknown.

Peripheral and intrathyroidal T-cell responses to thyroid antigens have been analyzed *in vitro* and epitope mapping carried out using overlapping peptides. Because TG generates only a weak proliferative response in AITD-derived T cells, attention has focused on TPO and TSH-R, the latter considered in detail in the section on Graves disease that follows. T-cell proliferation in response to TPO epitopes is heterogeneous both within and between patients (81,82 and 83), and this makes therapeutic intervention with modified TPO peptides unrealizable. The immunodominant T-cell epitopes on TPO are predominantly, although not exclusively, located on amino acid residue 100 through 119 and 625 through 644 in patients with Hashimoto thyroiditis (84). The diversity of T-cell responses to TPO peptides presumably is due to determinant spreading whereby diversification of the immune response to antigenic epitopes occurs as disease progresses (85).

IMMUNE-MEDIATED TISSUE DESTRUCTION

Cellular events leading to tissue destruction as well as thyroid cell dysfunction have been considered already. Antibodies to thyroid antigens exacerbate but do not initiate this process. Deposition of early components of complement was identified in the thyroid of Hashimoto thyroiditis patients and elevated levels of thyroidal and circulating terminal complement complexes are found in ATD (36). The contribution of complement activation to thyroid cell destruction is limited by the expression of a number of membrane attack complex regulatory proteins expressed on thyroid cells, most importantly CD59 (86). Sublethal complement attack can impair thyrocyte function, for instance, resulting in suppression of the cAMP response to TSH (87). Also, complement-attacked thyroid cells release a range of proinflammatory molecules, including reactive oxygen metabolites, prostaglandin (PG)-E_2, IL-1, and IL-6 (88); thus, the complement system could play an important role in tissue damage in ATD indirectly by mediating the release of a variety of inflammatory mediators.

Antibody-dependent cellular cytotoxicity is mediated by the interaction of NK cells with the Fc portion of the antibodies that are, in turn, attached to thyroid follicular cells, resulting in cell lysis. Initially, antibodies that mediate ADCC were detectable in Hashimoto thyroiditis but not in Graves disease (89), and an inconsistent correlation between anti-TPO titers and cell lysis has been reported (90). Although a unique antibody, not reactive with TPO, has been proposed to account for the detection of ADCC activity in Hashimoto thyroiditis but not Graves disease sera (90), it now seems clear that TPO antibodies in any type of ATD sera, including Graves disease, mediate the bulk of ADCC activity (91).

IMMUNOPATHOGENESIS OF DISEASE

The main factors involved in the immune pathogenesis of autoimmune hypothyroidism are T-cell-mediated cytotoxicity directed against thyroid cells, including thyroid cell apoptosis and thyroid cell dysfunction, particularly that from cytokines. Antibodies play a secondary role except in a small proportion of patients with TB Abs. The role of thyroid cells may be critical in these processes. ATD-derived thyroid follicular cells show increased expression of HLA class I and II molecules, which is regulated by cytokines, most importantly IFN-g (92). Class II-expressing thyroid cells may enhance the activation and proliferation of certain autoreactive lymphocytes, but the inability of thyroid cells to express costimulatory signals, necessary for naive T-cell activation, may also render these latter cells anergic and thus induce peripheral tolerance (93). Class II-expressing thyroid cells may perpetuate ATD after autoimmunity is firmly established and B7 dependence is no longer important for continued T-cell responses.

Thyroid cells also express a number of adhesion molecules, such as intercellular adhesion molecule (ICAM-1) and lymphocyte function antigen-3 (LFA-3) (69), which have a central role in disease pathogenesis through leukocyte activation and localization to the inflammatory sites. Cytokine-mediated modulation of such adhesion molecule expression in the thyroid could be crucial to the perpetuation, if not initiation, of the disease. Furthermore, the ability of thyroid cells to produce cytokines and nitric oxide (94) emphasizes the potential role of these cells in augmenting the inflammatory process. In addition to these proinflammatory effects, thyroid follicular cells express complement-activation regulatory proteins, enhancing resistance to complement-mediated cell lysis. The possible effector mechanisms operating on thyroid follicular cells in ATD are summarized in Fig. 49.5.

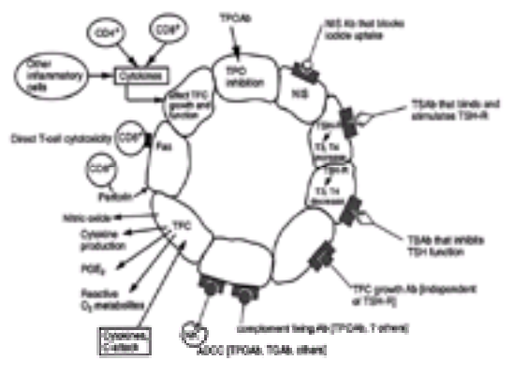

Figure 49.5. Mechanisms underlying thyroid cell destruction and dysfunction in autoimmune thyroid disease. Ab, antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; C-attack, complement attack; NIS, Na^+/I^- symporter; T_3, triiodothyronine; T_4, thyroxine; TBAB, thyroid blocking antibody; TFC, thyroid follicular cells; TG, thyroglobulin; TPO, thyroid peroxidase; TSAB, thyroid-stimulating antibody; TSH, thyroid stimulating hormone; TSH-R, TSH receptor.

Animal Models

In some animal models, EAT occurs either spontaneously or is immunologically induced. The Buffalo and Bio-Breeding (BB) strains of rats, the nonobese diabetic (NOD) mouse, and the obese strain (OS) of chicken develop spontaneous autoimmune thyroiditis (95,96). The incidence of thyroiditis in Buffalo rats does not exceed 25%; however, this can be enhanced by immunologic modulation (i.e., neonatal thymectomy) and toxin administration (3-methylcholanthrene). A higher incidence of spontaneous thyroiditis in seen in BB rats, which is usually, but not necessarily, associated with diabetes in these animals. The animal model closest to Hashimoto thyroiditis remains the OS chicken (96). These birds develop severe thyroiditis with overt hypothyroidism requiring T_4 supplementation to thrive. Thymectomy at birth

prevents thyroiditis, indicating that the disease is T cell dependent. All the above models develop TG antibodies, but TPO reactivity is generally absent. MRL-*lpr/lpr* mice develop thyroiditis with both TG and TPO antibodies as well as a lupuslike syndrome (97).

Thyroiditis can be induced in monkeys, rabbits, guinea pigs, rats, and mice by immunization with TG in complete Freund's or other types of adjuvant or even by TG alone (98). The strength of the thyroid autoimmune response is influenced by class II and, to a lesser extent, class I genes. Nonmajor histocompatibility complex (MHC) genes, such as those encoding immunoglobulin heavy chains, also can play a role in the susceptibility of the disease in rats and mice (99). Disease can be transferred by T cells but inconsistently or not at all by TG antibodies, showing that TG antibodies are not directly involved in the pathogenesis of the disease (100). The importance of T cells to pathogenesis is further demonstrated by the development of thyroiditis after neonatal thymectomy (with or without irradiation) in genetically susceptible strains of mice and rats, presumably due to failure of central tolerance or the generation of immunoregulatory T cells (101). Treatment of neonatal mice with cyclosporin A also can induce thyroiditis (102). The development of thyroiditis in these animals can be prevented if they are kept in pathogen-free conditions; disease then can be induced by the transfer of normal gut flora (103), emphasizing the importance of environmental factors in development of the disease.

Future Directions

Although treatment of autoimmune hypothyroidism is relatively simple, it is not curative. Establishing the exact pathogenic mechanisms that result in autoimmune hypothyroidism may allow the development of new therapeutic strategies that offer a cure, not only for autoimmune hypothyroidism but also for other organ-specific autoimmune disease. It is well accepted now that numerous genetic and environmental factors are important in the susceptibility to autoimmune hypothyroidism. The exact contribution of each and the pattern of combination that leads to the emergence of the disease remain largely unknown. The recent development of automated linkage analysis should allow a precise identification of the genes that are directly involved in disease pathogenesis.

T cells play a central role in the pathogenesis of autoimmune hypothyroidism. Most studies found a polyclonal T-cell response to thyroid antigens, probably because study of these cells was performed when the disease is well advanced and earlier epitope-specific T-cell responses are lost. If the genetic basis of the disease is established in the future, it should be possible to predict the development of the disease, allowing study of the evolution of T-cell autoreactivity. More work is also needed to establish the exact role of thyroid antibodies in disease pathogenesis. The investigation of the role of NIS antibodies is of particular interest. These antibodies have been shown to inhibit the symporter function *in vitro*, potentially resulting in clinical hypothyroidism even before tissue destruction becomes evident.

Postpartum Thyroiditis

Transient thyroid dysfunction during the year after childbirth is termed *postpartum thyroiditis* (PPT). The population prevalence is around 5%, although this figure includes many patients diagnosed during surveys of biochemical changes postpartum; clinically apparent disease is much less common. The pathology resembles that of Hashimoto thyroiditis or primary myxedema. PPT starts usually with a thyrotoxic phase 2 to 3 months postpartum, followed by a hypothyroid phase at 5 to 6 months. The thyrotoxic or hypothyroid phase can occur alone. After a euthyroid interval, up to a quarter of patients become hypothyroid 3 to 4 years after onset of postpartum thyroiditis (12,104), indicating that this disease represents an early manifestation of autoimmune hypothyroidism.

The usual feature of this disorder is a postpartum rise in TPO Abs; occasionally, TG Abs accompany TPO Abs, and rarely they represent the only marker of the disease. The presence of TPO Abs antepartum can serve as a predictive marker of the disease because it has been shown that 50% to 75% of TPO Ab-positive pregnant women will develop PPT (105).

The complement system is activated in postpartum thyroiditis PPT, and this activation is related to the extent of thyroiditis and correlates with the severity of thyroid dysfunction (106,107). Therefore, TPO Abs may play an important role in this disease through their complement-fixing properties, but the occurrence of PPT in the absence of TPO Abs in some women suggests that T-cell-mediated events are at least as important. In keeping with this, PPT is associated with an elevated ratio of $CD4^+$:$CD8^+$ circulating T cells, both during pregnancy and in the postpartum period, and an increase in the circulating $CD45RA^+$ lymphocyte subset has been found in pregnant women who subsequently developed PPT (108).

GRAVES DISEASE

Although Caleb Parry first described a case suggestive of Graves disease and its associated eye signs, it was not until 10 years later that Robert Graves identified the disease in 1835. An autoimmune basis of the disease was subsequently revealed by the discovery of a long-acting thyroid stimulator (24), now known as the thyroid-stimulating antibody (27). This antibody binds the TSH-R, mimicking the effects of TSH and resulting in hyperthyroidism. More than 90% of patients with Graves disease have thyroid-associated ophthalmopathy (TAO), which can be detected using a number of scanning techniques. Clinically apparent TAO is evident in about 50% of patients with Graves disease. Thyroid dermopathy is found only in a minority of patients with Graves disease (1%–5%), and this usually occurs in the presence of severe TAO.

Epidemiology

Hyperthyroidism affects up to 2% of women and 0.2% of men, with a peak age of onset in the fifth decade of life (3). About 80% of hyperthyroid patients have Graves disease.

GENETICS

Recent data suggest that the role of genetic factors in the susceptibility to Graves disease is less than previously thought (109,110). There is only a 22% concordance in monozygotic twins and 0% in dizygotic twins (111). The risk in HLA-identical siblings is generally less than 10% (112), indicating that HLA has a modest genetic effect on disease pathogenesis. A detailed review of HLA associations with Graves disease can be found elsewhere (9,113), and only key features are summarized here. An association of HLA-DR3 with Graves disease has been recognized in most studies of white subjects, with a relative risk of 2 to 3. Studies of white populations have described HLA-DQA1*0501 as an important risk factor in Graves disease over and above that of HLA-DR3, with which there is linkage disequilibrium (114). In contrast, inheritance of HLA-DRB1*07 appears to be protective (115). As with autoimmune hypothyroidism, the association of Graves disease with polymorphisms of immunoglobulin or TCR genes has been conflicting, but, on balance, there are no firm data to indicate a true association (5,6 and 7,116). Association between Graves disease and TSH-R polymorphisms has been inconclusive, and association with TNF polymorphisms have not been reproducible (111). In contrast, an association between polymorphisms of the CTLA-4 gene and Graves disease has been repeatedly shown (9,117,118). Furthermore, the inheritance of thyroid autoantibodies has been recognized, suggesting that immunoglobulin genes may play a role in the susceptibility to the disease (119).

Linkage studies showed that Graves disease is linked to a locus on chromosome 14 (GD-1; 99cM) and chromosome 20 (GD-2; 56cM), with an MLS of 2.5 and 3.5, respectively, and, in addition, a locus on the X chromosome has been linked (GD-3; 114 cM) with an MLS of 2.5 (10). This latter finding poses the question of whether the X-inactivation mosaic is involved in disease pathogenesis (120).

ENVIRONMENT

As in autoimmune hypothyroidism, Graves disease affects mainly women, indicating a role for sex hormones in disease pathogenesis. Iodine intake exacerbates thyroid autoimmunity in general (13), and increased iodine intake can precipitate Graves disease by the synthesis of excess thyroid hormones (the Jod–Basedow phenomenon).

Some patients with Graves disease have antibodies to *Yersinia* antigens, proposed to be attributable to TSH-R cross-reactivity (121). Most patients with *Yersinia* infection, however, do not develop Graves disease, and the role of this organism in the pathogenesis of Graves disease is still controversial. Retroviral sequences have been detected in both the thyroid and peripheral blood mononuclear cells of patients with Graves disease (122), but others subsequently failed to confirm these findings (123). Thyroid biopsies from Graves disease patients showed reactivity to human foamy virus antibodies (124,125), but again this could not be repeated (126). Although these data are interesting, such viruslike particles also have been detected in normal thyroids and other tissues, and therefore a role in the development of Graves disease is unlikely.

Stress appears to be an important factor in the development of Graves disease (127), but the evidence is indirect because studies are retrospective and unfavorable life events could be a consequence rather than a cause of the disease. Disease-independent life events are more frequent than disease-dependent life events in patients with Graves disease, however, implying a real effect of stress (128). Stress causes a wide array of neuroendocrine changes that may alter the immune response through loss of suppressor cell function, T-helper cell subset imbalance, and defective clonal inactivation of self-reactive, immature B-lymphocytes.

Clinical Presentation

SYMPTOMS AND SIGNS

The common symptoms and signs of Graves disease are summarized in Table 49.4. Many symptoms are similar to those of adrenergic excess. The clinical presentation of the disease may be dramatic or subtle. In its extreme form, namely thyroid storm, it may manifest with atypical symptoms, including fever, cardiovascular collapse, confusion, psychosis, severe weakness, or even coma. This is a life-threatening emergency that requires urgent medical treatment. On the other hand, Graves disease may manifest atypically, particularly in elderly patients, with apathetic hyperthyroidism that can be confused with depression.

Symptoms	Frequency (%)
Symptoms	
Nervousness, irritability	>95
Increased perspiration	90
Change in temperature preference	90
Palpitations and dyspnea	90
Fatigue and weakness	90
Increased frequency of stools	80
Weight loss with increased appetite	78
Symptoms of TAO	50
Insomnia	30
Pruritis	20
Menstrual irregularities	20
Increased pigmentation or vitiligo	<5
Signs	
Hyperkinetic behavior, thought, and speech	>95
Tachycardia or atrial fibrillation	>95
Restlessness, hoarseness	>95
Goiter	90
Fine, warm, moist skin	90
Thrill and bruit	70
Signs of TAO	50
Thinning of hair and loss of curl	50
Hyperreflexia	50
Tachypnea on exertion	30
Pretibial myxedema	5
Onycholysis and clubbing	<5
Lymphadenopathy	<5
Splenomegaly	<5
Hyperpigmentation or vitiligo	<5

TAO, thyroid-associated ophthalmopathy.

TABLE 49.4. Symptoms and Signs of Graves Disease

PHYSICAL FINDINGS

A diffuse goiter of variable size can be found in most patients. The presence of TAO with hyperthyroidism is diagnostic of Graves disease. The most common signs of TAO are lid retraction or lag and periorbital edema (Table 49.5.) Exophthalmos can be demonstrated in about one third of patients, whereas diplopia and visual field loss resulting from optic nerve compression are unusual. Thyroid dermopathy occurs in fewer than 5% of patients and almost always in the presence of TAO. It is found most frequently over the anterolateral aspects of the shin but can occur anywhere in the body, especially at sites of trauma.

Symptoms
Irritation of the eye or pain in the globe
Limitation of movement of the eyeballs, especially upward
Diplopia
Blurred vision due to inadequate convergence and accommodation
Decreased visual acuity due to papilledema, retinal edema, retinal hemor
images or optic nerve damage
Signs
Lid reaction and lag
Wide palpebral opening
Periorbital swelling and puffiness of the lids
Staring or frightened expression
Infrequent blinking
Absence of forehead wrinkling on upward gaze
Inability to keep the eyeballs converged
Chemosis, corneal injection, or ulceration
Visible and palpable enlargement of the lacrimal glands
Visible swelling of lateral rectus muscles as they insert into the globe and
injection of the overlying vessels
Exophthalmos

TABLE 49.5. Symptoms and Signs of Thyroid-Associated Ophthalmopathy

DIAGNOSTIC STUDIES

The diagnosis of primary hyperthyroidism biochemically relies on demonstrating high T_4 levels with undetectable TSH levels. Because T_4 levels may be normal in the early phases of the disease, T_3 measurement should be carried out if TSH is suppressed with normal T_4.

Radionuclide scans with ^{123}I, ^{131}I, or ^{99m}Tc are helpful in difficult cases (e.g., without TAO), showing a diffuse goiter typical of Graves disease. The detection of TPO antibodies (positive in 80% of Graves' disease patients) further aids the diagnosis. The measurement of TS Ab is not done in many centers, but as assays improve and become less expensive, they will become the test of choice for diagnosis.

Usually, TAO is diagnosed clinically, but computed tomography or magnetic resonance imaging is indicated in cases of uncertainty or to rule out other pathologies, such as a retroocular mass or arteriovenous malformation. Several methods, such as octreotide scanning, were developed to assess orbital disease activity, which may help in selecting patients for immunosuppressive treatment, but further evaluation still will be necessary.

PATHOLOGY

The thyroid gland is diffusely enlarged from twice to several times normal size. Thyroid follicles show marked epithelial hypertrophy and hyperplasia (Fig. 49.6). Blood vessels are large and congested. A variable degree of lymphoid infiltration of the gland with both T and B cells is usually seen. The formation of germinal centers is not uncommon, whereas fibrosis is unusual (26). These changes reverse with antithyroid drug treatment.

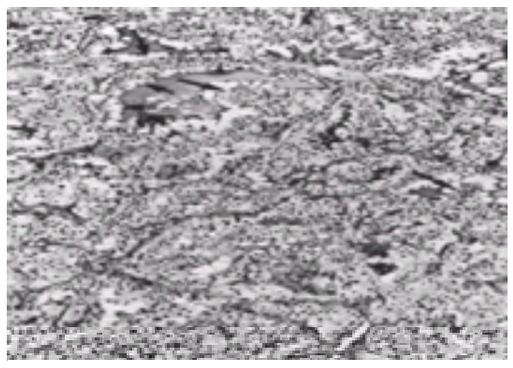

Figure 49.6. Histology of Graves disease. The thyroid is markedly hyperplastic. The follicular cells are tall, cuboidal to columnar, with pale cytoplasm. Colloid is depleted and shows a moth-eaten appearance where present *(short arrows)*. Papillary projections in the follicles are noted (*long arrows*). (From Mendelsohn GF. Pathology of thyroid disease. In: Mendelsohn GF, ed. *Diagnosis and pathology of endocrine diseases.* Philadelphia: JB Lippincott, 1988:65, with permission.)

Treatment

Reversal of the autoimmune process in Graves disease, thereby restoring thyroid function, would be the ideal treatment of the disease; however, this is not an option at present. Current treatment of the disease consists of antithyroid drugs, radioiodine, or surgery.

ANTITHYROID DRUGS

These drugs can be given either as reducing doses (*titration regimen*) or as a high dose with the combination of T_4 (*block-and-replace regimen*). The former usually is not applied for more than 24 months, whereas in the latter, treatment is limited to 6 months. Long-term remission is achieved in up to 50% of patients. The drugs not only inhibit TPO activity, thereby lowering T_3 and T_4 levels, but also alter the autoimmune response and lower TS Ab levels, which explains this remission. The mechanisms are discussed subsequently here. The most serious, albeit rare, complication of these drugs is agranulocytosis. Hepatotoxicity that may continue despite stopping the drug is rare but life threatening. Antithyroid medications may cause thrombocytopenia and lupuslike syndromes. Less serious side effects include urticaria, arthralgia, and gastrointestinal symptoms (129).

RADIOIODINE (^{131}I) THERAPY

This is an increasingly popular first-line treatment for Graves disease. Contraindications are pregnancy and breastfeeding, and caution is required in TAO as ^{131}I may worsen eye signs. Early fears of an increase in malignancies after radioiodine treatment is unfounded (130); however, a minimal increase in death rates from thyroid cancer and other individual malignancies after radioiodine treatment has been documented, which may be related to the disease itself rather than the treatment. This type of treatment is probably best avoided in children with Graves disease until the risks are clearly established in this group. In most patients, the main long-term side effect of radioiodine is the development of hypothyroidism.

SURGERY

Some patients prefer subtotal thyroidectomy, especially in the presence of a large goiter or the coexistence of a nodule whose nature is unclear. In specialized centers, the cure rates are usually more than 95% with low operative complication rates. It is worth noting that surgery has no effects on TAO.

Immunopathology

HUMORAL IMMUNITY

It is now accepted that TSH and TS Ab interact with the extracellular domain of the receptor (27), but the existence of epitopes on the extracellular surface of the transmembrane domains cannot be ruled out completely. Several difficulties have been encountered in the study of TSH-R antibodies. The absence until recently of an animal model of Graves disease and the inability to purify the native, functional TSH-R in sufficient quantities have hampered research into TSH-R antibody interactions. Rather than using primary cell cultures or cell lines as a source of autoantigen (which would provide very little TSH-R protein), prokaryotic and eukaryotic expression systems for TSH-R have been developed, but none is ideal for the assay of antibodies. TSH-R expressed in *Escherichia coli* does not bind TSH (131), whereas TSH-R extracellular domain expressed in baculovirus remains intracellular and must be purified from cell homogenates and does not bind TSH (132). In addition, TSH-R expression in different systems has often shown inconsistent or insufficient binding of autoantibodies, recently attributed to the important relationship between autoantibody recognition and TSH-R maturation associated with glycosylation (27).

Substitution studies have shown that epitopes for TS Ab and TBII in Graves disease are located primarily on the N-terminal region of the extracellular domain (27,133,134). TB Ab binding sites are found mainly on the C-terminal portion of TSH-R and, to a lesser extent, on the N terminus and midregion of the extracellular domain (27,133). TSH-R Abs are often light-chain restricted and predominantly of the IgG_1 subclass (135), suggesting an oligoclonal B-cell origin for these antibodies in at least some patients. On the other hand, TS Ab activity (based on cAMP production and PI activity) can differ from one patient to another, with antibodies activating both pathways found in patients with the most severe disease, implying a heterogeneity in the activity of TS Ab (136). Attempts at a molecular analysis of TSH-R antibodies have been both limited and disappointing (137,138), probably because of low levels of TSH-R Abs compared with TPO Ab and TG Ab levels (139) as well as the lack of a pure TSH-R molecule suitable for the study of antibody interactions.

Recent studies of TSH-R-antibody interactions should be interpreted with care. Most TSH-R B-cell epitopes appear to be conformational, rendering the results of the peptide studies questionable (27). In addition, chimeric substitution studies in a particular region of TSH-R may induce overall conformational changes in the remaining receptor molecule, with the possibility of obtaining false-negative results in antibody binding assays. Moreover, recent evidence suggests that glycosylation of the receptor is an important factor in antibody recognition, which casts doubts on the validity of bacterial expression systems and peptide studies. Finally, recombinant TSH-R is “sticky” and may bind to irrelevant immunoglobulin molecules.

A recent bioassay using Chinese hamster ovary (CHO) cells transfected with NIS showed that 30% of Graves disease sera possess antibody-mediated NIS inhibitory activity (140). Using an *in vitro* translation and transcription system for NIS synthesis, 22% of Graves disease sera were found to bind this recombinant form of the symporter (55). Antibodies against TG and TPO can be detected in up to 80% of Graves disease patients, and their properties are similar to those found in autoimmune hypothyroidism (discussed previously).

CELLULAR IMMUNITY

A defect in TSH-R-specific T-suppressor cells was proposed to underlie Graves disease, akin to the thyroid-specific defect proposed in autoimmune hypothyroidism (56), but clear evidence using recombinant TSH-R is so far lacking, and the exact mechanism of suppression is poorly defined. As in Hashimoto thyroiditis, TCR restriction of the intrathyroidal T cells has been investigated in Graves disease with contradictory results (58,59). Similarities in the intrathyroidal, pretibial, and retrobulbar Va and Vb TCR repertoire were shown in two patients, but there was heterogeneity in TCR use between the patients (141), which suggests recognition of similar antigenic determinants at the different sites of Graves disease complications in the same patient, but it is obvious that more patients at different stages of disease need to be studied to obtain a comprehensive picture of T-cell restriction in Graves disease. The antigens recognized by these T cells are unknown.

Thyroid-infiltrating $CD8^+$ cells in Graves disease do not seem to have a role in cell-mediated cytotoxicity because they fail to express perforin, whereas Graves disease-derived ab^+, $CD4^-$, $CD8^-$ T cells do contain perforin. This pattern of perforin expression differs from autoimmune hypothyroidism, and of course in Graves disease there is no clinical ground to suspect that tissue destruction occurs, although 10 years after onset, up to 15% of Graves patients successfully treated with antithyroid drugs develop autoimmune hypothyroidism. In addition, thyroid follicular cells and reactive cytotoxic gd^+ T cells have been grown from the Graves disease infiltrate; these cells are not HLA restricted and preferentially express IL-4, indicating a Th2-like phenotype (142). The importance of these cells in pathogenesis is unclear.

Thyroid-derived T cells from Graves disease patients produce IL-4, indicating a Th2 response, which one would predict in this exemplar par excellence of autoantibody-mediated disease. Furthermore, quantitative RT-PCR has shown predominance of Th2 cytokines in Graves disease tissue samples (72). The failure to detect IL-4 in some Graves disease tissue samples (69), together with the detection of IL-13 expression, indicates that the latter is also involved in priming toward a Th2 response in Graves disease. The importance of the Th2 response is demonstrated by the development of Graves disease in a patient treated with IL-4 for metastatic malignant melanoma (143). Moreover, more than 30% of multiple sclerosis patients treated with a monoclonal antibody to CD52 (resulting in an immune deviation away from Th1) developed Graves disease (144), further emphasizing a pivotal role for the Th2 cells in this disease.

Using recombinant techniques, T-cell responses to full-length TSH-R extracellular domain have been shown in Graves disease, but positive reactivity (albeit at lower levels) was also evident in patients with focal thyroiditis (145). To define T-cell epitopes on TSH-R, peripheral and intrathyroidal T-cell responses to a series of overlapping peptides covering the entire extracellular domain of TSH-R have been investigated in proliferation assays. A heterogeneous response has generally been detected, and occasional responses are found using T cells obtained from normal individuals (146,147). TSH-R amino acids 158 through 176, however, have been defined as an immunodominant T-cell epitope in Graves disease in a number of studies (84,148). Furthermore, a loss of T-cell reactivity to this peptide was demonstrated after the successful treatment of Graves disease with thyroidectomy or radioiodine administration (149). Residues 207 through 222 and 343 through 376 also contain important T-cell epitopes in Graves disease (84), which are also recognized by T-cell clones from apparently healthy subjects (150).

Antithyroid drugs may induce remission by a number of mechanisms, detailed elsewhere (13). In brief, treatment with antithyroid drugs leads to a fall in thyroid but not other antibody levels, and a reduction in thyroidal lymphocytic infiltration that is independent of any change in thyroid hormone status produced by the action of these drugs on TPO. The most likely explanation of these immunologic effects is that the drugs are concentrated by thyroid cells and reduce their expression of a number of proinflammatory molecules, including cytokines and reactive oxygen metabolites (88). This will have the effects of suppressing the intrathyroidal autoimmune process, specifically reducing thyroid autoantibody production (Fig. 49.7).

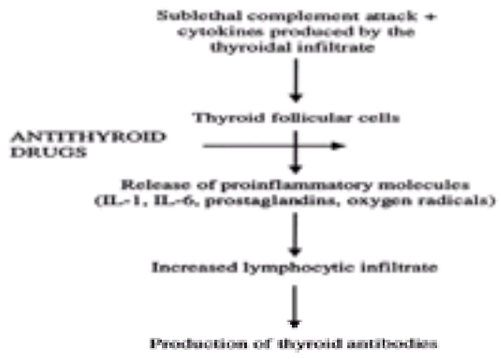

Figure 49.7. Mechanism for the thyroid-specific immunomodulatory action of antithyroid drugs in Graves disease.

IMMUNOPATHOGENESIS

It is clear that Graves disease is caused by TS Abs, which stimulate thyroid cell growth and overproduction of thyroid hormones via TSH-R. There is good evidence for a predominant Th2 response in the thyroidal lymphocytic infiltrate, a major source of TS Ab production, and a number of genetic (HLA-DR and CTLA-4 polymorphisms) and nongenetic (stress, iodide intake) factors have been identified that predispose individuals to develop Graves disease. This is not simply a disease mediated by TS Abs, however, as autoimmune responses to other thyroid autoantigens also occur in almost the same frequency as in autoimmune hypothyroidism, and occasional patients develop Graves disease after autoimmune hypothyroidism, whereas others become spontaneously hypothyroid early or late after disease onset. Moreover, autoimmune hypothyroidism and Graves disease frequently occur in different members of the same family, pointing to a shared etiology. Antithyroid drugs induce remission by modulating the intrathyroidal autoimmune response but are successful in only about one half the patients who have Graves disease. Further definition of the intrathyroidal events that perpetuate Graves disease may allow improved remission rates from medical therapy.

Animal Models of Graves Disease

Research into Graves disease has been severely handicapped by the absence of an animal model of the disease. Traditional approaches to induce Graves disease in animals have been unsuccessful, and more complicated immunologic manipulation has been necessary (reviewed in detail elsewhere: 151).

Classic immunization methods with recombinant receptor (expressed in prokaryotic or eukaryotic cells) induced TSH-R binding antibodies in mice, with some antibodies having blocking properties but no TS Ab could be produced (152,153). A minor derangement of thyroid function (both a fall and a rise in T_4) may occur in these animals, although these are inconsistent (154). Instead of recombinant receptor peptide fragments, Hidaka et al. used peptide fragments for immunization (155). Injection of DBA/1J mice with TSH-R peptides (known to stimulate T-cell proliferation *in vitro*) induced low amounts of TS Abs in some animals, but none had clinical or biochemical hyperthyroidism, and their thyroids remained histologically normal.

A novel and increasingly popular method for immunization involves the injection of animals with cDNA of the antigen of interest (rather than protein). Immunization of BALBc mice with full-length TSH-R cDNA induced antibodies that bound TSH-R in 14 of 15 animals (156). Most had TBAb activity with only one developing TS Ab. Histologically, all mice displayed severe thyroiditis with signs of epithelial hypertrophy, but none developed thyroid dysfunction. Interestingly, when the same method was applied to the NMRI outbred strain of mice, 4 of 30 female mice developed hyperthyroidism, both clinically and biochemically, with circulating TS Abs (157). This protocol was unusual in its use of outbred animals, providing a more diverse genetic background (similar to humans). Furthermore, some of these animals developed eye signs, thereby mimicking the full clinical picture of Graves disease.

Another promising approach involves the treatment of mice with fibroblasts transfected with MHC class II and the full-length TSH-R (158). The fibroblasts were MHC identical to recipient mice, and the transfected TSH-R was shown to be functional (by measuring cAMP production after TSH stimulation). Injection of these transfected cells into mice resulted in the development of TBII antibodies. Sera from one fifth of these mice showed TS Ab activity with an increase in T_4 levels. In these animals, the thyroid was enlarged, with hypertrophy in the thyroid follicles, but there was no lymphocytic infiltration, indicating that TS Abs are not necessarily produced locally in the thyroid.

Finally, T-cell transfer experiments have shown that treatment of BALBc and NOD mice with TSH-R-primed, $CD4^+$-enriched syngeneic T cells results in the induction of TSH-R antibodies (159). A Th2 response was identified in the thyroid of BALBc mice, whereas a Th1 response with thyroid destruction was evident in NOD mice. The orbit of BALBc, but not NOD, mice displayed changes consistent with TAO, suggesting that this complication of Graves disease is Th2-mediated. No convincing evidence of hyperthyroidism was found in these animals (only a minority of animals had elevated T_4), however, and no data were provided on TS Ab activity of their sera (160).

Thyroid-Associated Ophthalmyopathy and Pretibial Myxedema

The close clinical association between these conditions and Graves disease (and, less commonly, autoimmune hypothyroidism) suggests a common pathogenesis, most likely the result of an autoimmune response against an autoantigen shared between thyroid, orbit, and skin. In both TAO and pretibial myxedema (PTM), there is an infiltration of the affected site (extraocular muscle or skin) by activated T cells (Fig. 49.8) and a variety of cytokines can be detected, which can activate fibroblasts to secrete glycosaminoglycans (GAGs). The accumulation of GAGs is a cardinal feature of TAO and PTM, which in turn causes water trapping and edema. The resulting swelling accounts for most of the clinical features of these complications. Late in disease, continued fibroblast activation may lead to fibrosis, and at this stage some muscle-fiber damage may be detectable in TAO. It is noteworthy that in the early stage of the disease, muscle cells are intact; the key target is therefore most likely the fibroblasts of the extraocular muscles and the skin. Enhanced susceptibility to cytokine-mediated induction of GAG production may be a feature of fibroblasts from these particular sites, explaining the localization of the autoimmune process.

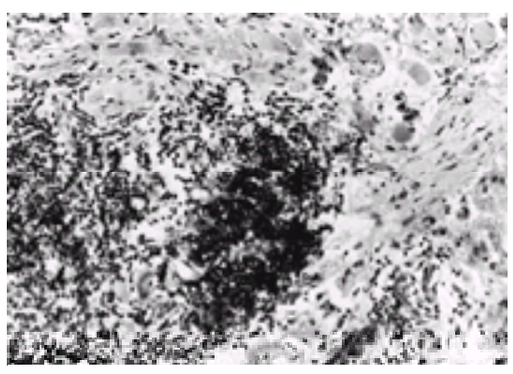

Figure 49.8. Histology of thyroid-associated ophthalmopathy. The extraocular muscles are infiltrated by a diffuse and focal lymphocytic infiltrate. The muscle fibers are intact but separated by edema secondary to glycosaminoglycan accumulation.

Treatment of mild to moderate TAO is usually supportive. In severe cases, high-dose corticosteroids, cyclosporin A, intravenous immunoglobulin, radiotherapy, and surgery can be used (161). PTM is usually left untreated, but troublesome lesions may benefit from corticosteroid ointment or the somatostatin analog octreotide.

Studies on B-cell responses in TAO have faced major difficulties, largely because the autoantigen in TAO is still unknown. The conventional thyroid antigens TG and TPO do not seem to have a role in TAO, whereas the role of TSH-R is controversial. The search for orbital antibodies has generally used nonhuman and human crude orbital tissue homogenate or extraocular muscle (EOM) membranes as a source of autoantigen. By immunoblotting, antibodies against eye-muscle (55-, 64-, and 95-kd antigens) and fibroblast (23- and 66-kd antigens) membranes have been detected more frequently in patients with TAO than in controls (162). The 64-kd reactivity created particular interest because it is shared between eye muscle and thyroid and now is known to comprise a number of different molecules, including a so-called

D1 protein, calsequestrin, and succinate dehydrogenase (163). Kromminga et al. (164) demonstrated that 82% of patients with TAO have antibodies that react with nondenatured D1 protein compared with 5% of controls, indicating a high specificity and sensitivity. Immunoreactivity against denatured protein was not detected, indicating that epitopes are conformational. Calsequestrin (63 kd) was recognized by sera from 40% of patients with TAO and only 5% of controls (165). Succinate dehydrogenase (67 kd) reacted with 67% of sera from active TAO patients, 30% of chronic disease, 30% of Graves disease without associated TAO, and 7% of controls, suggesting that antibodies to this molecule may correlate with the severity of eye disease (166). Other nonspecific candidate autoantigens in TAO include acetylcholinesterase, actin, tubulin, and acetylcholine receptor, but none has been confirmed as a key autoantigen (162).

Attention has focused on TSH-R as a potential autoantigen in TAO. Full-length TSH-R transcripts have been detected in retrobulbar tissue but not EOM or fibroblasts, suggesting that TSH-R mRNA is present in orbital fat (167). Fragments of TSH-R ECD have been detected in fibroblasts from retrobulbar muscle and connective tissue (168), but most studies used RT-PCR for TSH-R mRNA detection and applied more than 30 cycles for amplification, making the physiologic importance of TSH-R in the orbit questionable (168). Burch et al. (169) demonstrated that rabbit anti-TSH-R polyclonal antiserum reacted with retrobulbar fibroblasts, but a drawback of this study is the absence of an appropriate negative control. Other studies also detected TSH-R in orbital tissue and PTM lesions by immunostaining (170,171), thereby confirming the translation of detected mRNA into protein. This occurs particularly in the preadipocyte subpopulation of fibroblasts (161), but how this minor population of cells participates in the autoimmune process is unknown. The recent development of an animal model of Graves disease and TAO using TSH-R immunization emphasizes the possible role of TSH-R as a major autoantigen in TAO (see preceding).

Analysis of TCR gene use by the infiltrating T cells in the retrobulbar tissues revealed a strikingly limited heterogeneity in eight patients with recent-onset TAO (172). By contrast, four patients with long-standing, clinically inactive TAO had a lesser degree or an absence of restriction. Comparison of TCR use in orbital connective tissue and extraocular muscle tissue from the same patient did not show major differences, suggesting similar antigenic recognition in both compartments. Similar results were obtained by analyzing TCR restriction in seven patients with early and late stages of PTM (172). Moreover, the same group found similarities in the intrathyroidal, pretibial, and retrobulbar TCR repertoire use in two patients (141), indicating that T-cell reactivity is restricted in the early phases of TAO and PTM, whereas in the later stages recruitment of a more diverse spectrum of T cells occurs (spreading of the immune response), and these T cells can react to a variety of tissue-specific and tissue-nonspecific antigens. It remains possible that the restriction described in such studies is an artifactual finding related to the small size of samples analyzed (in particular, samples from orbital tissue), which may harbor only a limited number of lymphocytes resulting in “pseudorestriction.”

Orbital-derived T cells from patients with TAO recognized autologous orbital fibroblasts in one study but not crude eye-muscle extracts or autologous peripheral blood lymphocytes (PBLs) (173). These T cells were mainly $CD8^+$, $CD45RO^+$, and they secreted a mixture of cytokines but had little cytotoxic activity, ruling out the possibility of direct tissue destruction by these cells and favoring an indirect role in disease pathogenesis mediated by cytokine production. More recently, it was shown that orbital-derived TAO lymphocytes are mainly of the $CD4^+$ phenotype and can proliferate in response to thyroid and EOM extracts and membrane preparations (174). In addition, TAO-derived lymphocytes, but not PBLs, were shown to proliferate in response to TSH-R extracellular domain (ECD) in one patient, whereas both TAO-derived lymphocytes and PBL proliferated to TSH-R ECD in another patient, further indicating that TSH-R is an autoantigen involved in TAO.

Future Directions

Several lines of evidence indicate that autoimmune hypothyroidism and Graves disease have similar immunopathogenesis and probably represent two clinical presentations of the same broad disease process. The most important distinguishing factor is the presence of TS Abs in Graves disease; these are infrequently detected in autoimmune hypothyroidism.

In contrast to autoimmune hypothyroidism, treatment of Graves disease is problematic because of the side effects of medications used, complications of surgery, or the development of permanent hypothyroidism. Furthermore, adequate control of thyroid function is not always achievable, and treatment of extrathyroidal complications can be notoriously difficult.

The identification of genetic markers of the disease is of utmost importance. Linkage analysis studies should allow the identification of susceptibility genes involved in Graves disease, and these can be compared with genetic markers in autoimmune hypothyroidism to establish whether a real difference exists between these two clinical presentations of what seems to be a spectrum of disease. In particular, immunoglobulin V gene use by TS Abs is a major focus of study because it is possible that germline V gene differences could explain why some patients develop Graves disease and others develop autoimmmune hypothyroidism. The study of T cells early in the disease may identify restricted T-cell clones, potentially offering curative treatment by immunologic manipulation. A major breakthrough was the development of animal models of Graves disease, which have eluded researchers for the past four decades. These should help to shed some light on the initiation and progression of the disease and may allow identification of immunologic interventions that can halt the disease process.

More than 90% of Graves disease patients have TAO, with clinically obvious disease apparent in 50%. Treatment strategies for TAO are unsatisfactory at present, and almost certainly the only curative treatment of TAO will be immunologically directed. Therefore, the identification of the pathogenic mechanisms and the major autoantigens involved in TAO is a pressing task. Although several studies suggested that TSH-R is a major autoantigen in TAO, the data are not conclusive at present. The recent development of animal models of Graves disease with associated ophthalmopathy is of great importance in helping to establish the exact role of TSH-R in TAO and it is hoped will help to understand the complex pathogenesis of this disease.

ADDISON DISEASE

When Thomas Addison first described primary adrenocortical failure in 1855, the main cause was tuberculosis, and yet two of the 11 original cases were probably autoimmune in origin. In developed countries, the most common cause of Addison disease is now autoimmunity, accounting for around 80% of cases. The prevalence is between 30 and 60 per million population, but this may underestimate the current frequency as a result of either a true increase in prevalence or improved diagnosis (175). In about half of the autoimmune cases, the disease occurs in isolation, but in the remainder there is an associated autoimmune disorder, often as part of one of the two autoimmune polyglandular syndromes (APS) (Table 49.6 and Table 49.7). These syndromes are discussed in detail in the next section; the present section focuses on autoimmune Addison disease, but it considers, where appropriate, the different autoimmune features associated with APS.

Features	Frequency (%)
Major components	
Chronic mucocutaneous candidiasis	80–100
Hypoparathyroidism	80
Addison disease	70
Other autoimmune features	
Gonadal failure	10 men, 60 women
Vitiligo	30
Alopecia	30
Malabsorption	20
Insulin-dependent diabetes mellitus	10
Hypothyroidism	10
Pernicious anemia	10
Chronic active hepatitis	10
Ectodermal features	
Nail dystrophy	50
Keratopathy	40
Tympanic membrane calcification	30

TABLE 49.6. Clinical Features of Autoimmune Polyglandular Syndrome Type 1

Major endocrinopathies
Autoimmune thyroid disease (Graves disease, primary myxedema, Hashimoto thyroiditis, postpartum thyroiditis)
Type 1 diabetes mellitus
Addison's disease
Other endocrinopathies
Premature ovarian failure
Lymphocytic hypophysitis
Lymphocytic infundibuloneurohypophysitis
Isolated ACTH deficiency
Other autoimmune features
Vitiligo
Alopecia or leucotrichia
Pernicious anemia
Celiac disease/dermatitis herpetiformis
Myasthenia gravis
Serositis
Chronic autoimmune hepatitis/primary biliary cirrhosis

ACTH: adrenocorticotrophic hormone.

TABLE 49.7. Clinical Features of Autoimmune Polyglandular Syndrome Type 2

Epidemiology

The average age of onset is 30 to 35 years, and there is a female excess of twofold to threefold, although this sex difference is absent in the first two decades of life. The association of Addison disease with other autoimmune disorders in families suggests genetic susceptibility but also would be compatible with shared environmental factors; however, to date, no environmental factors have been identified that confer susceptibility. The major genetic association is with the HLA-DR3 specificity, both in the spontaneous form and that in APS type 2 (the genetic basis of APS type 1 is considered subsequently), with a relative risk of about 3.5 (176). Some studies found an association between HLA-DR4 and Addison disease, but this association may be a distortion produced by a linked susceptibility to type 1 diabetes mellitus; subtypes DRB1*0401 and DRB1*0402 are associated with limited progression to adrenal failure in predisposed subjects compared with DRB1*0404, which confers a high risk (177).

Certain polymorphisms in the CTLA-4 gene also are associated with Addison disease in some populations, implying a relatively modest effect on susceptibility akin to that found in autoimmune thyroid disease and type 1 diabetes mellitus (178). It is unknown whether these associations reflect differences in CTLA-4 expression or function.

Clinical Presentation

SIGNS AND SYMPTOMS

Addison disease has a range of presentations that depend on the degree of adrenal failure (Table 49.8). The most dramatic symptoms and signs occur in adrenal crisis, with dehydration, severe hypotension, vomiting, diarrhea, coma, and, if untreated, death. Such crises are usually precipitated by an acute illness, increasing the demand for glucocorticoids, against the background of chronic adrenal impairment. More commonly, there is an insidious onset over months or even years; the autoimmune process generally destroys the adrenal cortex, leading to impaired glucocorticoid and mineralocorticoid production (179). In women, the failure of androgen secretion may result in loss of body hair and impaired libido, but in men the testicular contribution to androgen production ensures that these effects are not seen. The main presenting features with chronic destruction of the adrenal cortex are lethargy, fatigue, weakness, postural hypotension (sometimes with syncope), and gastrointestinal disturbance. Salt craving may be prominent. Subclinical Addison disease sometimes is detected when adrenal antibody testing reveals the presence of an autoimmune process and further investigation (see later) reveals mild impairment of adrenal reserve (180).

Symptoms and Signs	Frequency (%)
Symptoms	
Weakness, fatigue	95
Anorexia	95
Nausea	90
Vomiting	70
Abdominal pain	30
Diarrhea	15
Postural symptoms	10
Myalgia, arthralgia	10
Signs	
Weight loss	95
Increased pigmentation	95
Hypotension	90
Associated autoimmune disease	25

TABLE 49.8. Main Signs and Symptoms in Addison Disease

PHYSICAL FINDINGS

Increased pigmentation is distinctive and is due to the rise in pituitary secretion of adrenocorticotrophic hormone (ACTH) as a result of impaired negative feedback from low cortisol levels. It is seen particularly in sun-exposed skin or areas subject to mild trauma, such as the elbows and knuckles. It also can occur in the mouth as a result of trauma from the teeth. The blood pressure is usually low, with a postural drop. Evidence of an associated autoimmune disorder, particularly vitiligo, occurs in 10% to 25% of cases.

LABORATORY TESTS

The diagnosis may be suggested by the presence of hyponatremia and hyperkalemia, the typical electrolyte disturbances of mineralocorticoid deficiency (hypoaldosteronism), which are present in up to 90% of patients. Hypercalcemia, normochronic, normocytic anemia, and eosinophilia also can occur. Random serum cortisol measurements are insufficient to make the diagnosis because there is a pronounced diurnal variation and an overlap with values in healthy individuals. Nonetheless, if a cortisol value lower than 100 nmol/L is obtained, this finding is virtually diagnostic, as is a value within the reference range in a stressed individual, such as a patient who has features of adrenal crisis.

In most cases, the diagnosis is confirmed by demonstrating a failure of serum cortisol to rise adequately (normal response, >550 nmol/L) 30 to 60 minutes after intramuscular or intravenous synacthen (synthetic ACTH). Because this test simply confirms adrenocortical failure, which could be primary or secondary to a hypothalamic or pituitary problem, the next step is to demonstrate that there is intrinsic adrenal disease, either by measuring ACTH and finding an elevated level or by performing a prolonged synacthen test. After continuous adrenal stimulation with ACTH, usually over 5 days, cortisol production is initiated in secondary adrenal failure but not in Addison disease.

Once the diagnosis of Addison disease is made, the cause must be established. An autoimmune cause is suggested by the presence of other autoimmune disorders and is confirmed by detecting adrenal antibodies (see later). Depending on the test used, 70% to 90% of patients with autoimmune Addison disease will be identified (181,182). Adrenal antibodies may disappear over an extended period.

PATHOLOGY

The adrenal glands in end-stage Addison disease are atrophic, and the cortex is replaced by fibrous tissue. When the disease is evolving, there is a variable but widespread mononuclear infiltrate of lymphocytes, plasma cells, and macrophages in much greater number than in the normal adrenal gland: lymphocytic infiltration of healthy adrenals increases with age, and there may be expression of MHC class II molecules on 10% to 20% of adrenocortical cells under normal circumstances. The distinctive layers of the adrenal cortex (the zona glomerulosa, zona fasciculata, and zona reticularis) disappear in Addison disease as the steroidogenic tissue is destroyed. Further pathologic analysis, including immunohistochemical characterization of the infiltrating lymphocytes, has not been undertaken because access to adrenal tissue in the active phase of disease is impossible during life, and mortality rates have declined markedly with improved recognition and treatment.

Treatment

Immunologic intervention is not indicated because properly monitored hormone replacement restores normal health and life span with little or no adverse effect (179). Hydrocortisone is given in doses of 10 mg first thing in the morning and then 5 to 10 mg at lunchtime and late afternoon. Mineralocorticoid replacement is provided by fludrocortisone, 50 to 200 µg daily. Women may benefit from dehydroepiandrosterone, which improves well-being and sexuality, although long-term studies have not yet been performed (183). Adrenal crisis is an emergency that requires intravenous saline and hydrocortisone and supportive measures; treatment must commence without delay on clinical suspicion of the diagnosis, biochemical confirmation being retrospective.

Immunopathology

HUMORAL RESPONSES

Autoantibodies to the adrenal cortex in Addison disease were first identified by complement fixation and immunofluorescence; with these techniques, about 60% of

patients were positive (184). About 25% of women and 5% of men with autoimmune Addison disease have antibodies that react not only with adrenocortical cells by immunofluorescence but also with steroidogenic cells in testis, ovary, or placental trophoblast, and this subset of antibodies was identified as being against “steroid cells.” In women with Addison disease, a strong correlation exists between the presence of steroid cell antibodies and premature ovarian failure, defined as menopause below the age of 40. The obvious implication is that autoreactivity against shared antigen(s) in the adrenal and ovary leads to both conditions, although it remains unexplained why so few men with steroid cell antibodies develop testicular failure.

Molecular approaches have clarified the nature of the adrenal and steroid cell antibody reactivities in Addison disease. Initially, the cytochrome P450 enzyme, 17a-hydroxylase (P450c17), was identified as an autoantigen when a fetal adrenal library was screened with sera from a patient with type 1 APS (185), but another adrenal autoantigen, 21-hydroxylase (P450c21), was quickly recognized thereafter (186). Antibodies to side-chain clearing enzyme (P450scc) were found in another subset of patients (187). Although there was initial confusion over this plethora of autoantigens, it is now clear that the key autoantigen in Addison disease is P450c21, which is specific to the adrenal, whereas antibodies to P450c17 and P450scc correspond to the steroid cell antibodies previously identified by immunofluorescence (181,188,189). Because steroid cell antibodies are more frequent in Addison disease associated with type 1 APS than in isolated or APS type 2-associated disease, P450c17 and P450scc are most frequent in APS type 1 (Table 49.9).

	Antibody reactivity (%)		
	P450c21	P450c17	P450scc
Isolated Addison disease or with APS type 2	80–100	<10	<10
Addison disease in APS type 1	60–70	40–50	40–50
APS type 1 without Addison disease	5–10	5–10	5–10
Other endocrinopathies	1–2	<1	<1

APS, autoimmune polyglandular syndrome; P450c17, cytochrome P450 enzyme 17a-hydroxylase; P450c21, adrenal autoantigen 21-hydroxylase; P450scc, side-chain-cleaving enzyme.

TABLE 49.9. Distribution of Autoantibodies against P450 Enzymes in Addison Disease

Other steroidogenic enzymes (11b-hydroxylase, 3b-hydroxysteroid dehydrogenase, aromatase, and adrenodoxin) have been tested for autoreactivity in Addison disease with negative results (189), and the high frequency of P450c21 antibodies in some series (182) indicates that the major autoantigens have been found. An alternative explanation for Addison disease was suggested by the finding, in bioassays, of blocking antibodies to the ACTH receptor in a high proportion of patients (190), but the most probable explanation for these results is the use of only partially purified immunoglobulin preparations. Pure IgGs from Addison patients do not block the ACTH receptor (191).

Epitope mapping studies suggest that P450c21 antibodies bind to at least three different epitopes in the C-terminal part of the enzyme, most likely part of more complex, conformational epitopes (192,193). The most important remaining question is whether antibodies to P450c21 and the other enzymes have a pathogenic role or are simply markers of cell-mediated adrenocortical destruction. The steroidogenic enzymes are intracytoplasmic, and it is difficult to see how antibodies could gain access to their targets *in vivo*. There is certainly no evidence that the inhibitory effects of P450c21 antibodies on enzyme activity and steroid synthesis *in vitro* are manifested *in vivo*, supporting the notion that the antibodies cannot react with the enzymes in living cells (194). Complement-mediated cytotoxicity has been demonstrated using steroid cell antibodies and granulosa cells *in vitro* (195), but much further work is needed using adrenal cells and carefully defined antibodies before a role for these antibodies in pathogenesis can be accepted. It should be noted that pregnancy is uneventful in women with Addison disease and that there is no evidence of adrenal insufficiency in neonates, arguing strongly against a role for adrenal antibodies alone in pathogenesis.

CELLULAR RESPONSES

In common with many other autoimmune disorders, an increased proportion of circulating T cells are activated in Addison disease, as judged by their surface expression of HLA-DR (196). The circulating population of T cells is obviously the only one that is accessible for the purposes of analyzing functional responses in this condition, although it is likely that autoantigen-specific lymphocytes accumulate within the diseased adrenal. T cells from one half of a group of patients with autoimmune Addison disease reacted to a crude adrenal antigen in a migration inhibition assay, such reactivity being absent when controls or patients with tuberculous Addison disease were examined (197).

T-cell proliferation to adrenal antigens also has been demonstrated, but the nature of the autoantigen(s) has not been defined (198). We used P450c21 in proliferation assays but found consistent positive responses only 3 of 18 patients; two additional patients had T cells that proliferated in response to P450c17 (unpublished observations). This low frequency of T-cell reactivity to what is believed to be the key adrenal antigen may be due to patients being studied at a late stage of disease and there being a low frequency of reactive T cells in the circulation. These observations and the analogy provided by autoimmune hypothyroidism strongly suggest that T-cell-mediated responses are involved in Addison disease, but it must be said that final, conclusive proof is not available and that the exact mechanism of adrenocortical cell destruction is unknown.

IMMUNOPATHOGENESIS OF DISEASE

A major breakthrough in the understanding of any autoimmune disease is the delineation of the autoantigens, and it is now clear that P450c21 is a major autoantigen in isolated Addison disease and APS type 2. The same autoantigen is implicated in APS type 1, but in this form of Addison disease, some cases appear to be the result of autoimmunity against P450c17 and P450scc. The latter two enzymes are the targets of steroid cell antibodies and, because of their tissue distribution, steroid cell autoimmunity is associated with premature ovarian failure. It is likely that the occasional occurrence of P450c17 and P450scc antibodies in isolated and APS type 2-associated Addison disease reflects a widening of the autoimmune responses following P450c21 autoreactivity.

Good but indirect evidence of T-cell involvement exists, and, by analogy with other autoimmune endocrinopathies, it seems most likely that adrenocortical destruction is cell-mediated. Whether this is mediated by perforin/granzyme, Fas-induced apoptosis or cytokines is unknown. Direct effects of P450c21 antibodies are unlikely. Previous reports of antibodies against adrenal cell-surface antigens (199) are unconfirmed, but if separate cell-surface autoantigens do exist, these would clearly be targets for complement-mediated or antibody-dependent NK cell-mediated cytotoxicity.

Animal Models

Immunization of monkeys, rabbits, guinea pigs, rats, and mice with adrenal gland extracts and adjuvant induces lymphocytic infiltration of the adrenal gland and adrenal cell antibodies, which may cross-react with ovary (200,201). The disease is presumably T cell-dependent because it is transferable with lymph node-derived cells (202). Evidence of adrenal failure, however, is poorly documented and indirect. Lowering of blood pressure has been reported in immunized rats (203), and hypoglycemia, salt wasting, and reduced plasma corticosterone have been observed in another set of experiments using rats (204). Because it is difficult to produce P450c21 in sufficient quantities, no reports have yet appeared of immunization with this antigen. The approach of cDNA immunization may be one way to circumvent this problem and to establish an animal model of Addison disease that can be investigated using modern biochemical and immunologic techniques.

The only other animal model that has been reported consists of neonatal thymectomy in mice, combined with administration of cyclosporin A (205); thymectomy alone has no effect, although it can induce autoimmune oophoritis, thyroiditis, and gastritis. This model has not been studied in detail, but adrenocortical antibodies and adrenalitis result. Certainly, the results support the involvement of regulatory T cells in preventing adrenal autoimmunity in normal circumstances.

Future Directions

Despite its rarity and the relative ease of hormone replacement, autoimmune Addison disease is important as a serious, and at times life-threatening, disorder that requires lifelong management. Recent observations, which demonstrate that many patients are overtreated with conventional doses of glucocorticoids, point to the inadequacy of current methods to determine individual hormone requirements (206). The short-term benefits of dehydroepiandrosterone replacement in women (183) also warrant more detailed study. Immunologic treatment seems most unlikely to offer any benefits because adrenal destruction is probably too great by the time Addison disease is recognized, but conceivably it may have a role in subclinical disease. The question then is whether screening is justified. The low frequency (~ 1%) of adrenal cell antibodies in patients with another autoimmune endocrinopathy suggests that this will not be cost-effective in the near future, but combined approaches

with screening for genetic susceptibility may identify particularly high-risk patients (177).

Any environmental factors leading to Addison disease remain unidentified, although an effect is suggested by the possible rise in disease frequency (175). Developments in our understanding of the immunogenetic contribution to Addison disease are most likely to come from the characterization of candidate genes from studies in the associated endocrinopathies, but this approach may leave untouched the question of why the adrenal cortex (rather than, say, thyroid or beta cell) is targeted. The development of new animal models is also a pressing need because this approach will produce fundamental insights into disease pathogenesis, particularly with regard to the involvement of antibodies and the mechanisms behind the production of cross-reactive autoimmunity to the ovary.

AUTOIMMUNE POLYGLANDULAR SYNDROMES

The main clinical features of APS types 1 and 2 are listed in Table 49.6 and Table 49.7, and these syndromes are discussed in more detail in this section. APS type 2 is also known as *Schmidt syndrome,* but many patients have only one of the three major components (autoimmune thyroid disease, type 1 diabetes mellitus, Addison disease) originally described in this syndrome and have either another presumed autoimmune endocrinopathy (premature ovarian failure, lymphocytic hypophysitis, isolated ACTH deficiency) or another autoimmune disorder (Table 49.7). Sometimes patients with autoimmune thyroid disease plus another endocrinopathy are described as having type 3 APS, but this term has not become established. The mechanism behind these nonclassic associations is probably the same as that underlying APS type 2, namely, shared environmental or genetic susceptibility factors, although there have been attempts to implicate shared sequence homologies in the different target antigens as a cause (207). This latter mechanism has no experimental support. The nonendocrine components of APS type 2 are detailed elsewhere in this book, and this chapter concludes with a brief review of the autoimmune basis for premature ovarian failure and autoimmune hypophysitis.

Type 1

This rare autosomal recessive condition is also known as *Blizzard syndrome* and *autoimmune polyendocrinopathy-candidiasis-ectodermal dysplasia* (APECED). There are clusters of APS type 1 in Finland and Sardinia. The syndrome is caused by one of at least nine identified mutations in the autoimmune regulator (AIRE) gene on chromosome 21 (208,209). This gene encodes a nuclear protein with two zinc finger domains that is expressed in thymus, lymph nodes, and fetal liver. Mutations presumably affect T-cell development, but the exact basis for the disease will become apparent only from further studies, including knockout mice, currently being developed. The phenotype varies greatly, and so far there is no clear correlation with genotype.

Clinically, the disease pattern is usually the appearance of chronic mucocutaneous candidiasis in early life, followed by hypoparathyroidism in infancy or childhood, with Addison disease occurring 5 to 10 years later (210). If unrecognized, these endocrinopathies can be fatal. Patients may be more prone to infection in childhood, and fulminant hepatic failure is an important cause of death later in life. Malabsorption is another clinical feature that can cause difficulties in management because of erratic absorption of drugs.

The pattern of autoantibodies against the adrenal cortex in APS type 1 with Addison disease is wider than in other, more common forms of the disease (Table 49.8). The frequent occurrence of P450c17 and P450scc autoreactivity is thought to underlie the gonadal failure that is often found in both sexes. The pathogenesis of the hypoparathyroidism is unclear. Antibodies against the calcium-sensing receptor have been identified in 6 of 17 APS type 1 patients with hypoparathyroidism, but these had no functional effect on receptor function (211). So far, these observations have not been repeated in another group of patients. The cytochrome enzyme P450 1A2 is a hepatic autoantigen for antibodies in APS type 1 and is associated with hepatitis (212). Autoantibodies to glutamic acid decarboxylase (GAD) occur in type 1 APS patients with diabetes mellitus as they do in the far more common sporadic disorder, but in some APS type 1 diabetic patients, these antibodies are absent, and, instead, antibodies to aromatic L-amino acid decarboxylase occur (213). It is unclear whether these antibodies are linked directly to the development of diabetes; their presence in APS type 1 is also associated with the presence of hepatitis and vitiligo. The malabsorption and diarrhea that affect about one fourth of patients with APS type 1 are associated with the presence of antibodies to tryptophan hydroxylase and the disappearance of enterchromaffin cells from the intestinal mucosa (214).

The exact basis for the tissue damage and for the associated ectodermal features in APS type 1 remains unclear. The wide spectrum of antibodies is striking and has led to identification of the likely target autoantigens, but a pathogenic role for these antibodies has not been established. A recent case report demonstrated some improvement in exocrine pancreatic failure, alopecia, and keratoconjunctivitis when cyclosporin A was given to a patient with APS type 1 (215), suggesting the possibility of using immunomodulation to treat this syndrome in the future.

Type 2

The prevalence of APS type 2 is not established; surveys have concentrated on taking one of the index disorders and determining the frequency of the others. For instance, about 6% of patients with type 1 diabetes mellitus have autoimmune thyroid disease and 0.1% have Addison disease (216). In a long-term survey of Addison disease, 30% had clinically evident autoimmune thyroid disease, 5% had subclinical hypothyroidism, and 6% had type 1 diabetes mellitus (217). It is therefore clear that APS type 2 is much more common than type 1, and, of course, point prevalence studies such as those quoted do not take full account of later onset of an associated disease. In turn, this implies that clinicians should be vigilant for the development of APS type 2 in any patient with an index disorder and most would offer patients with Addison disease annual screening, at least for thyroid disease. The place of regular screening in the other two index disorders is uncertain except that pregnant women with type 1 diabetes mellitus should be offered antenatal screening because of the high frequency of postpartum thyroiditis in these patients (218).

There is nothing unique clinically or immunologically in the individual disease components in a patient with APS type 2. The syndrome is inherited as an autosomal dominant disorder with incomplete penetrance and variable disease expression in different family members. Affected siblings share one or both HLA haplotypes in 95% of cases. The HLA-A1, B8, DR3 haplotype shows a stronger association with APS type 2 than with the individual disease components, and HLA-DR4 is associated with type 1 diabetes in the usual way.

OTHER ENDOCRINOPATHIES

Premature Ovarian Failure

Premature ovarian failure (POF) usually is defined as the onset of menopause before the age of 40; 0.3% to 1% of women are affected. As well as its effects on fertility, POF leads to early onset osteoporosis and an increased risk of cardiovascular disease resulting from the loss of estrogens. Occasional cases reverse spontaneously, and uncontrolled trials of glucocorticoids suggest possible benefit, but further work is needed in this area (219). For most women, hormone-replacement therapy is given.

Only a small proportion of women with POF have Addison disease; in these cases, an autoimmune basis for the ovarian disorder is now established, as discussed already, even if the exact pathogenic mechanisms are uncertain. More than 95% of women with POF, however, do not have Addison disease and, whereas other factors (smoking, cytotoxic drugs, irradiation, cytogenetic abnormalities, and metabolic disorders) can be identified in some of these, many women have so-called idiopathic POF. An autoimmune basis for idiopathic POF has been repeatedly suggested, but the evidence for this is, at best, incomplete and often circumstantial (220,221).

Between 9% and 38% of affected women have an associated autoimmune disorder, particularly autoimmune thyroid disease, suggesting that POF may be part of an APS-like syndrome, but it is also possible that the disturbed sex hormone milieu in POF is responsible for an increased frequency of autoimmunity. There is no significant HLA association with idiopathic POF, and animal models of oophoritis do not closely resemble the human counterpart (220,222). Antibodies against poorly characterized ovarian antigens have been found in about 50% of women in various studies (although there is considerable variation), but these antibodies are not all specific, being found in POF that clearly has a non-autoimmune basis. Antibodies to 3b-hydroxysteroid dehydrogenase, a steroidogenic enzyme, recently were found in 20% of patients with idiopathic POF, suggesting that this is a novel ovarian autoantigen (223), but it is difficult to see why steroid cell antibody positivity by immunofluorescence is generally rare in idiopathic POF. Moreover, 23% of patients with diabetes had antibody reactivity to this enzyme. We have been unable to confirm these results using an *in vitro* translation assay that demonstrated that the frequency of 3b-hydroxysteroid antibodies was only 2% in POF (Peterson et al., submitted for publication). Thus, a small proportion of women with idiopathic POF may have antibodies against this enzyme, but even then we have no proof that such autoreactivity is important in pathogenesis.

Autoimmune Pituitary Disease

Pituitary autoimmunity is rare and takes at least three forms (224). Lymphocytic hypophysitis usually presents as a pituitary mass with hormonal disturbance, in two thirds of cases occurring during or after pregnancy. About 25% of patients have an associated autoimmune disease, especially thyroiditis. Detailed systematic analysis of the immunologic features has not been performed because the condition is so rare, and access to the pituitary for research purposes is limited. Antibodies to the pituitary are detected by immunofluorescence infrequently, but reactivity to 49-kd and 40-kd pituitary proteins in immunoblots has been reported in 50% to 70% of patients (225). A second likely autoimmune disorder of the pituitary is the rare isolated deficiency of ACTH. Almost all these patients have evidence of thyroid

autoimmunity (226). Finally, lymphocytic infundibulo-neurohypophysitis may be a rare cause of diabetes insipidus (227).

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50 IMMUNOLOGIC RENAL DISEASE

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The kidney is a frequent target of humoral and cellular immune responses and is highly susceptible to immune-mediated damage. A heterogeneous group of human and experimental nephritides that have diverse clinical and pathologic phenotypes is characterized by immunoglobulin (Ig) deposition and mononuclear cell infiltration of the glomerulus and renal interstitium. The development and progression of many of these entities can be blocked by immunosuppressive agents, thus providing further evidence of direct immunologic involvement. This propensity for immune injury and mediator localization may be related to the specialized excretory function of the kidney. The extensive blood flow, daily filtration of an estimated 150 to 180 L of plasma across an open (nondiaphragmatic) fenestrated endothelium, and reabsorptive and endocytic functions continuously expose the glomerular capillary wall, mesangium, and downstream tubular epithelium to circulating antigens, soluble mediators, Ig, and lymphocytes. Notably, with rare exceptions, our understanding of the immunopathogenesis of human renal immune injury remains speculative, limited in part by the restricted availability of human renal tissue and patient pedigrees, uncertain correlation between peripheral blood sampling and processes in the kidney, and the complex genetic heterogeneity of human populations. Fortunately, a growing cadre of spontaneous and induced experimental models shares many clinical and histopathologic characteristics with human disease. These animal surrogates have proven highly instructive and continue to yield important insights into human nephritis (1,2).

Pioneering work in early experimental models of glomerular injury, most notably nephrotoxic serum nephritis (NSN) and acute and chronic serum sickness, implicated intrinsic glomerular epitopes as well as circulating foreign proteins as antigenic targets in nephritogenic humoral responses (3,4 and 5). These models, in conjunction with examination of human renal biopsy specimens using immunofluorescence and electron microscopic techniques introduced in the 1950s, revealed considerable morphologic heterogeneity in glomerular disease, suggesting that diverse pathogenic pathways are operative. Only after meticulous dissection of the mechanism of immune deposit (ID) formation in the rat Heymann nephritis (HN) model of membranous nephropathy (6) was *in situ* engagement of autoantigen recognized as a dominant mechanism of renal Ig deposition, accounting for granular as well as linear IF patterns. In the past two decades, it has become clear that a plethora of structurally diverse antigens distributed throughout the kidney, including both protein and nonprotein soluble antigens, basement membrane (BM) components, and cell-surface molecules, can serve as targets of renal immune injury. Appreciation of the importance of cellular immunity in initiating and mediating renal injury lagged behind these discoveries but ultimately emerged with the development of animal models of tubulointerstitial nephritis (TIN) and of immunologic techniques to characterize T-cell subsets. This field was further advanced by parallel investigations in renal transplantation and elucidation of the immunobiology of acute rejection mediated by infiltrating sensitized T cells. With this came evidence that resident renal parenchymal cells participate in antigen presentation and local amplification of immune responses and inflammation.

During the past decade, an explosion of investigation focused on basic mechanisms of tissue injury and repair. These studies led to the isolation and characterization of a broad array of downstream mediators and revealed an intricate network of soluble and cell-bound molecules derived from resident renal and infiltrating cells that facilitate cell–cell and cell–matrix communication, modulate injury, and ultimately promote disease resolution or progression to fibrosis. Local host factors (i.e., parenchymal cell expression of Fc receptors and complement (C) regulatory proteins) were demonstrated to influence profoundly the renal response to immune attack and inflammation. Elucidation of this network also provided a conceptual framework for the rational design of an array of novel therapeutic interventions. It is of note that, in contrast to the substantial insight gained regarding the nature of disease mediators, our understanding of events that initiate nephritogenic immune responses remains rudimentary and conjectural. Nonetheless, increasing availability of novel mutant mouse lineages and refined molecular and immunologic techniques are beginning to shed light on these processes, including the interplay of environmental factors and genetic susceptibility that induces nephropathic immunity.

INITIATION OF NEPHROPATHIC IMMUNE RESPONSES

Humoral or cellular immune effectors that target a wide variety of foreign or self-antigens (Table 50.1) can mediate renal injury. These nephritogenic effectors may arise in the course of a protective immune response mounted against a microbial pathogen as part of an allergic response to normally innocuous foreign antigen (e.g. antibiotic) or during an autoimmune response targeting normal or altered self-antigen (Table 50.2). Nephropathic Igs are also occasionally inadvertently produced as a by-product of unregulated monoclonal lymphocyte expansion. What is not clear in most cases, however, is the relationship between immunizing and tissue target antigen because it appears that they are distinct, at least in some situations. This issue is particularly germane to understanding the relative roles of autoimmune versus antiforeign responses in mediating renal injury and the mechanisms by which pathogenic Ig localizes to the kidney [i.e., *in situ* versus circulating immune complex (CIC) deposition].

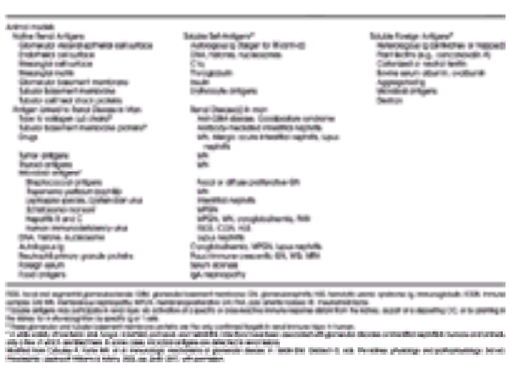


TABLE 50.1. Antigens Implicated in Immunologically Mediated Renal Diseases

TABLE 50.2. Development of Nephropathic Immune Responses

Observations over the past two decades in animal models of renal immune injury (Table 50.3) precipitated a paradigm shift that now recognizes intrinsic kidney self-antigen as a primary target of nephritogenic responses (7). Three of the most extensively studied animal models involve Ig interactions with native glomerular proteins. Pioneering studies exploited active immunization of animals with heterologous or autologous kidney extracts enriched for different renal antigens to induce nephritis marked by linear glomerular BM (GBM) Ig deposits (e.g., autoimmune anti-GBM Ig-mediated disease) or disease dominated by granular subepithelial deposits (e.g., active HN) (4,8). Subsequent investigations ultimately determined that these pathogenic Igs arose as induced autoimmune responses to GBM a3(IV) collagen and to glomerular epithelial cell membrane antigens, respectively (6,9). A third model is induced by passive administration of Ig against a rat mesangial cell phosphatidylinositol-anchored glycoprotein, termed *Thy-1 antigen* (10). Numerous additional renal or nonrenal self-antigens encompassing a diverse group with regard to molecular structure and tissue distribution have since been implicated in induced and spontaneous models of renal-restricted and systemic autoimmune nephritis (Table 50.1) and provide considerable insight into potential targets and mechanisms in humans.

TABLE 50.3. Particularly Instructive Experimental Models of Renal Immune Injury

Despite the litany of self-antigens engaged in renal injury, our understanding of the events that initiate and regulate autoimmune responses remains rudimentary. Insight into human autoimmune nephritis is limited in particular by a lack of information regarding the disease-relevant antigens. This issue will remain difficult to resolve because it will require careful and detailed analysis of Ig and T cells recovered intact from affected kidneys early in the disease course (11). Models of induced nephritis that depend on passive transfer of differentiated effectors or induction of an autologous Ig response to planted foreign antigen (e.g., the heterologous and autologous phases of NSN) (Table 50.3) provide minimal insight into the more proximal events that promote autoimmunity; nonetheless, they are excellent tools with which to study downstream mediator pathways (12). Models induced by active immunization with autoantigen-in-adjuvant are instructive in elucidating genetic factors that control disease and strain susceptibility. The relevance of these last models to disease initiation in humans has yet to be determined because they are dependent on adjuvant, a potent activator of innate immunity, to overcome immunoregulatory mechanisms that normally prevent the emergence of autoreactivity. Conversely, rodent models of spontaneous autoimmune nephritis have provided important insights into the proximal events that initiate nephritogenic responses (reviewed in 13). The common murine lupus strains [e.g., MRL/lpr, New Zealand black (NZB), BXSB] and several of the recent mutant variants or novel lineages are particularly useful for dissecting complex immunologic interactions and genetic mapping of nephritis susceptibility loci. Insight into the basis of renal-restricted disease also is extrapolated in part from observations in other models of organ-restricted autoimmunity.

It is likely that distinct disturbances in immunoregulation predominate in kidney-limited (i.e., anti-GBM disease, HN) versus systemic (e.g., lupus nephritis) autoimmune diseases (Table 50.2). Renal-restricted localization of immune effectors suggests a targeted immune response requiring activation of a specific population of autoreactive lymphocytes (via specific immunization with self- or cross-reactive foreign antigen) as well as effector localization to the kidney in response to appropriate attractants and recognition of renal-restricted ligand. Lymphocyte activation may occur in distant lymphoid tissues or locally by antigen presented in an immunogenic manner by resident macrophages, dendritic, or renal parenchymal cells. The requirement for both lymphocyte activation and recognition of locally expressed antigen is demonstrated in the rat model of induced antitubular basement membrane (TBM) TIN. The target TBM antigen is polymorphic, and in rat strains in which renal antigen is either lacking or inaccessible, nephritis does not develop despite production of a vigorous peripheral anti-TBM immune response (14). Shared kidney and microbial epitopes may provide the basis for renal localization of effectors in certain infection-associated renal diseases. Alternatively, normally inaccessible antigens may be exposed or modified by local injury or inflammation induced by infection or actions of industrial chemicals or pharmaceuticals (1), as proposed for exposure of normally privileged a3(IV) collagen epitopes in Goodpasture syndrome (GPS) (15).

The T-cell repertoire against cryptic self may be particularly important in renal-restricted disease. Normally, cryptic self-peptides are not processed or presented and thus do not tolerize reactive T cells. Lymphocytes reactive to dormant antigens, in which expression is restricted to certain developmental or differentiation states, similarly may escape tolerance induction. If conditions arise to expose previously hidden kidney self-antigen and permit its presentation with appropriate costimulation, however, normally ignorant but immunologically competent kidney-reactive T cells will be activated. This latter phenomenon is perhaps most elegantly illustrated in experimental cadmium-induced TIN (16). Cadmium-induced expression of heat shock protein-70 (HSP-70, a stress-induced neoantigen) in murine renal tubular cells recruits nontolerant cytotoxic HSP-70-autoreactive T cells to the renal interstitium. Antigen-nonspecific activation of immunologically ignorant or anergic renal-specific T cells by T-cell superantigens or bystander help are also possible but have not yet been documented as a cause of nephritis. Finally, the possibility is raised that epitope spreading during disease induction may contribute to the final nephritic phenotype, based on the observation that more severe glomerular injury accompanies the production of a more diverse autoantibody (autoIg) response in experimental anti-GBM disease and rat HN (17,18 and 19).

In contrast, more global defects in immunoregulation that alter lymphocyte activation and tolerance pathways likely contribute to the onset of nephritis in systemic autoimmunity. Genetic studies in lupus-prone mice indicated that the products of multiple susceptibility genes must interact to induce full-blown nephritis and implicate generalized defects in immune regulation that unleash autoreactivity to multiple diverse self-antigens (see later). These studies also indicated that disparate immune abnormalities can lead to a similar nephritis phenotype (reviewed in 13).

Considerable experimental and epidemiologic evidence indicates that the kidney is also frequently involved in immune responses induced by foreign antigen or exposure to exogenous agents (Table 50.1 and Table 50.2). Microbial or drug antigen is identified within renal deposits or infiltrates in some affected humans and is taken as presumptive evidence of a direct role in pathogenesis. The immunogenicity of drugs, many of which are small chemicals and poorly immunogenic in isolation, may be enhanced by *in vivo* conjugation with host proteins or direct binding to major histocompatibility complex (MHC) molecules, thus promoting antigen processing and recruitment of T-cell help and, in some cases, creating neoself-antigen as target of the nephritogenic response (20). The induced T cells, and occasionally Ig, localize to the kidney, presumably by recognition of drug “trapped” in the kidney during metabolism and excretion. Direct infection of renal cells or renal trapping of microbial antigens may similarly target antimicrobial immune effectors to the kidney (21,22). It is notable, however, that many infections [e.g., hepatitis C virus (HCV), human immunodeficiency virus (HIV), malaria, staphylococcus] are accompanied by activation of autoreactive lymphocytes (23), and a variety of pharmaceutical and

chemical agents can induce systemic autoimmunity and nephritis in susceptible individuals (Table 50.2) (24). Therefore, it remains unclear to what degree renal immune injury arising in association with exogenous agents is antiforeign versus autoimmune in origin.

IMMUNOGENETICS OF RENAL IMMUNE INJURY

Familial predisposition and reproducible differences in strain susceptibility clearly point to an important contribution of heredity in the development of immune nephritis (25). This is not surprising because selective breeding experiments in animals indicated strong genetic influences on the type and magnitude of immune and inflammatory responses. Immunogenetic studies in human nephritis are difficult to perform and interpret, however, because of problems of an outbred population, racial and ethnic differences in genetic makeup, heterogeneity in disease phenotypes (pathology, stage, and severity), uncertainty regarding pathogenesis, and the rarity of many nephritides. Nonetheless, numerous studies of human leukocyte antigen (HLA) allele association have been performed (25), and a strong association of HLA class II alleles with disease susceptibility has been reported for several disorders (Table 50.4). Isolated studies report MHC associations with disease severity or prognosis or disease associations with other immune-relevant genes with functional polymorphisms [e.g., C receptors, Fcg receptors, IgGm allotypes, Ig switch regions, T-cell receptor (TCR) constant a-chain region, tumor necrosis factor (TNF) production]. Results are often difficult to interpret, inconsistent, or lack independent confirmation.

	Population	
	Caucasian	Japanese
Disease	Class II specificity	
Goodpasture syndrome	DR2(w15)	
Membranous nephropathy	DR3	DR2(w15)
IgA nephropathy	None	DR4
Minimal change nephrotic syndrome	DR7	DR8
SLE	DR3, DR2	

Modified from Rees AJ. Immunogenetics of renal disease. In: Neilson EG, Couser WG, eds. Immunologic renal diseases. Philadelphia: Lippincott-Raven, 1997:99–125, with permission

TABLE 50.4. Major Histocompatibility Complex Associations in Human Glomerulonephritis

The most rigorous studies in humans have necessarily been performed in anti-GBM Ig-mediated disease, the only human renal disease with a relatively well-characterized pathogenesis in which the renal antigen is known. Four series from the United States, United Kingdom, Australia, and France found that more than 80% of white patients with GPS carry a DR2 haplotype bearing DRB1*1501 and DRB5*0101 alleles, which encode b-chains of HLA class II molecules DR15b and DR15a, respectively (25). Less strong positive and negative associations have been reported with additional alleles. It was determined that DR15 molecules are capable of binding to many a3(IV) noncollagenous 1 (NC1)-derived peptides; however, most of the naturally presented peptides bind to DR15 with only intermediate affinity (26). Further investigation is needed to elucidate the basis of DR15 susceptibility and to determine the relative roles of intermediate versus high-affinity peptides in the induction of self-tolerance and autoimmunity.

No consistent class II allele associations have been reported in white patients with Ig A (IgA) nephropathy (IgAN) (25), the most common glomerulonephritis worldwide. Disparate results have been reported for antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides. At least six independent studies found an association in patients with Wegener granulomatosis or antiproteinase 3 Ig with the Z allele, associated with relative enzyme deficiency, at the a_1-antitrypsin locus. Most studies in human systemic lupus erythematosus (SLE) confirmed associations with DR3, DR2, C4A, and C4B null and deficiencies of components of the classical C pathway (i.e., non-MHC-linked C1q and MHC-linked C2 and C4) or regulatory proteins (C1 inhibitor, factors H or I) (27). A potential basis for the association between C deficiency and autoimmune susceptibility was suggested by observations in mice indicating a complex role for C in the initiation and regulation of humoral immune responses, including B-cell activation and tolerance induction (28). An association of SLE with FcgR allele FCgR2A is described, and a recent directed linkage analysis of sibpairs with SLE, based on a locus syntenic to murine nephritis susceptibility locus Sle1 (see later), linked human SLE to chromosome 1q41-q42 (29).

Linkage studies and more recent genomewide screening in experimental autoimmune renal diseases in rodents (Table 50.3) concur that nephritis is a complex trait and that both MHC- and non-MHC-linked genes are essential for full expression of disease (25). As noted earlier, in rat anti-TBM Ig TIN, disease susceptibility depends on genetically determined renal expression of the relevant TBM antigen and the ability to mount an anti-TBM response (14). An unknown gene inherited in an autosomal recessive fashion with complete penetrance appears to be responsible for disease in spontaneous autoimmune TIN in kd/kd mice (30) and is currently being mapped.

Extensive genomewide linkage studies in lupus-prone mouse strains (e.g., MRL, NZB F1 hybrids, and BXSB) indicated that murine lupus nephritis is inherited as a complex polygenic trait (reviewed in 27,31). Each strain carries a unique constellation of MHC- and non-MHC-linked susceptibility loci, the products of which confer additive disease risk or protection, with certain gene combinations being particularly potent disease modifiers. No single gene is necessary or sufficient for disease but may contribute to distinct phenotypes and different gene combinations produce similar phenotypes. The position of at least 12 non-MHC susceptibility loci have been mapped in the New Zealand hybrid model. Several loci link to severe nephritis and death, i.e., Sle1/nba2/Lbw7, Sle2/nba1/Lbw2, and Sle3/Nba3/Lbw5/Lrdm1 on chromosmes 1, 4, and 7, respectively. Sle1 and Sle3, loci derived from New Zealand white (NZW) mice that lack severe autoimmunity, mediate lupus nephritis on a C57BL/6 background. Four NZW modifier loci have been identified that suppress nephritis in that strain (32). B6.NZM mice rendered bicongenic for loci Sle1 and Sle3 develop a polyreactive autoIg response and highly penetrant GN not observed in the monocongenic lines (33). Nephritis susceptibility similarly links to both MHC- and non-MHC-linked loci in induced lupus models (34). Candidate susceptibility genes include those involved in various apoptosis, cell survival, immune response, and lymphocyte signaling, selection, and adhesion pathways.

It is of note that five murine SLE susceptibility genes have been identified to date: MHC class II alleles and Src-homology 2-domain phosphatase-1 (SHP-1), molecules involved in antigen presentation and lymphocyte signaling, respectively, and three spontaneously arising autoimmunity accelerator genes, *Yaa*, *lpr*, and *gld*. The function of *Yaa* is not yet determined; the *lpr* and *gld* mutations encode nonfunctional Fas antigen and Fas ligand proteins, respectively, leading to defects in apoptosis and peripheral tolerance. Notably, the congenic MRL^{++} background is responsible for autoimmune kidney disease (35); a superimposed Fas mutation accelerates disease onset and severity. The Fas mutation expressed on the non-autoimmune C3H or C57Bl/6 background does not cause autoimmune renal damage. Lupuslike autoIg and nephritis are also reported in mice with targeted disruptions of genes encoding lyn, a Src protein-tyrosine kinase, PD-1, an inhibitory membrane protein on activated lymphocytes and monocytes, or the immunosuppressive cytokine transforming growth factor (TGF)-b1, and in mice transgenic for Bcl-2 or the cytokine interferon (IFN)-g under control of the involucrin promoter (reviewed in 13). The impact of genetic susceptibility on the expression of nephritogenic autoimmunity has been examined directly using mice rendered transgenic for Ig reactive with nephritis-associated autoantigens (DNA and BM laminin). Transgene-expressing autoreactive B cells developing in a non-autoimmune background appear to be regulated, whereas at least some specificities escape regulation in autoimmune-prone MRL mice (36,37 and 38).

ENVIRONMENTAL CONTRIBUTIONS TO NEPHROPATHIC IMMUNE RESPONSES

Synergy between inherited and environmental susceptibility is suggested by reports of monozygotic twin sets discordant for disease in the various nephritides and SLE. Experimental models suggest that infectious agents, chemicals, and pharmaceuticals may induce nephritis by diverse mechanisms (Table 50.2), many of which lead to acquired defects in immune tolerance. Inducible heat shock proteins, in particular, are highly immunogenic; they are expressed in the setting of imposed stress (ionizing radiation, heavy metal toxicity, infection), epidemiologically associated with development of TIN in humans and targets of immune attack in rodent TIN (16,39). Exposure of hidden BM epitopes by environmental insults also is implicated in the anti-GBM diseases (1). Procainamide and hydralazine induce systemic autoimmunity in humans and rodents; *in vitro* they inhibit DNA methylation, upregulate T-cell lymphocyte function-associated antigen-1 (LFA-1) expression, and induce T-cell autoreactivity. Adoptive transfer of T cells overexpressing LFA-1 induces lupuslike autoreactivity and GN in recipient mice (40). Heavy metals and allogeneic responses may preferentially induce Th2-type autoreactive $CD4^+$ T cells (reviewed in 24). Mercuric chloride is reported to trigger T-cell interleukin (IL)-4 production directly (41). Notably, however, mercuric chloride induction of nephritis in IL-4-deficient but not IFN-g-deficient mice implicates an IFN-g-dependent mechanism in this model (42). Polyclonal B-cell activation induced by these agents as well as microbial mitogens may activate nontolerant (ignorant) autoreactive B cells or in appropriate concentrations reverse anergy in tolerant B cells. Superimposed specific-antigen immunization, perhaps by nucleosomes, bacterial DNA, or apoptotic cell antigens (reviewed in 43,44 and 45), is implicated in the development of full-blown lupus nephritis. Notably, exacerbation of experimental nephritis by bacterial lipopolysaccharide or adjuvant suggests that microbial products may modulate the effector as well as inductive limb of nephritogenic responses (1). In this regard, synpharyngitic exacerbations of hematuria and renal injury are characteristic of several human GN.

RENAL PARENCHYMAL CELL ANTIGEN PRESENTATION AND LOCAL IMMUNE RESPONSES

Systemic and local immune responses and a variety of nonimmune renal injuries (e.g., acute ischemia, toxins, gentamicin, mercuric chloride) can dramatically induce class II expression on renal cells *in vitro* and *in vivo* (46). Proximal tubular epithelial cells can process and present self-antigen and foreign antigen *in vitro* for recognition by $CD4^+$ T cells (reviewed in 47). In the absence of concomitant upregulation of costimulatory molecules, however, isolated class II expression likely induces tolerance in reactive $CD4^+$ T cells. Antigen-specific nonresponsiveness (*anergy*) is induced in T cells exposed to murine tubular cells transfected with class II antigens alone, and transplantation of kidneys from transgenic mice bearing high levels of class II molecules fails to initiate autoimmune injury (47). Although less readily induced, immune accessory molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), CD40, or B7-1 are expressed on renal tubular cells exposed to IFN-g or other cytokines and in some settings support *in vitro* T-cell proliferation and IL-2 production (47,48). Mesangial cells (49), glomerular visceral epithelial cells (50), and vascular endothelium (51) have inducible antigen-presenting functions, as do resident renal fibroblasts, interstitial dendritic cells, and macrophages. Activated resident renal cells also express a broad array of proinflammatory cytokines, chemokines, and adhesion molecules (52). The relative role of renal parenchymal cells in initiating versus amplifying local immune responses remains unclear.

MECHANISMS OF IMMUNOGLOBULIN LOCALIZATION TO THE KIDNEY

In Situ Binding to Renal Self-Antigen

Elucidation of mechanisms leading to renal Ig deposition, a major feature of glomerular immune disorders, has provided considerable insight into disease pathogenesis (1,2). Observations in two classic animal models, experimental anti-GBM disease and HN (Table 50.3), as well as in human anti-GBM disease/GPS were instrumental in documenting direct binding of autoIg to intrinsic renal antigens as a dominant mechanism of Ig deposition. These models also revealed that the location and nature of the Ig–antigen interaction is an important determinant of disease phenotype because *in situ* deposition can lead to diverse histopathologic manifestations that mimic a variety of human glomerulopathies (12). Most subepithelial deposits are thought to arise by *in situ* mechanisms. The site of IC formation also may be a determinant of the inflammatory response because subepithelial deposits generally do not cause inflammation (7,53).

Experimental anti-GBM disease is induced in susceptible mammals by active immunization with heterologous or autologous GBM-in-adjuvant (Table 50.3) as well as with a variety of other antigenic preparations [alveolar BM, choroid plexus, a3(IV) NC1 dimers, and recombinant a3(IV) collagen] (1,54). Hosts develop characteristic linear GBM IgG and C deposits and severe proliferative GN. Passive transfer of disease using anti-GBM serum or renal eluate Ig from diseased animals or patients confirmed a direct pathogenic role for induced anti-GBM Ig (55,56). A passive form of anti-GBM disease, NSN, also can be induced by administration of heterologous anti-GBM Ig, leading to both an immediate heterologous and a subsequent autologous (anti-heterologous Ig) disease phase. The major pathogenic epitope targeted by renal eluate Ig in both experimental and human anti-GBM disease has been identified as a3(IV)NC1 collagen (9,57), a highly tissue-restricted epitope found predominantly in GBM and alveolar BM. Notably, lung binding by heterologous anti-GBM usually occurs only after lung injury is experimentally induced (oxygen toxicity, gasoline exposure, cytokine administration) (1), suggesting that lung epitopes are less accessible than GBM epitopes to circulating Ig.

Whereas *in situ* binding readily accounts for linear Ig staining of the GBM, most human Ig-mediated glomerular diseases are characterized by discontinuous granular deposition of Ig. Dissection of pathogenesis in the rat HN model of membranous nephropathy revealed that *in situ* mechanisms also can induce this pattern of injury (6). In active HN (8), rats immunized with a suspension of their own kidney in Freund adjuvant develop nephrotic syndrome with a histopathologic picture similar to human membranous nephropathy. The lesion is noninflammatory and is characterized by granular Ig deposits in the glomerular subepithelial region. Disease induction by antigen preparations (termed fraction 1A, or Fx1a) enriched for tubular proteins initially prompted investigators to attribute pathogenesis to deposition of CICs composed of autoIg and tubular antigen. In now classic experiments, however, it was demonstrated that the early histopathologic findings, including granular capillary wall deposits found exclusively in the subepithelial space, could be reproduced by perfusion of isolated rat kidneys with anti-Fx1A Ig (58). It was subsequently shown that Ig eluted from kidneys with active disease binds to glomerular antigens in tissue sections and when infused into isolated kidneys, confirming *in situ* binding to an antigen expressed in the glomerulus.

The major antigen target in HN was subsequently determined to be the HN antigenic complex (HNAC) (59,60) expressed in renal proximal tubular epithelial microvilli and clathrin-coated pits on the glomerular epithelial cell. This multiple ligand endocytic receptor is composed of the 516-kd membrane glycoprotein megalin (initially gp 330), a member of the low-density lipoprotein receptor gene family, and the 44-kd receptor-associated protein, RAP (6). Several non-overlapping pathogenic epitopes were mapped on megalin and RAP using renal eluate Ig. Immunization with intact megalin, RAP, or peptides or administration of heterologous Ig to any component can induce subepithelial IDs in rats (61). IDs are thought to form by circulating anti-HNAC Igs that penetrate the GBM and bind HNAC at the base of the foot processes. ICs are then fairly rapidly shed from the cell surface and attach to the GBM. RAP has a heparin-binding site, which has been proposed as one mechanism by which the ICs bind to the GBM.

It is noteworthy that anti-HNAC Igs, although responsible for immune deposition in HN, are inefficient in inducing proteinuria, the major clinical manifestation in this disease. The crude anti-Fx1A antisera also contains additional autoIgs that recognize unrelated podocyte antigens, including a3b1 integrin receptors and glycolipid, which appear to be responsible for proteinuria and in situ C activation (18). Anti-glycolipid IgG does not form IDs independently but may associate with anti-HNAC deposits. Additional specificities have been reported (1,62), including two, dipeptidylpeptidase IV (DPPIV) and laminin, that also have been reported in experimental lupus nephritis. Although megalin/gp330 is not detected in human glomeruli, human homologs of both megalin and RAP are reported as well as an undefined glomerular epithelial antigen that is cross-reactive with nephritogenic Ig in spontaneous membranous-like GN in rabbits (63). It is not yet clear whether these human proteins are targets for autoIg. Nonetheless, similarities between the experimental and human disease suggest common pathogenic mechanisms.

When directed at a variety of additional intrinsic renal antigens, autoIgs are capable of inducing renal injury or proteinuria in experimental models (Table 50.3). An extensively studied rat model of immune mesangial injury is induced by passive administration of polyclonal antisera or monoclonal antibodies (Mabs) against the Thy-1 antigen expressed on rat (but not other species) mesangial cells (1,10). Administration to rabbits of heterologous Ig reactive with rabbit endothelial angiotensin converting enzyme (ACE) induces granular deposition of IgG on glomerular and arterial endothelium and renal injury. Notably, over several days, repeated injections lead to subepithelial IDs, interpreted as dissociation of subendothelial ICs *in vivo* with redistribution and local reassociation in the subepithelial space (64). Renal artery perfusion with antiendothelial Ig induces thrombotic microangiopathy (reviewed in 65). Injections of Ig specific for collagen IV, laminin, or heparan sulfate proteoglycan induce renal morphologic and functional abnormalities in rodents, and Ig with these specificities are isolated from rodents with spontaneous or mercuric chloride-induced lupuslike nephritis (66,67,68 and 69). Serum reactivity with a variety of renal antigens (glomerular epithelial, mesangial, and endothelial cells) has been reported in selected patients with a variety of nephritides, but their role in disease pathogenesis in humans and specificities of renal-bound Ig remain unknown. The only confirmed autoIg targets in human nephritis are a3(IV) collagen and two TBM glycoproteins, in anti-GBM diseases and certain forms of TIN, respectively (9,70,71).

In Situ *Binding to Planted Antigen*

Glomerular deposition or “trapping” of foreign antigen followed by *in situ* binding by specific Ig has been validated as a mechanism of renal immune injury in numerous experimental models (1,2). Susceptible animals immunized with foreign protein bearing affinity for the glomerulus mount a humoral immune response against the foreign protein; antigen that localizes to the kidney serves as “planted” target for induced circulating Ig. The renal disease phenotype is dependent on multiple factors, including location of the planted antigen, affinity and isotype of the autologous response, engagement of C or Fc receptors, and donor and recipient species and strain. An unambiguous example of planted antigen GN is the autologous phase of NSN. Heterologous (i.e., foreign) anti-GBM Igs induce an initial (heterologous) disease phase mediated by the injected antiserum binding to the GBM and a subsequent autologous phase mediated by induced host anti-Ig binding to deposited (planted) heterologous Ig (72). In a classic variant of this model, rodents injected with a subnephritogenic dose of polyclonal sheep anti-GBM antiserum develop a high-affinity anti-sheep Ig response and florid nephritis. A variety of plant or microbial lectins [concanavalin A (Con-A), hemagglutinin] and cationized antigens [ferritin, IgG, bovine serum antigen (BSA), avidin, myeloperoxidase], including intact ICs, bind to glomerular endothelial cells, polyanionic GBM components or podocytes to deposit in the subendothelial or subepithelial space and serve as planted antigen in experimental models (73,74).

Circulating Immune Complex Deposition

Soluble antigens combine with specific Igs to form CICs that can lodge in the kidney. The prototype CIC nephritis is acute serum sickness induced in rabbits by injection with a single large bolus of BSA. In this model, free BSA does not accumulate in glomeruli; rather, glomerular deposition and GN develop only after a substantial Ig response is established and coincides with the appearance of large cross-linked ICs between days 9 and 10 after BSA injection (5,12). It is difficult to reproduce the severe histopathologic lesions of serum sickness using passive transfer of preformed ICs. Glomerular trapping of ICs in the mesangium and subendothelial space is common, but clinical and morphologic changes are typically mild (75). Systemic or oral immunization with a variety of foreign antigens (dextran, dinitrophenol (DNP)-conjugated BSA, gluten) has met with similarly limited success in inducing florid GN. These observations have lessened enthusiasm for passive entrapment of CIC as the dominant mechanism of renal immune deposition in humans. Chronic serum sickness is induced by daily injection, often for weeks, with native BSA (5,12). The delayed onset of renal lesions (several weeks) has raised the possibility that additional mechanisms (e.g., neoantigen exposure, epitope spreading, auto-antiidiotypic Ig, autoIg) are recruited in disease induction. In both models, lesion morphology is dependent on the magnitude and nature of the immune response, including antigen dose, isotype, and epitope specificity; physicochemical properties (antigen:Ig ratio and Ig avidity, which in turn determine IC size, charge, and solubility); as well as nonimmunologic factors, such as IC clearance and renal hemodynamic parameters. CIC deposition based on a particular physicochemical

affinity of the CIC for glomerular structures also has been proposed (e.g., binding of IgA fibronectin complexes to collagen) (76).

Mechanisms of Immunoglobulin Deposition in SLE Nephritis

Lupus nephritis develops as part of a spontaneous multisystem autoimmune syndrome with a remarkably heterogeneous phenotype. As with other disease manifestations, the initial renal manifestations and course are highly variable. Although considerable controversy exists, current thinking favors the notion that nephritogenic Igs of diverse specificities, isotype, avidity, and charge arise in the course of disease and localize to the kidney by a variety of mechanisms (reviewed in 77,78 and 79). Anti-DNA IgGs are present in nephritic kidneys in both murine and human SLE and clearly implicate anti-DNA in nephritis pathogenesis. A select subset of murine and patient monoclonal anti-dsDNA IgG are capable of forming IDs and induce a variety of renal histopathologic lesions in naive animals (80,81). Nonetheless, the relative importance of reactivity with DNA versus other antigens and the mechanism by which anti-DNA localize to the kidney are the subject of much debate (82) because it appears that anti-DNA activity is neither necessary nor sufficient to induce SLE nephritis. Anti-dsDNA titers and affinity can be dissociated from the presence and severity of renal disease in both patients and experimental models, and many Igs eluted from SLE kidneys do not bind to DNA.

In situ IC formation may be a dominant mechanism of renal Ig deposition in SLE (reviewed in 77,78 and 79). Both direct binding to intrinsic renal components and Ig reactivity with planted self-antigen are implicated. Anti-DNA cross-reactivity with non-DNA antigens may be involved. Anti-DNA eluted from nephritic kidneys as well as serum and monoclonal SLE Ig cross-react with polynucleotides, phospholipids, cytoskeletal proteins, and nonnuclear glomerular antigens. Subsets of SLE Ig bind *in vitro* to isolated glomeruli, crude renal extracts, cultured endothelial and mesangial cells, and purified glomerular antigens. *Ex vivo* renal perfusion with polyclonal human SLE IgG and both human and murine SLE monoclonal Ig result in glomerular Ig deposition. Some nuclear antigens (i.e., small 100–200 base pair DNA fragments) and soluble proteins have an intrinsic affinity for glomerular cell or BM surfaces. Collagen, laminin, and fibronectin possess binding sites for DNA, and some cells express DNA-binding receptors. Cationic ICs and nuclear histones (pI ~10–11) and nucleosomes (DNA–histone) can bind polyanions in the GBM or negatively charged glycosaminoglycans in cell membranes and serve as binding sites for anionic DNA, DNA-anti-DNA ICs, antihistone or antinucleosome Ig. These autoIgs and corresponding antigens have been recovered from human and murine lupus renal lesions. Rheumatoid factors, certain Ig idiotypes, and anti-C1q Ig are reported in high frequency in SLE serum and glomerular deposits, suggesting Ig binding to previously deposited Ig or C1q.

Antigen Nonspecific Mechanisms of Immunoglobulin Deposition

Mechanisms independent of antigen specificity may underlie Ig deposition in certain renal disorders. Abnormalities in hinge region O-glycosylation, possibly related to a defect in B-cell b-1,3 galactosyltransferase activity, are reported in circulating IgA_1 of patients with IgA nephropathy (83). It is postulated that the presence of abnormal carbohydrate moieties may alter IgA_1 interactions with matrix and mesangial cell IgA FcRs or interfere with normal degradation and contribute to ID and injury; however, a similar O-glycan defect in mesangial deposited IgA_1 has not yet been proven. A role for mesangial cell IgA FcR- or FcgR-mediated trapping of IgA or IgG is also postulated.

Physicochemical properties (e.g., amyloidogenicity, self-aggregability, tissue affinity, tubular toxicity) determined by particular Ig variable region sequences or chains, are presumed to underlie renal deposition of Ig or Ig fragments in the dysgammaglobulinemias (see later). Nephritogenicity of IgG_3 in MRL/lpr murine lupus is attributed in part to the unique capacity of murine IgG_3 constant regions to self-associate through nonimmunologic Fc–Fc interactions that contribute to decreased solubility and cryoglobulin formation (84).

T CELLS IN NEPHRITOGENIC IMMUNE RESPONSES

Cellular and humoral immune responses collaborate in the pathogenesis of most immunologically mediated renal disease (85). Cellular immunity is dominant in most forms of TIN (reviewed in 39). T cells (variably $CD4^+$ dominant) and monocytes/macrophages often dominate the mononuclear interstitial infiltrate, accompanied by B cells, plasma cells, eosinophils, and natural killer (NK) cells; ICs and anti-TBM Igs are uncommon. TIN also frequently accompanies primary glomerular disorders and nonimmunologic renal lesions, such as urinary tract obstruction. Although glomerular immune injury is often dominated by Ig deposition, numerous studies indicated direct cellular contributions (reviewed in 86,87). In experimental models, the relative contributions vary by species, strain, and disease.

T-cell involvement in the pathogenesis of renal disease can occur at several levels: (a) through provision of T-cell help for B-cell production of nephritogenic Ig; (b) indirectly via release of cytokines or permeability factors that affect renal function (88); (c) indirectly through immunoregulatory functions (89); and (d) directly as activated renal-infiltrating and renal-specific effectors causing injury by direct cytotoxicity or recruitment to the kidney of activated macrophages or NK cells via a delayed-type hypersensitivity (DTH)-like response. A role for T-cell priming in inducing experimental anti-GBM Ig responses is suggested by T-cell transfer and depletion studies (90) and the ability of costimulatory blockade via interference with CD28/B7, CD40/CD40L, or ICAM-1/LFA-1 interactions to ameliorate T-dependent Ig responses and disease (91,92). Direct T-cell involvement in mediating injury is suggested by the presence of activated T cells in renal lesions (39,86), experimental transfer of disease by renal antigen-reactive T cells in the absence of Ig (93,94), and amelioration of disease by T-cell depletion in the absence of effects on the Ig response (95,96).

Most of what is known about T-cell pathogenesis in renal disease has derived from experimental models. Early work focused predominantly on spontaneous and induced models of TIN mediated by sensitized T cells reactive with TBM proteins (reviewed in 39,97,98). In the induced antigen-in-adjuvant anti-TBM TIN model in SJL mice, renal anti-TBM Ig deposition precedes a mononuclear cell infiltration, but renal injury appears to arise primarily from cellular immunity. Pharmacologic interventions can ameliorate disease without diminishing the anti-TBM Ig response or renal Ig deposition. $CD4^+$ and $CD8^+$ antigen-reactive T cells can be isolated from lymph nodes of immunized mice. The TBM-reactive $CD4^+$ T cells do not directly mediate nephritis, but they are required for induction of effector TBM-reactive MHC class I restricted $CD8^+$ T cells that mediate antigen-specific DTH responses and renal tubular cell cytotoxicity (99). $CD8^+$ effectors induce TIN after adoptive renal subcapsular transfer to naive recipient mice. Notably, nonpathogenic TBM-reactive $CD4^+$ T cells from nonsusceptible mice can induce TIN on adoptive transfer to host with cytokine-upregulated renal class II MHC expression.

Spontaneous autoimmune TIN develops in kdkd mice mutated from the non-autoimmune CBA/Ca strain (30). Analogous to the anti-TBM model, class I-restricted $CD8^+$ effector T cells mediate TBM-specific DTH and induce TIN on adoptive transfer. Antigen-reactive $CD8^+$ T cells can be demonstrated in peripheral lymphoid organs of both kdkd and CBA/Ca strains. In experimental cadmium-induced TIN, $CD4^+$ HSP-70-autoreactive a/b T cells cytotoxic for heat-shocked or cadmium-treated renal tubular cells can transfer TIN in mice expressing tubular epithelial HSP-70 (16). Cytotoxic $CD4^+$ effectors capable of transferring disease can be isolated from renal lesions in murine chronic graft-versus-host disease (GVHD) glomerulopathy (100).

A major role for sensitized T cells in mediating glomerular injury only recently became widely appreciated (101,102). Macrophages and, to a lessor extent, activated $CD4^+$ and $CD8^+$ T cells are present in glomerular lesions in most experimental GN and in human proliferative and crescentic GN traditionally attributed to humoral immunity as well as in noninflammatory lesions (e.g., rat HN) (102,103). Severe anti-GBM disease can be induced in bursectomized chickens, which lack humoral immunity, and adoptive transfer of antigen-reactive T cells, in some cases derived from diseased kidneys, induces glomerular lesions in naive recipients in the absence of Ig (93). Depletion of $CD8^+$ or $CD4^+$ T cells inhibits proteinuria, macrophage infiltration, and skin DTH responses in rat NSN and HN without modifying Ig titers or C deposition (95,96,104). In the rat active HN model of membranous nephropathy, proteinuria develops coincidentally with glomerular infiltration by Th1 $CD4^+$ and cytotoxic $CD8^+$ T cells and macrophages and is abrogated by $CD8^+$ depletion (105). Underlying strain and antigen-dependent Th1/Th2 polarization has been suggested, based on differences in glomerular and systemic cytokine, Ig isotype, and T-cell effector profiles. In experimental GN, this suggestion is based on differences in DTH responsiveness and lesion responsiveness to cytokine manipulation (i.e., administration, inhibition, or gene-targeted deletion of IL-4, IL-10, INF-g, or IL-12) (reviewed extensively in 106). Interpretation of these studies is complicated by the *in vivo* production of counterregulatory cytokines by resident renal cells as well as lymphocytes, Th1 dependence of some pathogenic Ig isotypes, and variable success using therapeutic immune deviation. Polarization to a Th1 response may be most prominent in patients and models with strong evidence of glomerular DTH (T cells and macrophages), that is, crescentic GN. A genetically determined tendency to differentiate toward a Th1 effector subset in response to heterologous Ig (the nephritogenic antigen in NSN models) is implicated in autologous anti-GBM disease induced in rabbits and certain rodent strains (Wistar Kyoto rats, C57BL/6 mice) that are particularly susceptible to crescent formation, Th1 cytokine production, and prominent glomerular mononuclear cell infiltrates (104,106,107 and 108). Susceptibility to autoimmune crescentic GN by active immunization with a3(IV)NC1 collagen also is associated with a Th1-like immune response, characterized by glomerular IL-12 and IFN-g and induction of antigen-specific IgG2a Igs that transfer disease (109).

Regulatory T-cell subsets, cytokines (TGF-b in particular), and nitric oxide (NO) play an active role in regulating the expression of cell-mediated renal injury (89,110,111). The retrieval of antigen-reactive T cells from nonsusceptible mice and humans (39,112) suggests that peripheral mechanisms are crucial in maintaining nonresponsiveness. An intricate network of suppressor and/or contrasuppressor T cells regulate autoimmunity in rodent anti-TBM TIN (reviewed in 39). TGF-b1, a potent T-cell modulator, downregulates perforin expression and T-cell proliferation and inhibits the ability of T cells to transfer TIN (110). TGF-b-resistant $CD4^+$ nephritogenic T cells induce severe interstitial lesions (113). NO also has potent T-cell antiproliferative effects (111).

Cellular and humoral responses collaborate in lupus nephritis pathogenesis. Nephritis is ameliorated in lupus mice rendered deficient in a/b $CD4^+$, a/b $CD8^+$, CD28, or B cells (114,115 and 116) or subjected to MAb interruption of CD40/CD40L or CD28/B7 costimulation (117). AutoIg production and severe GN are variably attributed to

a/b or g/d T-cell subsets in TCR gene-targeted mice (114,118). Studies using pharmacologic or genetic alteration of Th1- and Th2-type cytokines have yielded conflicting results and fail to implicate selective disease dependence on a single $CD4^+$ T-helper subset (119,120). Differential modulation of renal mononuclear infiltrates and vasculitis in MRL mice deficient in B cells or secreted Ig indicates a crucial role for antigen presentation by autoreactive B cells (121); DNA-protein conjugates (nucleosomes in particular) are proposed antigen intermediaries (43).

MECHANISMS AND MEDIATORS OF TISSUE INJURY IN IMMUNE NEPHRITIS

Classic experimental models and novel mutant mouse strains, particularly knockout (KO) mice bearing selective deficiencies in critical molecules involved in inflammatory responses, have been useful in dissecting effector mechanisms in renal immune injury (12,53). These models revealed that proteinuria in noninflammatory lesions of the glomerulus is usually due to injury to the podocyte initiated by Ig, complement, or other soluble factor. Inflammatory renal lesions are mediated by infiltrating leukocytes or proliferation of resident glomerular cells induced by Ig, activated T cells, or both, with release of numerous inflammatory mediators. Each of these cells, molecules, and signaling pathways poses a promising target for novel therapies directed at renal immune injury (122,123 and 124).

It cannot be overemphasized, however, that the responsible mediators and relative C, FcR, leukocyte, or T-cell dependence vary considerably between species, strains, and disease models, inviting considerable caution in extrapolating between models or to disease in humans. Moreover, the pathogenic potential of renal-localized Ig depends in large part on effector functions determined by Ig constant region domains, each subclass of which has a unique profile of interactions with C and FcRs, which in turn are regulated by a complex system of C regulatory proteins and cytokines. This may explain in part the observations that monospecific Igs do not reproduce the fulminant pathologic changes seen with polyclonal antisera or in native disease models and that substantial Ig deposition may occur in the absence of renal histologic or functional alterations. Finally, in addition to their effector functions, some of these intermediates are potent modulators of immune responsiveness and autoimmunity by way of their modulation of B-cell signaling (i.e., C receptors and FcgRIIB) and T-cell functions (i.e., NO, TGF-b). In some settings in which manipulation of these molecules markedly alters the nephritis phenotype, it may be difficult to determine the point of dominant effect (disease induction versus mediation) in the pathogenic response. In this regard, experimental immune complex glomerulonephritis (ICGN) induced by foreign protein immunization induces distinct humoral responses and nephritis phenotypes in wild-type, C3-, and C4-deficient mice (125). C-receptor CD21/CD35 deficiency prevents the induction of anergy in autoreactive B cells, which, when combined with Fas deficiency, leads to severe lupus nephritis (126). These complex interactions must be taken into consideration when interpreting studies involving manipulation of these molecules.

Direct Immunoglobulin-Mediated Injury

It was well documented in early models of NSN that the injection of substantial quantities of heterologous nephrotoxic serum could result in immediate massive proteinuria that was C and polymorphonuclear (PMN) independent (127). A direct effect of Ig alone was confirmed by the induction of heavy proteinuria in antiserum-perfused isolated rat kidneys (128,129). Immediate transient proteinuria also has been observed in rats injected with F(ab')2 or Fab' fragments of sheep anti-Fx1A, and heavy proteinuria is reported in active HN in C6-deficient rats unable to form the C5b-9 membrane attack complex (MAC) (130). Subsequently, it was determined that these heterologous polyclonal antisera contain pathogenic Ig reactive with glomerular podocyte surface antigens. MAbs reactive with some of these determinants are capable of inducing marked alterations in glomerular permeability, proteinuria, and ultrastructural alterations in podocyte morphology (131).

Proteinuria-inducing murine MAb 5-1-6 recognizes a 185-kd protein on rat podocyte slit diaphragms recently identified as rat nephrin, an Ig-like transmembrane protein and important regulator of glomerular permselectivity (132). Ig cross-linking induces antigen redistribution, which may contribute to proteinuria. Notably, mutations in human podocyte nephrin underlie the massive proteinuria seen in congenital nephrotic syndrome of the Finnish type (133). Injection of intact or F(ab')2 monoclonal anti-Thy-1 Ig into mice expressing human Thy-1.1 on podocytes similarly induces rapid foot process swelling and proteinuria (134). Ig reactive with podocyte a3b1-integrin alters glomerular permeability to albumin *in vitro*; active signal transduction rather than simple interference with cell–matrix adhesion is apparently necessary because glomerular permeability is not altered by non-cross-linking Fab fragments that inhibit adhesion (135). As yet, none of these target antigens has been shown to be involved in human renal disease.

Complement

Complement (C) deposition is a prominent feature of many Ig-mediated glomerular diseases (136,137). Deposited C may derive from the circulating pool or from local synthesis by intrinsic renal cells. The presence of early C components (C1q, C2, C4) indicates activation of the classical pathway (e.g., anti-GBM disease), whereas deposits are often restricted to C3 or terminal components in certain nephritides [e.g., IgA nephropathy, membranoproliferative GN (MPGN) type 2], which indicates alternative pathway activation. A critical role for C in disease pathogenesis has been established in experimental models, although the mechanism of C-induced injury varies with the model. Acute C depletion with cobra venom factor markedly diminishes disease in each of the three most well-studied models of glomerular immune injury: NSN, passive HN, and anti-Thy-1 disease (138,139). Similar results have been seen with disease induced in C-deficient mice (C3, C4, C5), rabbits, or rats (C6) or using antisera containing only non-C-fixing Ig subclasses (140,141).

The MAC-induced renal injury is best characterized in the noninflammatory passive HN model. Proteinuria is largely dependent on *in situ* C activation by podocyte-bound antiglycolipid Ig (18), followed by insertion of the MAC into the podocyte membrane (142,143). Subsequent sublytic cell injury induces the transactivation of receptor tyrosine kinases; activation of extracellular signal-regulated kinase-2 (Erk-2) and cytosolic phospholipase A_2; and local release of proteases, oxidants, and eicosanoids that induce lipid peroxidation in the GBM, alter glomerular permeability, and induce proteinuria (144). Notably, the necessary lipid environment for peroxidation is provided in part by lipoproteins that accumulate in GBM IDs in this model; the anti-HNAC Igs that contribute to immune deposition (see earlier) also block HNAC-mediated endocytic uptake of apoproteins (145), a normal route of lipoprotein clearance from the glomerular filtrate. Thus, C- and non-C-fixing autoIgs of diverse specificities collaborate to induce this disease.

The MAC also mediates inflammatory and thrombotic renal injury. In the anti-Thy-1 model, mesangial cell lysis and proliferation, platelet and macrophage accumulation, and proteinuria are C dependent but PMN independent; all forms of injury are markedly reduced in C6-deficient rats (146). MAC-mediated lytic or sublytic mesangial cell activation releases inflammatory mediators and growth factors (147). C6 deficiency ameliorates Ig-induced endothelial injury in experimental rat thrombotic microangiopathy (148), and C5 blockade ameliorates renal disease in SLE-prone NZB/WF1 mice (149). Endothelial cells from human umbilical vein that were subjected to sublytic MAC assembly secrete chemoattractants (150). A role for MAC in human nephropathy is suggested by demonstration of terminal C components in renal biopsy specimens and in the urine of patients with membranous nephropathy (151).

The binding of C1q and C activation with release of anaphylatoxins C3a and C5a are largely responsible for PMN infiltration in NSN. PMN infiltration is reduced significantly in KO mice that are deficient in either C3 or C4 (140,152). Notably, C deficiency provides only partial protection from proteinuria in this model, indicating coexisting C-independent mechanisms. C also can recruit leukocytes via CD11/CD18-dependent leukocyte adhesion, C-induced renal cell injury with release of chemoattractants, or MAC-induced mobilization of P-selectin on endothelial cells (153,154).

A role for the alternative pathway in modulating lupus nephritis is suggested by the attenuation of nephritis in MRL/lpr lupus mice deficient in factor B; serum C3 levels remained normal in KO mice (155). Non-Ig-initiated C activation has been implicated in a porcine model of MPGN type 2 associated with hereditary factor H deficiency (156). Affected piglets develop spontaneous systemic C activation and massive *in situ* GBM deposition of C3 and MAC, but no Ig, associated with mesangial proliferation and BM electron-dense deposits analogous to human MPGN type 2. Factor H inhibits C activation, whereas its deficiency leads to uncontrolled C3 activation. Notably, human MPGN type 2 and hemolytic uremic syndrome (a thrombotic lesion) occasionally are associated with factor H deficiency, but pathogenesis is not established. H_2O_2 released by activated cells can itself activate C, amplifying injury. C filtered at the glomerulus into the tubular lumen during heavy proteinuria is a postulated source of downstream tubular cell injury (157).

Renal protection from C-induced injury depends in part on circulating, soluble, and renal cell membrane-associated C regulatory proteins that limit C consumption and bystander damage (158). C-bound plasma proteins can be found in renal lesions in human GN, and depletion of plasma clusterin exacerbates proteinuria in a C-dependent isolated perfused rat kidney model of HN (159). Activated murine tubular cells also synthesize clusterin, and C-activated human mesangial cells secrete soluble decay-accelerating factor (DAF). The human cell-bound regulatory protein C3/C5 inactivators, DAF, membrane cofactor protein (MCP), complement receptor type 1 (CR1), and CD59, an inhibitor of MAC formation, are constitutively expressed in the kidney and variably modulated by immune injury *in vitro* and *in vivo*. C-regulatory proteins Crry and CD59 are expressed in virtually all types of renal parenchymal cells in rodents. The disease relevance of these molecules is demonstrated by maneuvers that modulate their *in vivo* expression or function in models of C-dependent renal injury. Soluble forms of CR1 or Crry, a rat CR1-like C regulatory protein, block C activation *in vivo* and reduce glomerular injury in experimental ICGN (19,160). Overexpression of membrane-bound Crry protects against C-mediated mesangial injury *in vitro* (161), and MAb neutralization of CD59 exacerbates injury in rat antiendothelial Ig thrombotic microangiopathy (162). Recent studies also indicated a role for these molecules in tubulointerstitial defense against autologous C attack under normal and disease conditions (163,164).

In the absence of C3 activation, C1q may play a protective role in GN through the clearance of glomerular apoptotic cells. Whereas C1q-deficient mice develop autoimmunity and GN with IgG and C3 deposits and glomerular apoptotic bodies, C1q-deficient mice that also lack C2 and factor B develop similar lesions but without glomerular C3 deposition (165). C itself has antiinflammatory effects because of its ability to solubilize and inhibit deposition by ICs that may modulate renal injury. It is noteworthy, however, that preferential renal uptake of IgG2a-containing ICs was abrogated by C depletion, suggesting that enhanced renal deposition was C

dependent (166).

C-pathway components also can serve as targets of autoIg responses. Nephritic factors (NeFs) are spontaneously arising autoIgGs that bind and stabilize alternative or classical pathway C3 convertases (C3 NeF and C4 NeF, respectively), permitting continuous C activation. NeFs are reported in association with human MPGN types 1 and 2 and occasionally in other nephritides. Their presence and serum levels do not correlate with disease activity, and it is unresolved whether they contribute to ongoing renal injury. Anti-C1q Igs are common in SLE and may contribute to nephritis by binding renal-deposited C1q. Experimental evidence suggests that neutralizing anti-Crry autoIgs develop in active HN in Fx1A-immunized rats; these autoIgs impair the C-regulatory activity of Crry, permitting unrestricted C activation and renal injury by other subsets of pathogenic Ig (167).

Fc Receptors

The FcRs constitute a complex family of constitutive and inducible polymorphic cell-surface proteins that vary in cell distribution, Ig affinity, signaling, and function. The relative balance of inhibitory (FcgRII) versus activating (FcgRI and FcgRIII) FcgRs expressed on macrophages, PMNs, and renal parenchymal cells is a major regulator of the inflammatory response to IC deposition and a major determinant of disease susceptibility (168). Mice rendered deficient in activating FcgRs by KO of the common FcRg-chain gene (FcRg-KO) are generally protected from renal immune injury, demonstrating a pivotal role for FcgRs in regulating inflammation *in vivo*. Nephritis is dramatically attenuated despite prominent glomerular IgG and C3 deposition in FcRg-KO lupus-prone NZB × NZW mice (169) and in two models of NSN induced in FcRg-KO mice (170,171); conversely, disease is accelerated in mice deficient in inhibitory FcgRII (171). Renal inflammation is similarly relieved in SLE-prone MRL/lpr mice subjected to high dose granulocyte-colony stimulating factor (G-CSF) infusions, an effect attributed to G-CSF-induced down-modulation of glomerular FcgRIII (172). Notably, however, marked proteinuria was not attenuated (170) and C-independent, angiotensin II-dependent chronic renal injury with mesangial proliferation and mononuclear cell infiltration developed in FcRg-KO mice with anti-GBM disease (171), indicating the presence of ongoing non-FcgR-dependent injury.

The IC engagement of activating FcgRs promotes leukocyte adhesion and triggers proinflammatory functions in leukocytes. An IgG-induced anti-GBM GN dependent on FcR-mediated accumulation of macrophages was described in rabbits (173), and Fc-dependent, C-independent PMN infiltration was described in a murine NSN (174). FcgR are expressed on renal parenchymal cells, and their cross-linking can induce synthesis of multiple inflammatory mediators (175,176). A novel IgA receptor distinct from CD89, the myeloid IgA receptor, is expressed on human mesangial cells (177); its engagement by IgA triggers cytokine and chemokine expression, cell proliferation, and extracellular matrix synthesis. Additonal, as yet poorly understood, renal effects of FcgR modulation are possible because novel aspects of FcgR biology continue to emerge. In some model systems, targeted deletion of FcgR dramatically increases IC deposition, presumably by an altered IC clearance. FcgRII and III on mast cells are potent regulators of IgE-mediated allergic responses and may modulate renal allergic diseases. Intracellular collaboration involving FcgRs and C-receptor MAC-1 (CDIIb/CD18) in mediating sustained PMN adhesion in acute experimental GN has been reported, suggesting complex interactions between FcgR and complement systems (178). The relative roles of FcgR and C in mediating experimental and human nephritis remain to be determined. FcR-blocking strategies have been used successfully in ameliorating experimental ICGN (179).

Additional Mechanisms of Immunoglobulin-Mediated Renal Injury

Direct Ig reactivity with PMNs and monocytes may contribute to pathogenesis in ANCA-positive vasculitides (reviewed in 180,181). ANCAs react with soluble enzymes, primarily proteinase-3 (Pr3) and myeloperoxidase (MPO), contained in PMN primary granules. Their role in the pathogenesis of vascular and renal lesions remains unclear because Ig deposition is typically absent (termed *pauciimmune*) from glomeruli and vessel walls. *In vitro* studies indicated that these enzymes become exposed on the surface of activated PMNs, monocytes, or endothelial cells, permitting antigen-specific binding of ANCA and subsequent PMN respiratory burst, degranulation, and lipid-mediator generation, endothelial damage, apoptosis, and inflammation. Binding mediated by PMN FcR engagement is also reported. ANCA stimulation of primed human PMNs in the presence of free arachidonic acid (prevalent in inflammatory microenvironments) induces strong activation of the 5-lipoxygenase pathway and release of chemoattractants and proinflammatory leukotrienes (182). Heterologous MPO induces an autoimmune anti-MPO response in rodents that contributes by an unknown mechanism to severe necrotizing crescentic GN in rats injected with a subnephritogenic dose of anti-GBM serum. Anti-MPO Igs also are expressed in several rodent models with induced or spontaneous renal vasculitis. Cationic MPO has a natural affinity for anionic GBM and can serve as a planted glomerular antigen target in MPO-immunized rats to induce an ICGN and vasculitis. A subset of cANCA (anti-Pr3) from patients with active vasculitis cross-react with a 130-kd lysosomal-associated membrane protein, h-lamp-2, expressed on renal microvascular endothelial cells and PMNs, potentially bridging PMN and endothelium (183). Direct endothelial toxicity of released proteinase 3 also has been postulated.

Recent studies suggested that SLE autoIgs may induce renal injury by a variety of mechanisms in addition to IC deposition. Disruption of cell functions by autoIgs that gain intracellular access has been postulated. Select human and murine SLE Igs are able to penetrate living cells in culture (184). Cell entry typically involves the antigen-specific binding (myosin is one putative membrane target) and may depend on the endocytosis of Ig-bound cell-surface receptors (185). Subsequent Ig localization to distinct intracellular compartments is attributable to either Ig specificity for subcellular antigens or expression of nuclear localization sequences within the Ig-variable region. A pathogenic role is suggested by the association of *in vivo* renal nuclear localization by SLE Ig with proteinuria and renal injury (184). Several mechanisms have been proposed as being involved in the pathogenesis of anti-heparan sulfate Ig, including alterations in glomerular permeability from the blockade of BM anionic sites, thrombus formation from Ig binding to endothelial cell-surface heparan sulfate, a physiologic ligand for antithrombin III, and promotion of a procoagulant state resulting from cross-reactivity with anionic phospholipids and interference with phospholipid-dependent coagulation. Selected anti-dsDNA Igs initiate C-mediated cytotoxicity of cultured kidney cells (186).

Cellular Mediators

The magnitude and relative proportion of cell types in the inflammatory infiltrate vary with the disease model and stage, species and strain, and renal expression of cytokines and adhesion molecules that attract and retain the different cell populations. Differentiated T-cell effectors (see earlier) exert their functions by interactions with other cells. Experimental models indicate that nephritogenic $CD4^+$ and $CD8^+$ T cell clones can mediate MHC-restricted cytotoxicity toward cultured renal proximal tubular cells (187). Cytotoxic clones express perforin and granzymes B and C (188), and granzyme C inhibition blocks both *in vitro* cytotoxicity and *in vivo* tubular cell destruction (188). $CD4^+$ T cells eluted from renal lesions in experimental TIN mediate DTH responses to challenge with renal antigen (39,97,98). Renal antigen-reactive T-cell clones isolated from mice with induced anti-TBM TIN or GVHD variably express a Th1, Th2, or a mixed cytokine profile (39,98,100). *In situ* studies similarly suggest a mixed cytokine profile in glomerular lesions (106). Nephropathic T cells secrete inflammatory and cytotoxic cytokines capable of upregulating renal adhesion and MHC class II molecules (IFN-g), amplifying inflammation (TNF-a, IL-1b) or promoting fibrosis (TGF-b) (189). Antigen-nonspecific T cells attracted to the inflammatory locus by chemokines may further amplify injury.

MACROPHAGES

Macrophage accumulation is prominent in TINs of all causes and in aggressive forms of GN (190). A role in renal injury has been demonstrated in experimental models in which monocyte depletion inhibits renal macrophage accumulation and abrogates disease and induction of renal lesions by passive transfer of macrophages to leukopenic rabbits during autologous anti-GBM GN (191,192). Monocytes and macrophages are recruited, retained, and activated in the kidney by a variety of Ig-, C- and T-cell-dependent mechanisms. Macrophages bind to ICs through their cell-surface Fc or C (CD11b/CD18, MAC-1) receptors and migrate in response to C5a. Activated $CD4^+$ T cells, platelets, endothelial and renal parenchymal cells release chemoattractants, upregulate adhesion molecules, and release cytokines [IFN-g, macrophage migration-inhibiting factors (MIFs)] to bind and activate macrophages (Table 50.5). Local macrophage proliferation, possibly driven by local macrophage colony-stimulating factor (M-CSF) production, contributes to macrophage accumulation in severe renal injury (193). Florid interstitial macrophage accumulation also occurs in nonimmune injury, such as obstructive nephropathy, presumably because of tubular dysfunction and chemoattractant release (194). Abnormal macrophage sensitivity to renal-expressed CSF factor 1 (CSF-1) is implicated in MRL-lpr lupus nephritis (195). Macrophages are a major source of IL-1b and release a variety of products that mediate direct cell cytotoxicity and promote mesangial cell proliferation and fibrosis (Table 50.5).

TABLE 50.5. Cell Sources of Mediators and Modulators of Renal Inflammation and Injury[a]

Macrophages are the dominant cell type in cellular crescents associated with GN. IL-1b, TNF-a, and $CD4^+$ T cells promote crescent formation, which involves macrophage migration into Bowman space, deposition of fibrin and fibronectin (a fibroblast chemoattractant), and local proliferation of macrophages and glomerular epithelial cells. Fibrin deposition depends in part on macrophage release of coagulant tissue factor-like activity; macrophage depletion prevents fibrin deposits (196). Crescent formation can be prevented in experimental GN by depletion of fibrin or $CD4^+$ T cells or blockade of ICAM-1, IL-1b macrophage MIF, or osteopontin (190,197). Rupture of Bowman capsule, mediated by infiltrating periglomerular macrophages and $CD4^+$ T cells, permits crescent infiltration by fibroblasts, leading ultimately to monocyte chemoattractant protein 1 (MCP-1)-, TGF-b- and basic fibroblast growth factor (bFGF)-mediated glomerular sclerosis.

POLYMORPHONUCLEAR NEUTROPHILS

In several models of inflammatory GN (e.g., NSN) and in exudative human GN, primarily in association with subendothelial or GBM injury, PMNs are prominent. A pathogenic role has been demonstrated by reduced injury in experimental GN by PMN depletion and reconstitution of injury with PMN repletion (198). PMNs are recruited by both C-dependent (C5a, MAC-1, C3b R adherence) and C-independent (chemoattraction, FcgR) mechanisms. Blockade or knockout of these molecules decreases glomerular PMN infiltration in experimental GN. Experimental evidence indicates that PMN-mediated renal damage occurs primarily through the release of oxidants and proteases. PMNs also have been demonstrated to collaborate with glomerular cells and platelets in transcellular biosynthesis of leukotrienes and lipoxins; lipoxins are endogenous inhibitors of PMN recruitment in GN (199).

Secondary Mediators of Renal Immune Injury

A long list of secondary mediators contributes to renal injury in immune mediated disease (Table 50.5). Insight into their actions derives from animal models; information regarding their role and importance in human disease is fragmentary, derived from *in vitro* and correlative studies. Nonetheless, they provide an abundance of potential targets for therapeutic intervention. Most are produced locally in the kidney and exert their effects at the site of synthesis in an autocrine or paracrine fashion. *In vivo* studies thus necessarily focus on changes in tissue synthesis and receptor expression, evaluation of *in vivo* pharmacologic blockade of mediator synthesis or receptor engagement, and genetic manipulation to assess the effect of overexpression or deficiency.

EICOSANOIDS

The eicosanoid family of oxygenated fatty acids derived from arachidonic acid (AA) metabolism in infiltrating leukocytes, platelets, and resident renal cells plays a major role in pathogenesis of renal immune injury (200). A long list of cytokines, hormones, physical stresses, and inflammatory mediators induce renal eicosanoid synthesis, increased production of which is described in inflammatory and noninflammatory experimental and human immune-mediated GN (201). The type and relative amounts of counterregulatory eicosanoids [i.e., proinflammatory leukotriene B4, antiinflammatory prostaglandin E_2 (PGE_2), prostaglandin I2 (PGI2), 15-S-hydroxyeicosatetraenoic acid, lipoxin A, vasodilatory PGI2, vasoconstrictive thromboxane B2, and leukotriene D4 (LTD4)] vary with the model and stage of disease. Prostaglandins, leukotrienes, and lipoxins exert a variety of often opposing effects on mesangial cell growth, contraction, and matrix formation as well as opposing effects on inflammation and renal hemodynamic function; interruption of AA metabolism pathways in experimental GN using thromboxane blockade, cyclooxygenase (COX) inhibition, LTD4 receptor blockade, or 5- or 15-lipoxygenase inhibitors has variable renoprotective or injurious outcomes (202,203; reviewed in 204). Favorable effects from PGE_1 infusion and dietary fish oil are reported in experimental and human GN in some studies; amelioration of experimental GN by 5-lipoxygenase inhibitors or transfection of rat kidney with the human 15-lipoxygenase gene confirm an *in vivo* antiinflammatory role (205).

PLATELETS

Evidence of systemic platelet activation and destruction and glomerular localization of platelet products or aggregates is observed in experimental and human glomerular injury (206). Platelets release a plethora of products that contribute to inflammation, coagulation, adhesion, cell proliferation, chemotaxis, and matrix production. Biologic relevance is suggested by disease models in which infusion of platelet-associated products alters glomerular permeability or induces glomerular cell proliferation or matrix accumulation. Platelet depletion and antiplatelet therapy are efficacious in certain experimental GN (206).

CHEMOKINES AND ADHESION MOLECULES

A diverse group of potent chemoattractants with overlapping functions can be synthesized by activated renal cells, monocytes, and T cells to regulate leukocyte trafficking (154). Renal expression of chemokines is upregulated in experimental and human renal injury; it is noteworthy, however, that the pattern of expression does not necessarily indicate the cellular composition of the infiltrate. Blockade of a variety of chemoattractants by neutralizing Ig or antagonists abrogates glomerular inflammation in experimental ICGN (207,208, reviewed in 154). KO mice revealed complex and unexpected *in vivo* roles for chemokines in renal injury. Deficiency of chemokine receptor CCR1 enhances Th1 immune responses and worsens NSN nephritis (209), and MCP-1-deficiency reduces tubular but not glomerular injury in NSN (210). The importance of chemokines to renal immune injury in humans is suggested by the correlation of urinary chemokine levels with disease activity and glomerular leukocyte infiltration.

Renal adhesion molecule expression (ICAM-1, VCAM-1, endothelial P- or E-selectin) is similarly upregulated in human and experimental proliferative GN and often correlates with inflammation (153). Numerous antiadhesion therapies have been assessed in experimental renal disease (153); results are generally positive but are ligand, species, model, and timing dependent. The role of adhesion molecules in mediation of GN is complex because they modulate not only leukocyte trafficking but also multiple other immune and inflammatory processes (e.g., antigen presentation, phagocytosis, cytotoxicity) (168). In this regard, P-selectin-deficient mice paradoxically demonstrated more severe glomerular damage in experimental anti-GBM disease; this has been attributed to disruption of production of lipoxin A4, an endogenous inhibitor of PMN recruitment, and to decreased levels of circulating soluble antiinflammatory P-selectin (168). Antiadhesion MAbs block T-cell or phagocyte-mediated cytotoxicity directed at renal cells (187) and block transcellular eicosanoid synthesis (199).

PROTEASES

Metalloproteases and serine proteases secreted by infiltrating leukocytes and activated renal cells synergize with oxidants in degradation of GBM, leading to proteinuria, and play a key role in tissue remodeling (211,212). Renal artery infusion of elastase or cathepsin G induces marked proteinuria, and proteinuria is absent in NSN induced in beige mice with PMN deficient in these enzymes (213). Cysteine proteinase inhibitors significantly reduce proteinuria in a C- and PMN-independent model of NSN. Gelatinase A directly modulates mesangial cell proliferation and differentiation *in vitro* and *in vivo*, promoting an inflammatory phenotype (214). Matrix metalloproteinase activity is regulated in part by tissue inhibitors (TIMPs).

RENAL OXIDANT STRESS

Generation of reactive oxygen species (ROS) by activated renal cells or infiltrating leukocytes is increased in noninflammatory as well as inflammatory forms of experimental glomerular injury, and administration of ROS scavengers or antioxidants in these models reduces proteinuria (reviewed in 212,215,216). Upregulation of the NADPH-oxidoreductase complex in glomerular cell membranes is reported in the HN model (216). Powerful oxidants injure the kidney by multiple mechanisms: lipid peroxidation, BM destruction in concert with proteases to alter glomerular permeability, conversion of latent proteinases to active forms, altered production of inflammatory mediators (eicosanoids, TNF-a), direct cell cytotoxicity, inhibition of glomerular cytokine-induced isoform of nitric oxide synthase (iNOS) activity, and induction of mesangial cell proliferation, activation, or apoptosis (212,215,217,218). Renal perfusion with MPO–hydrogen peroxide causes proteinuria and significant glomerular morphologic changes and platelet activation followed by marked proliferative glomerular lesions (212,219). Dysregulated renal antioxidant defenses may be an important component of severe injury (220).

NITRIC OXIDE

Intrarenal activation of iNOS in macrophages and intrinsic renal cells in experimental acute GN generates NO, which in turn mediates or modulates renal immune injury (111,221). NO executes a variety of dose-dependent proinflammatory and antiinflammatory effector functions; it mediates macrophage cytotoxicity, promotes apoptosis, promotes mesangial cell relaxation, lysis and matrix production, inhibits platelet activation and aggregation, inhibits leukocyte-endothelial adhesion, downregulates proinflammatory cytokines and chemokines, inhibits T-cell proliferation and Th1 expansion, inhibits MHC class II expression, and promotes vasodilatation. NO can neutralize or enhance superoxide toxicity, and it is variably reported to promote or inhibit intrinsic cell proliferation. Its role in pathogenesis of GN and TIN remains unclear, and is likely dependent on the particular micromilieu. Interventions in experimental GN and TIN using nonselective NO inhibition have yielded

conflicting results (221). Mixed results have been reported with selective iNOS inhibition; it had little effect on rat NSN (221), whereas disease severity increased in association with Th1-type skewing of the immune response in induced TIN in rats, suggesting a protective role for renal iNOS in the latter model (222). Knockout of iNOS did not alter the course of renal injury in induced ICGN in 129SV mice (221) or in MRL/lpr murine lupus nephritis (223); it did, however, attenuate renal vasculitis in the latter model (223).

Heme oxygenase 1 (HO-1), a cytokine- and stress-inducible heat shock protein, has antiinflammatory functions that may be renoprotective in immune injury. HO-1-catalyzed degradation of the oxidant heme generates the antioxidant bilirubin and biliverdin, the vasodilator and antiplatelet mediator carbon monoxide (CO), and iron. Bilirubin also has anticomplement activities; CO and iron are negative modulators of iNOS activity and expression. NO is reported to enhance HO-1 expression in mesangial cells (224). Administration of HO-1 inducers reduces proteinuria and glomerular macrophage and PMN infiltration in rodent models of NSN in association with downregulation of renal iNOS expression (225,226).

CYTOKINES AND GROWTH FACTORS

A wide variety of pleiotropic growth factors (GFs) and cytokines produced by activated leukocytes and renal cells can modulate biologic responses in the kidney and contribute to mediation or recovery in renal immune injury (Table 50.5) (52,106,227,228 and 229). Clear insight into their role in disease pathogenesis is lacking because of conflicting observations reported in the literature. These conflicting results reflect in part the pleomorphism, redundancy, and complex regulation of cytokines, GFs, and their receptors, their synergistic and counterregulatory actions, their involvement at multiple stages (i.e., inductive and effector limbs) of immune responses and direct actions on parenchymal as well as lymphoid cells. Various abnormalities in systemically and local cytokine production are reported in human and experimental GN and TIN (52,106,227,228). An important role in renal injury and repair has been postulated based on *in vitro* studies that assessed cytokine production and the effects on cultured renal cells, the association of immunologic renal diseases with a variety of *in vivo* abnormalities in systemic or renal cytokine, GF or receptor expression, and modulation of normal or diseased kidney by *in vivo* manipulation of GF or cytokine networks.

Cytokines involved in Th1 and Th2 differentiation (IL-12, IFN-g, IL-4, and IL-10) have received particular attention due to their proinflammatory (IFN-g, IL-12) or antiinflammatory (IL-4, IL-10) actions (Table 50.5) and potential therapeutic efficacy (see earlier) (106). *In vivo* cytokine manipulation has met with variable success in predictably modulating experimental nephritis (reviewed in 106). Recent results from KO mice are mixed and in some cases are paradoxical to those predicted by *in vivo* interventions: Crescentic GN is exaggerated in mice with gene-targeted deficiencies of IL-10 (230), IL-4 (231), or IFN-g (232), whereas IFN-g receptor-deficient autoimmune MRL/lpr and (NZB × NZW)F1 mice are protected from lupus nephritis (233,234). Data regarding the relative importance of Th1-type versus Th2-type cytokines in murine lupus nephritis are particularly inconsistent and collectively suggest that both are required for full disease expression (13,106). A pioneering approach to examine the effects of sustained local cytokine production on renal immune injury uses implantation of genetically modified cytokine-secreting tubular cells under the renal capsule (235). Using this method, autoimmune renal injury is elicited in lupus mice by local secretion of IL-12 and macrophage growth factors [as well as by the chemokine regulated on activation normal T cell expressed and secreted (RANTES)], but not by IL-6 (235).

Both IL-1b and TNF-a are broadly proinflammatory and major regulators of acute inflammatory responses. They are produced in copious amounts by activated macrophages (a major source of intrarenal IL-1b) and are inducible in intrinsic renal cells. Renal cell expression of IL-1b, TNF-a, and their receptors increases in proliferative and inflammatory lesions. IL-1b and TNF-a are implicated in the development of crescentic GN, and they have numerous effects on cultured renal cells, inducing proliferation and synthesis of eicosanoids, NO, cytokines, chemokines, enzymes, ROS, C, adhesion and MHC molecules (229,236). Administration of IL-1b or TNF-a aggravates disease and blockade of either cytokine by neutralizing Ig, soluble receptors, or IL-1b receptor antagonist diminishes injury in various experimental GN (229,236). An exception is murine lupus, because chronic administration of TNF-a inhibits nephritis in NZB/NZW mice; it is unclear whether the dominant effect is on the autoimmune or the inflammatory response. Crescent formation and PMN influx are ameliorated in NSN induced in TNF-a-deficient mice (237).

Interleukin-6 has proinflammatory and antiinflammatory properties, and it is widely overexpressed in the kidney in a variety of immunologic and nonimmunologic renal diseases. Its role in inducing mesangial cell injury has been controversial; recent results in IL-6 KO mice suggest that IL-6 plays little role in mediating glomerular injury *in vivo* (238). Unconfirmed reports indicate inducible renal tubular expression of IL-13 and IL-15; their role in renal injury awaits definition.

Transforming growth factor-b1 stimulates matrix production and decreases matrix turnover, and it is a major mediator of fibrosis in experimental immunologic and nonimmune renal injury. Renal cell production of TGF-b1 contributes to matrix expansion via autocrine pathways. TGF-b1-based therapy (neutralizing anti-TGF-b Ig or decorin) arrests matrix accumulation and fibrosis in experimental GN (239). Renal TGF-b overexpression in a spectrum of chronic human glomerulonephritides suggests a similar role in man. TGF-b also has broad immunosuppressive and antiinflammatory properties that attenuate nephritogenic responses and renal inflammation (110,240). TGF-b inhibits glomerular cell mitogenesis *in vitro*, inhibits renal cell synthesis of proinflammatory cytokines IL-1b and TNF-a, downregulates iNOS, hinders leukocyte–endothelial adhesion, and regulates the plasminogen–plasmin system. TGF-b has complex actions on macrophages that include functional suppression and potent chemoattraction; however, the physiologic role of this latter function is unclear because TGF-b1-deficient mice develop progressive multifocal inflammation (241). TGF-b suppresses nephritogenic T-cell clones *in vivo* and downregulates tubular cell MHC class II and B7 expression (110,242).

Platelet-derived growth factor (PDGF) is a mesenchymal cell mitogen and chemoattractant that plays an important role in renal injury and repair (52). Renal PDGF and PDGF receptor expression is increased in a variety of experimental and human immune renal diseases. PDGF and bFGF are potent mesangial cell autocrine growth factors; neutralization of PDGF ameliorates injury in anti-Thy-1 nephritis. Basic FGF is fibrogenic; its production is linked to progressive glomerulosclerosis and tubulointerstitial fibrosis (243,244). Basic FGF and vascular endothelial GF (VEGF) are angiogenic and linked to glomerular endothelial repair.

COAGULATION

Fibrin deposition, increased procoagulant and plasminogen activator inhibitor (PAI) activity, and decreased PA are common in human and experimental crescentic GN. Enhanced macrophage and renal cell tissue factor procoagulant activity (PCA), IL-1b, and TNF-a promote an imbalance in endothelial proinflammatory and anticoagulant activity and in the plasmin fibrinolytic system that favors intravascular thrombosis and platelet adhesion and activation (245). Anticoagulant and fibrinolytic therapies decrease fibrin deposition and crescent formation in experimental GN (reviewed in 246). Notably, thrombin has proinflammatory effects in addition to its procoagulant activity that augments injury in crescentic GN (247).

Parenchymal Cell Responses to Immune Injury

Resident renal cells are crucial modulators of the renal response to immune injury. Resident cells activated by ICs, C, autoIg, inflammatory cytokines (IL-1b, TNF-a, IFN-g), angiotensin II, thrombin, GFs, and other stimuli undergo considerable morphologic and functional alterations and express an array of *de novo* cytokines, GFs, receptors, and other molecules that act in a paracrine or autocrine fashion to modulate injury (Table 50.5). In addition to inflammatory mediators, numerous endogenous defense systems are upregulated, the balance of which ultimately determines the outcome (progression or resolution) of renal injury (220).

Mesangiolysis, matrix deposition, and proliferation induced by a vast list of leukocyte, platelet, endothelial, and podocyte-derived agents are prominent components of the mesangial cell response. Disease resolution depends on the type and duration of injury, mesangial cell apoptosis, and upregulation of antiproliferative factors; persistent imbalance in control of proliferation and matrix production leads to glomerulosclerosis (248). Pharmacologic interference with mesangial cell proliferation (i.e., blockade of PDGF, IL1-b, endothelin) proved beneficial in disease models. Endothelial cell activation leads to phenotypic changes that promote leukocyte adhesion and infiltration, intravascular thrombosis, and platelet aggregation. bFGF and VEGF play an important role in renal capillary repair in resolving GN. Injury of the highly differentiated glomerular visceral epithelial cell (podocyte) is typically noninflammatory, presumably because the podocyte is separated by GBM from the capillary lumen. Ig, MAC, or mediator-induced damage to the podocyte interferes with its endocytic functions and maintenance of glomerular permselectivity and usually is accompanied by significant proteinuria. Podocytes normally have minimal proliferative capability; however, dedifferentiation and dysregulation of podocytes may contribute to the excess extracapillary cells observed in “collapsing” variant focal segmental glomerulosclerosis (FSGS). In contrast, activated parietal epithelial cells undergo substantial proliferation and constitute a major component of cellular crescents.

Much of the progressive functional deterioration in glomerular disease is attributable to the development of tubulointerstitial injury. It has been proposed that bioactive compounds (C, iron-generated free radicals, inflammatory fatty acids, lipid mediators, cytokines, GFs, albumin) derived from the injured glomerulus reach the tubulointerstitium via abnormal ultrafiltration, postglomerular capillaries, or diffusion from the mesangium (249). These agents directly stimulate tubular cell proliferation and matrix production, modulate epithelial cell–matrix interactions, and attract mononuclear cells into the interstitium. These injurious pathways may be balanced by glomerulus-initiated tubuloprotective mechanisms (i.e., heme oxygenase expression) (250).

CLINICAL ASPECTS OF IMMUNOLOGIC RENAL DISEASE

Immune responses directed at the kidney initially may target primarily the glomerulus, tubulointerstitium, or vasculature, but, as disease progresses, other compartments inevitably become affected. Distinguishing clinical, laboratory, and histologic features of major immunologic renal diseases in humans are shown in Table 50.6; additional comments are included below. Early injury leads to well-characterized clinical manifestations (Table 50.7) or some combination of these findings. The clinical presentation depends in large part on the degree and site of underlying renal inflammation and proliferation. Glomerular pathologic entities associated with minimal cellular reaction typically present with minimal findings or proteinuria that ranges from mild to nephrotic range (arbitrarily defined as greater than 3.5 g daily).

Those glomerular entities marked by inflammation and proliferation (i.e., GN) typically are associated with an active urine sediment (hematuria, pyuria, red blood cell casts, and proteinuria) and variably with acute renal insufficiency (elevated serum creatinine), oliguria, and hypertension. GN is a manifestation of several systemic and renal-limited (primary) diseases (Table 50.6), such that correlation of clinical, serologic, and pathologic evaluation of renal biopsy are usually necessary for definitive diagnosis. Notably, hematuria and acute renal failure are also common in allergic TIN in which interstitial inflammation is the pathologic hallmark. Two common disorders, minimal change nephrotic syndrome (MCNS) and FSGS, lack significant Ig and C deposits and cellular infiltrates and thus are not regarded as classical immune-mediated diseases. Several features suggest a role for the immune system in disease pathogenesis, however.

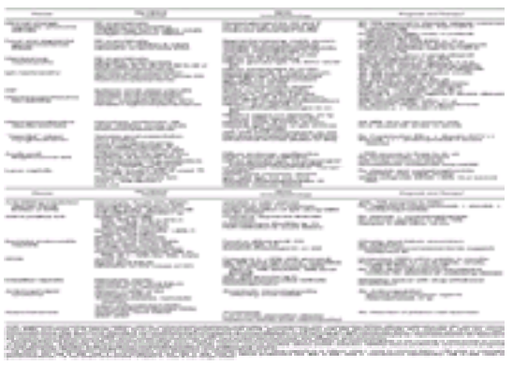


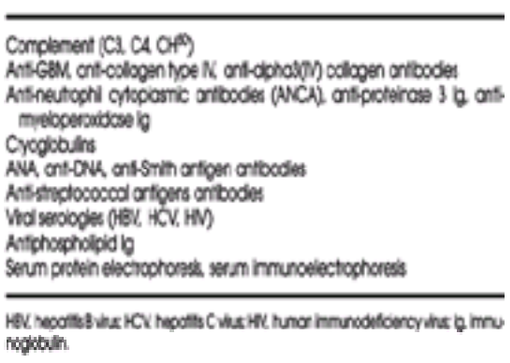
TABLE 50.6. Human Renal Diseases with a Presumed Underlying Immune Pathogenesis

Asymptomatic urinary abnormalities (proteinuria or hematuria)
Acute nephritic syndrome
Acute glomerulonephritis
Acute allergic interstitial nephritis
Nephrotic syndrome
Rapidly progressive glomerulonephritis
Chronic interstitial nephritis
Chronic glomerulonephritis
Symptoms related to underlying systemic disease
Thrombotic microangiopathy

Nephrotic syndrome is arbitrarily defined as greater than 3.5 g per day proteinuria with its attendant edema, hypoalbuminemia, and hyperlipidemia.

TABLE 50.7. Clinical Syndromes Associated with Immune-Mediated Renal Diseases

Serologic tests are useful in differential diagnosis (Table 50.8), particularly when kidney biopsy is delayed or contraindicated and in rapidly progressive GN (RPGN) and life-threatening pulmonary or renal syndromes (SLE, GPS, ANCA+ necrotizing vasculitis). Serologies must always be interpreted in the context of the clinical features. Serum C profiles for C3, C4, and CH^{50} are particularly useful in the setting of acute GN because of the frequent association of certain disorders with low or normal C levels (251). Notably, hypocomplementemia indicates only that C consumption exceeds production; it does not necessarily reflect disease pathogenesis. Hypocomplementemia may arise due to decreased C production, through activation and depletion of C by deposited ICs, via stabilization of C3 or C4 convertase by autoIg (NeFs), and by genetic C deficiency. Glomerular C deposition can be prominent in disorders associated with either low (e.g., APSGN) or normal (e.g., membranous nephropathy) C levels. Hypocomplementemia also occurs in nonimmunologic renal injury (e.g., atheroembolism, severe sepsis).



Complement (C3, C4, CH^{50})
Anti-GBM, anti-collagen type IV, anti-alpha3(IV) collagen antibodies
Anti-neutrophil cytoplasmic antibodies (ANCA), anti-proteinase 3 Ig, anti-myeloperoxidase Ig
Cryoglobulins
ANA, anti-DNA, anti-Smith antigen antibodies
Anti-streptococcal antigens antibodies
Viral serologies (HBV, HCV, HIV)
Antiphospholipid Ig
Serum protein electrophoresis, serum immunoelectrophoresis

HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; Ig, immunoglobulin.

TABLE 50.8. Serologic Tests Commonly used in Diagnosis and Monitoring of Immune Renal Diseases

Minimal Change Nephrotic Syndrome

This disorder (lipoid nephrosis, nil disease) gains its name from the lack of abnormal histopathologic findings on light or immunofluorescent examination of the kidney. Electron microscopy reveals glomerular podocyte changes (Table 50.6) (252). In some patients, T cells, monocytes, and IgM are found in the mesangium but appear not to have a significant impact on disease course. Although no causality is proven, there is a strong association with Hodgkin lymphoma, in which the NS may remit and relapse concurrent with the tumor. Several pharmaceuticals, atopy (asthma and eczema), and allergens have been associated with MCNS; nonsteroidal antiinflammatory drugs (NSAIDs) induce a unique renal syndrome with MCNS and marked interstitial T-cell infiltrate. HLA associations are weak. Various abnormalities in humoral and cellular immunity have been reported. Collectively, these associations and the steroid and immunosuppressant responsiveness of MCNS support an underlying immunologic basis. Humoral factors derived from stimulated lymphocytes, tumors, or serum of patients are capable of enhancing capillary permeability, but none has yet been convincingly linked to pathogenesis (252).

Focal Sclerosing Glomerulosclerosis

Focal sclerosing glomerulosclerosis derives its name from the observation that early pathologic involvement is restricted to only some tufts (segmental) of some glomeruli (focal). Biopsy sampling error may lead to a diagnosis of MCNS. A “collapsing” variant of FSGS is seen with HIV infection or idiopathic FSGS and is associated with more severe disease. The pathologic findings in more advanced disease are nonspecific and a frequent finding in progressive renal disease of diverse causes. Both sporadic disease and familial clustering are reported, as are racial differences in disease incidence and severity. Familial forms were reported; genome mapping suggests that at least three genes contribute to the familial FSGS phenotype (253). Mononuclear cells, including T cells, are often present in the glomerulus and interstitium. Sera from certain patients with an aggressive form of FSGS induce a marked increase in glomerular albumin permeability *in vitro*, which correlates with rapid recurrence (as early as 24 hours) in transplanted kidneys, implicating a circulating factor. This FSGS factor can be removed and proteinuria decreased by plasmapheresis in some patients (254).

Membranous Nephropathy

Membranous nephropathy (MN) is a pathologic diagnosis that depends on the demonstration of characteristic glomerular morphology with exclusive subepithelial localization of Ig deposits (Table 50.7); C3 and MAC are present, but cell proliferation and inflammation are absent (255). Increased urinary MAC excretion may reflect disease activity. MN occurs both in idiopathic form and in association with a wide variety of secondary causes, including infections [hepatitis B virus (HBV), malaria, leprosy, syphilis] and pharmaceuticals (gold, penicillamine, captopril). In 10% to 20% of patients initially diagnosed with idiopathic MN, an associated malignancy (gastrointestinal tract, lung, breast) eventually will be found. HLA associations vary by ethnic group. An autoimmune etiology of idiopathic MN is suggested by the

remarkable similarity to the rat HN model, although attempts to identify homologous human podocyte antigens so far have been unsuccessful. Other mechanisms of IC formation also may lead to subepithelial localization (see earlier). Microbial, DNA, thyroglobulin, tumor-associated, and additional nonrenal antigens have been identified in glomerular deposits; however, because of the rarity of suitable human biopsy tissue, little is known about the specificity of renal-bound Ig. MN occurs *de novo* after renal transplantation at relatively high frequency.

IgA Nephropathy

Immunoglobulin A nephropathy (Berger disease) is the most common primary GN worldwide. It occurs at all ages, although it is most common in adolescents and young adults. Renal presentations include macroscopic hematuria (40%–45%), which may be recurrent and synpharyngitic, asymptomatic hematuria, and proteinuria (35-40%) and, less commonly, acute GN, RPGN, or NS. The incidence and prevalence are highest in Asian countries and in Native Americans. Ten percent of cases have a family history. HLA associations differ considerably by geographic region. A subset of patients with DD genotype of ACE gene will progress more rapidly and may benefit particularly from ACE-inhibition therapy (256). Numerous links to bacterial or mucosal infections or food sensitivities are reported. A variety of nonspecific immunologic abnormalities involving IgA and mucosal immune responses are reported in patients and family members (257). Autoreactive IgA is identified in a minority of patients; specificities of renal-bound Ig have not been determined. Glomerular g/d T-cell infiltration, abnormalities in the gut mucosal g/dT-cell repertoires, and reduced terminal galactose on hinge region O-glycans of circulating IgA1 have been reported (see earlier).

Diagnosis relies on renal biopsy (Table 50.6); C3, properdin, and terminal C components are frequently present, consistent with IgA being an effective activator of the alternative pathway (258). The mesangial deposits are predominantly or exclusively IgA_1 polymers (J-chain positive). The cause of this subclass restriction is unknown. IgA subclass distribution normally varies with mucosa (e.g., 85% and 35%, of respiratory tract and colon mucosal plasma cells, respectively, are IgA_1); certain types of antigens (e.g., proteins) preferentially induce IgA_1. Mesangial IgA deposition is also prominent in Henoch-Shonlein purpura and SLE nephropathy as well as in a variety of systemic disorders (e.g., cirrhosis, dermatitis herpetiformis, gluten enteropathy, and ankylosing spondylitis) in the absence of renal injury. IgAN frequently recurs in renal allografts, implicating a systemic defect.

Pathogenesis and mechanisms of mesangial IgA localization remain speculative (257). Autoreactivity and antigen-independent mechanisms are postulated. The hinge region glycosylation abnormalities may contribute to abnormal IgA_1 deposition or clearance. An affinity of IgA for matrix fibronectin and of IgA-fibronectin aggregates for collagen have been reported. The expression of novel IgA Fc receptors, distinct from myeloid IgA FcRs, by human mesangial cells may facilitate mesangial IgA deposition and trigger cell activation (177). The antiinflammatory protein uteroglobin was demonstrated to play a crucial role in preventing murine IgA nephropathy (259). Uteroglobin-deficient mice develop most pathologic features of human IgA nephropathy, whereas administration of exogenous uteroglobin prevents glomerular accumulation of exogenous IgA. Heteromerization of fibronectin and uteroglobin prevented abnormal glomerular fibronectin and collagen deposition and abrogated formation and glomerular binding of IgA–fibronectin complexes (259). Numerous animal models of spontaneous or induced mesangial IgA deposition or, less commonly, IgAN exist and have contributed limited insight into pathogenesis (260).

Mesangioproliferative Glomerulonephritis

This primary glomerular disease is considerably more common in Asian than Western countries, with the exception of a relatively high incidence in Native Americans. It is a pathologic diagnosis based on characteristic mesangial disease in the absence of secondary causes (HSP, SLE, IgAN). Genetic and environmental contributions are suggested based on geographic distribution and a high incidence of prodromal infection in Chinese patients.

Anti-GBM Mediated Diseases

Anti-GBM Ig-mediated disease is defined as acute GN mediated by autoIgs that react with the GBM. When pulmonary hemorrhage resulting from Ig reactivity with alveolar BMs is present (50% of patients at presentation), the disease is referred to as Goodpasture syndrome (261). Hemoptysis is the most common presenting feature in GPS and often precedes development of overt GN by months, although laboratory evidence of GN (microscopic hematuria and proteinuria or red blood cell casts) is almost invariably present at presentation. Crescentic GN with variable endocapillary proliferation is common; tubulointerstitial infiltration is most marked in biopsies with significant TBM Ig deposition (262). This rare disease affects all age and ethnic groups. Familial clustering and associations with MHC class II genes, particularly DR2(w15) in white patients, suggest genetic predisposition. A variety of environmental triggers are suggested by clinical observations. The syndrome is preceded by upper respiratory tract infection or flulike illness in 20% to 60% of cases; seasonal clusters and association with influenza A2 outbreaks have been described. Pulmonary hemorrhage is more likely in cigarette smokers. Exposure to hydrocarbons, solvents, or chemicals have been reported anecdotally but has been documented in fewer than 5% of cases.

Diagnosis requires demonstration of serum anti-GBM Ig or characteristic linear immunostaining for Ig on kidney biopsy. Linear staining is variably demonstrable on lung biopsy. Numerous substrates are used as target antigen in available commercial assays; cruder preparations detect nonspecific, nonpathogenic Igs that bind collagen subunits (i.e., a1 or a4 chains of type IV collagen) other than pathogenic a3(IV)NC1 epitopes. These nonpathogenic anticollagen Igs are present at low titer in 15% of patients with GPS/anti-GBM disease and occasionally in patients with pulmonary disease of other causes. Conventional assays detect only IgG; there are isolated reports of anti-GBM disease due to IgA or IgM anti-GBM Ig. Anti-GBM titers do not predictably correlate with disease severity; however, serial titers within an individual are useful in monitoring disease activity. Prompt diagnosis is crucial because early therapeutic intervention (plasmapharesis, steroids, cytotoxic agents) is critical to renal survival. Because disease may recur in renal allografts, transplantation is routinely delayed until serum anti-GBM Igs are undetectable.

Anti-GBM disease is unique in fulfilling Koch's postulates that establish etiopathogenesis in human immune renal disease and its confirmation in an animal model (1). Anti-GBM Igs are present in the circulation and among Igs eluted from affected kidneys, including renal allografts with recurrent disease, and are capable of transferring GN to nonhuman primates (263). GPS patient sera and kidney eluate Igs were used to localize the major pathogenic epitopes to the noncollagenous (NC1) carboxyl globular domain of a3 chain of type IV collagen, that is, a3(IV) NC1 (9,57). Structural analysis suggested that the GPS epitope is normally sequestered in the hexameric NC1 structure and is exposed by chemical dissociation *in vitro* (264). Immunostaining demonstrates a3(IV) expression in kidney (predominantly GBM in humans), lung, eye, aorta, choroid plexus, cochlea, and neuromuscular junctions (265). The further restriction of clinical disease to kidney and lung, with rare reports of skin or eye involvement, are variably attributed to low-level expression of a3(IV) in certain organs and tissues, an “immunologically privileged location” of the epitope within the BM, and tissue-specific expression of variant a3(IV) chains due to alternative splicing (264,266,267 and 268). It is currently unclear whether potential tolerizing epitopes are sequestered from circulating anti-a3(IV) reactive B or T cells and whether exposure of the epitope is essential for disease initiation, Ig localization, or both. Notably, T cells reactive with GP antigen can be isolated from both patients and normal controls (112). Simple unmasking of the epitope by damage to the BM is highly unlikely to be sufficient for disease induction because anti-GBM disease is a rare complication of other forms of destructive glomerular injury. Nonetheless, unmasking may facilitate epitope access by previously differentiated immune effectors. It is possible that environmental toxins alter a3(IV) immunogenicity. The absence of pulmonary hemorrhage in a subset of patients has been attributed to restricted access to pathogenic Ig as a result of the absence of fenestrations in alveolar capillaries, a circumstance that may be overcome in experimental models by alveolar injury.

An anti-GBM-ANCA overlap syndrome characterized by pulmonary-renal syndrome, skin rash, arthritis, polyneuropathy, or uveitis has been described. Renal biopsy demonstrates variable amounts of necrosis, crescents, linear IgG deposition along the GBM, and renal vasculitis. The serum is positive for both anti-GBM and ANCA. As many as 10% to 30% of anti-GBM-positive sera are also positive for ANCA or anti-MPO Ig. It is unclear, however, whether the prognosis is different in these patients.

It is noteworthy that linear IgG staining of the GBM also commonly develops in patients with Alport hereditary nephritis receiving a kidney transplant. Patients with X-linked Alport disease, the most common type, have mutations in the a5-chain of type IV collagen. Development of Ig reactive with Goodpasture antigen is attributed to a requirement for a5 for normal incorporation of a3 into the GBM (269), such that lack of a5 disrupts expression of the pathogenic epitope. Transplantation of a normal kidney into a patient with Alport disease introduces these epitopes as foreign antigen and induces anti-GBM Ig; fortunately, development of severe crescentic GN is rare, perhaps as a result of immunosuppressive therapy.

Postinfectious Glomerulonephritis

Acute poststreptococcal GN (APSGN), a disease primarily of children over the age of 2 years, typically presents as an acute GN, with edema, hypertension, gross hematuria; RPGN is more common in adults (5%–10%). It occurs in epidemic and sporadic forms following skin or throat infections with certain strains of nephritogenic group A b-hemolytic streptococci (particularly type 49 in impetigo, the usual cause of epidemic APSGN, and type 12 or 4 in pharyngitis). Because of the latent period from infection (10 days with pharyngitis, more than 21 days with impetigo) and use of antibiotics, cultures are often negative. Anti-Group A streptococcal Ig may confirm a recent streptococcal infection if the patient did not receive antibiotics; however, antistreptococcal Igs are also frequently present in children with unrelated GN as a result of the normal high incidence of streptococcal infection in children and the persistence of elevated titers (3–6 months). Notably, the immune response to streptococcal extracellular antigens varies considerably, depending on the site of infection. Anti-DNAase B and anti-hyaluronidase titers are elevated in more than 80% to 90% of untreated patients with skin infection, whereas anti-streptolysin O (ASO) and anti-NADase titers are elevated in more than 90% of untreated patients with recent pharyngitis. The rapid, passive hemagglutination streptozyme test measures Ig responses to these four antigens and streptokinase. Titers do not correlate with the severity of renal disease. Most patients have a depressed C3 level in the first week, which typically normalizes within 2 to 6 weeks. Persistent

hypocomplementemia or significant depression of early C components (C1q, C4) suggests a different diagnosis (i.e., MPGN). Cryoglobulins, RF, and other autoIgs are reported. Renal biopsy is usually reserved for atypical presentations. C3 is present but early C components are usually absent on biopsy, suggesting C activation via the alternative pathway. RF activity is prominent among some renal-eluted Ig.

Although generally considered a prototype for human glomerular injury induced by glomerular trapping of circulating ICs, the pathogenesis of APSGN remains unclear (reviewed in 22). Several streptococcal antigens are found in affected kidneys but not among renal eluted Ig. Anionic endostreptosin and nephritis strain-associated protein (NSAP) have an intrinsic affinity for glomerular components. NSAP and preabsorbing antigen (PA-antigen) can activate C via the alternative pathway. Purified NSAP can induce glomerular lesions in rabbits and mice. Certain streptococcal M proteins share epitopes with glomeruli antigens, and patient sera antistreptococcal Igs and MAbs crossreactive with glomerular antigens are demonstrable. Streptococcal M protein and pyrogenic exotoxins can act as T- or B-cell superantigens.

Numerous additional infections are associated with glomerular or tubulointerstitial injury in humans (Table 50.2 and Table 50.6). GN occurs in about 20% of patients with infective endocarditis and 4% of children with infected ventriculoatrial shunts. Bacterial antigens have been found in deposits in some patients. RF, low-titer antinuclear antibodies (ANAs), and cryoglobulinemia are common. HIV is associated with a spectrum of lesions, including classic HIV nephropathy (FSGS and its collapsing variant) and a variety of ICGNs accompanied by dense interstitial infiltrates. HIV–anti-HIV Igs are eluted from affected kidneys. HBV is associated with polyarteritis nodosa (PAN) (see later); chronic HBV and HCV are associated with MN, MPGN, and cryoglobulinemia. It is postulated that chronic viremia may predispose to IC disease as a result of persistent but ineffective humoral response against an intracellular organism. AutoIgs are also common. Epstein-Barr virus (EBV) was linked to idiopathic chronic interstitial nephritis in humans (21). EBV genome was detected in renal proximal tubular cells, which express upregulated CD21, a known EBV receptor on B cells. These researchers postulated that tubular cell infection evokes or recruits a nephritogenic cellular immune response (21).

Membranoproliferative Glomerulonephritis (Mesangiocapillary Glomerulonephritis)

Idiopathic MPGN typically presents in adolescents and young adults and varies from asymptomatic urinary abnormalities to acute GN. MPGN that occurs in association with cryoglobulinemia is now known to be caused by HCV in most cases (see later). Recurrent synpharyngitic gross hematuria is common. Serum C3 levels are reduced (>70% at presentation, >85% during course) and tend to persist at low levels. The histologic findings are diagnostic. Two major variants that appear to be distinct entities are recognized (Table 50.7); the more common MPGN I is associated with depressed serum C4 and C1q levels, whereas these components are usually normal in MPGN II. C3 and C4 NeF may be present with either type of MPGN. The different C profiles are presumed to reflect differences in mode of C activation.

Pathogenesis is poorly understood. MPGN is associated with a variety of C-deficient states (partial lipodystrophy and genetic deficiency of C1q, C2, C3, C6, C7, C8, factor B, factor H, C1 esterase inhibitor). This may lead to defective C regulation of immune responses or defective IC clearance. Renal pathologic findings (Table 50.6) suggest that MPGN I is an immune deposition disease, possibly related to chronic antigen exposure (i.e., viral antigen). The BM-dense deposits in human MPGN II stain for C3; however, their complete biochemical nature remains unclear. Notably, MPGN II recurs almost invariably in renal allografts, suggesting a systemic factor is involved. Pigs genetically deficient in C factor H develop a lethal MPGN type II characterized by massive glomerular intramembranous dense deposits containing components of the alternative and terminal C pathways (see earlier) (156). It is postulated that the absence of factor H permits spontaneous C activation and *in situ* C deposition in the GBM. A role for factor H dysfunction in human MPGN type II is unclear.

Cryoglobulinemia

Membranoproliferative glomerulonephritis is also commonly seen in the syndrome known as *essential* or idiopathic mixed cryoglobulinemia (EMC), a small vessel vasculitis now recognized in many instances to be secondary to chronic infection with HCV. More than 50% of patients have evidence of GN, with a highly variable clinical presentation. Serum levels of cryoglobulins, usually type II mixed IgM–IgG with a monoclonal IgM, are extremely variable and may not correlate with disease activity. Early components of the classical C pathway, particularly C2 and C4, are often profoundly depressed, whereas C3 levels may be normal. C levels may not correlate with nephritis severity or normalize with clinical remission. Kidney biopsy confirms the diagnosis; pathology is typically MPGN type I, with associated intraluminal thrombi of precipitated cryoglobulins. The latter have a diagnostic fibrillar or crystalloid structure on EM. Skin biopsy of affected areas reveals hypersensitivity vasculitis and may contain HCV antigens. HCV antigen and anti-HCV Ig are often concentrated in the cryoprecipitate; polymerase chain reaction (PCR) testing for HCV RNA genome in serum and cryoprecipitate is indicated. HCV-associated renal disease is thought to result from deposition in the subendothelium and mesangium of circulating HCV antigen containing ICs and IgM RFs, with subsequent C activation. Infusion of human IgMk-IgG cryoglobulins into mice induces GN (270). Notably, however, diverse autoreactivity is common in chronic HCV infection (271) and raises the possibility of *in situ* autoIg deposition. In this regard, HCV interacts with cell membrane–bound coreceptors that modulate B-cell signaling and tolerance induction.

Rapidly Progressive GN

Rapidly progressive glomerulonephritis (RPGN) is a clinicopathologic syndrome characterized by acute crescentic GN and rapidly progressive renal failure (272). It may be secondary to any cause of systemic or primary acute GN or have no identifiable underlying cause (idiopathic RPGN). Clinical presentation, serologic tests, and renal biopsy confirm the diagnosis and underlying disease, if present. The findings on immunofluorescence microscopy divide RPGN into three major pathologic categories (linear, granular, or no Ig deposits), which are broadly associated with pathogenetic mechanisms and clinical syndromes (anti-GBM disease, non-GBM immune deposition, or vasculitis, respectively). The prognosis for untreated RPGN is poor regardless of the etiology, with the exception of RPGN due to APSGN, in which renal recovery is common.

Lupus Nephritis

Clinical renal involvement characterized by glomerular Ig deposition and mononuclear cell infiltration occurs in 70% to 75% of patients during the course of SLE; subclinical disease is typically evident on biopsy in the remainder. Clinically silent diffuse proliferative GN (DPGN) is described. The renal presentation is highly variable. New-onset heavy proteinuria or acute renal failure suggests transformation to a more severe form of nephritis (not an uncommon occurrence). Serum C levels (usually monitored as C3 due to the high incidence of C4 null genes in SLE) are usually depressed during active nephritis. Notably, however, neither C nor anti-dsDNA levels consistently correlate with disease severity, histopathology, clinical relapses, or response to therapy. Associations of SLE nephritis with MHC, particularly C4A and C4B null, and inherited C deficiencies are reported (see earlier). Severe nephritis is reported but rare in drug-induced SLE.

Renal biopsy defines histology and degree of activity (inflammation, proliferation) versus irreversible fibrosis and determines the need for immunosuppressive therapy. The World Health Organization (WHO) classification distinguishes major histopathologic patterns (Table 50.6, footnote); these correlate poorly with clinical or serologic parameters. IgG is the predominant isotype deposited, but IgM and IgA are also almost always present, as are C3, C4, and C1q. IgG1 and IgG3 often predominate in proliferative nephritis, whereas IgG_3 and IgG_4 are common in MN. Granular or linear Ig and C3 deposition in the TBM is common, especially in DPGN (class IV), as is a tubulointerstitial infiltrate composed of $CD4^+$ and $CD8^+$ T cells and monocytes. An occasional patient has severe TIN in the absence of glomerular disease. Patients with antiphospholipid Ig may have intraglomerular capillary thrombi; this is variably associated with poorer outcome. Patients with class V lupus nephritis (LN) have disease similar to idiopathic MN; pathologic clues to underlying SLE include staining for IgA and IgM as well as IgG, mesangial, subendothelial, and subepithelial deposits, and endothelial tubuloreticular structures (induced by IFN-a). SLE MN often runs an indolent course. Up to 50% of patients with MN may be ANA negative. Whereas therapy of SLE WHO class II and V disease remains controversial, the vast majority of clinicians use steroids and cytotoxic agents for induction therapy of class III and IV proliferative lesions, followed by indefinite maintenance suppressive therapy, because relapse frequently follows drug withdrawal even after years of quiescent disease. Experimental therapies show anecdotal benefit (intravenous Ig) or future promise (stem cell transplantation, costimulatory blockade). The factors that confer nephritogenicity on human SLE Ig remain unresolved (reviewed in 77,78 and 79). It is clear from study of humans and mice that a highly heterogeneous group of autoIg of diverse isotype, avidity, charge and specificity contribute to disease (see earlier).

Renal Vasculitis

Both small and large vessel vasculitis can affect the kidney. The subset of vasculitides associated with IC deposition (e.g., cryoglobulinemic, HSP, and SLE vasculitis) is discussed already and is shown in Table 50.6. A distinct subset of small vessel ANCA-positive vasculitides is associated with crescentic and necrotizing GN without significant immune deposition (i.e., pauciimmune) (Table 50.6); nonetheless, an immunologic etiology is suspected. Exposure to silica and pharmaceuticals (propylthiouracil, hydralazine, penicillamine) has been associated with necrotizing GN. Vasculitides associated with serum ANCA may affect the kidney either as part of a systemic vasculitis, Wegener granulomatosis (WG) and microscopic polyangiitis (MPA), or as renal limited disease with no evidence of extrarenal disease (considered by some to be renal-limited WG/MPA). Early in WG, urinalysis and renal function may be normal, but eventually most patients develop GN. Renal involvement is almost invariably present in MPA, and pulmonary hemorrhage due to alveolar capillaritis is common. Glomerular hypercellularity is minimal in contrast to IC deposition vasculitides.

The two ANCA subtypes, C-ANCA and P-ANCA, have distinct antigen specificities and disease associations (Table 50.6), although both can be found across the spectrum of ANCA-associated nephritis. Most patients with P-ANCA-positive crescentic and necrotizing GN, with or without systemic disease manifestations, have anti-MPO P-ANCA. It is noteworthy that most assays detect only IgG ANCA; a group of patients with an MPA-like illness with predominant IgM ANCA have been described. False-positive C-ANCAs are rarely observed in other systemic diseases, and additional specificities of P-ANCA are observed in other clinical settings. ANCA

testing is primarily useful in patients with pulmonary-renal syndrome or RPGN; ANCA titers do not necessarily correlate with renal disease activity. A restricted and shared idiotype among patients with ANCA has been reported.

Renal involvement also occurs in hypersensitivity vasculitis associated with drug or foreign antigen (viral, tumor) exposure, myeloproliferative, or autoimmune disorders. Nephritis is usually mild in Churg-Strauss syndrome (allergic granulomatosis) associated with anti-MPO ANCA in about 60% of patients. Henoch-Schonlein purpura (HSP) is a small vessel vasculitis typically affecting children or young adults (Table 50.6) and is characterized by palpable purpura, joint involvement, and gastrointestinal manifestations and nephritis in 40% to 60% of patients. HSP frequently is preceded by an upper respiratory infection. Biopsy of recent skin lesions or kidney reveals IgA deposits in dermal vessels and glomeruli. Although HSP and IgAN share immunologic and pathologic features, it remains unclear if they represent distinct entities or systemic and renal-limited variants of the same process.

Tubulointerstitial Nephritis

Human acute TIN generally occurs in association with drug hypersensitivity reactions, infection, or systemic autoimmune disease. Clinical features of hypersensitivity are often present in drug-induced disease, although fewer than 30% of patients have the complete triad (fever, rash, and eosinophilia). T cells and monocytes or macrophages dominate the mononuclear infiltrate. ICs and anti-TBM Ig are uncommon, with the exception of anti-GBM disease in which up to 70% of patients may have anti-TBM Ig. Several TBM antigens reactive with human anti-TBM Ig were isolated from rabbit or bovine TBM. One target antigen, a novel 58-kd proximal TBM molecule termed TIN-Ag, promotes BM adhesion via interaction with integrins on human proximal tubule epithelial cell membranes (273), fueling speculation that autoIg interactions with TIN-Ag interfere with matrix regulation of tubular cell function. Granular deposits along the TBM are also common in patients with SLE. Interstitial infiltrates also often accompany disorders classified as primary glomerular diseases and may be striking in certain glomerulopathies, such as HIV FSGS and SLE nephritis. The interstitial lesions are predictive of subsequent disease progression. Interstitial infiltrates are also seen in other renal lesions not traditionally associated with an immune etiology, such as urinary tract obstruction. The TIN seen in nonsteroidal antiinflammatory drugs (NSAID)-induced disease is unique in that it is frequently accompanied by NS due to a glomerular lesion, typically after prolonged drug exposure and in the absence of hypersensitivity features. As noted earlier, EBV infection of renal proximal tubular cells has been linked to chronic idiopathic TIN in humans (21).

Dysgammaglobulinemia/Dysproteinemias/Plasma Cell Dyscrasias

Renal deposition of Ig-derived protein can occur in the setting of abnormal production of excess monoclonal Ig or isolated Ig chains (paraprotein) due to a clonal B-lymphocyte expansion. The monoclonal Ig usually is detected by serum or urine immunoelectrophoresis. These are primarily diseases of the elderly, associated with multiple myeloma and other benign or malignant lymphoproliferative disorders. Only certain monoclonal Ig chains cause renal disease; nephrotoxicity and tissue deposition depend on physicochemical properties of the particular monoclonal Ig (charge, size, aggregability, isoelectric point). Proteinuria may be due to excreted L chains (Bence Jones proteinuria) or albuminuria in disorders with glomerular involvement. Therapy is directed at the underlying plasma cell dyscrasia.

A spectrum of renal pathology has been described. Nephrotoxic Ig chains that filter poorly tend to form nodular deposits in glomeruli, TBM, and other renal tissues (L- or H-chain deposition disease); amyloidogenic L chains capable of forming b-pleated sheets form amyloid deposits in glomeruli, vessel walls, and the tubulointerstitium. Extrarenal deposits are common in these systemic infiltrative diseases. Certain Igs form organized glomerular deposits with a unique fibrillar ultrastructure (immunotactoid glomerulopathy); extrarenal deposits are rare. Glomeruli stain for Ig (75% polyclonal) and C, but inflammation is uncommon. Pathogenesis is attributed to disruption of glomerular structure and function by deposited Ig. Certain L chains that pass the glomerular filter precipitate in the tubular lumens to form dense tubular casts (myeloma kidney or cast nephropathy); glomeruli are normal. Tubular cell reabsorption of toxic L chains and inflammatory reaction likely contributes to this pathology. Mixed cryoglobulinemia is typically associated with MPGN (see preceding discussion). Histopathologic findings have been reproduced by passive transfer of human paraproteins to animals (274).

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51 MYASTHENIA GRAVIS

John W. Rose, M.D., and Dale McFarlin, M.D.

> Nevertheless, those laboring with a want of Spirits, who will exercise local motions, as well as they can, in the morning are able to walk firmly, to fling about their Arms hither and thither, or to take up any heavy things; before noon the stock of the Spirits being spent, which had flowed into the Muscles, they are scarce able to move Hand or Foot. At this time I have under my charge a prudent and an honest Woman, who for many years hath been obnoxious to this sort of spurious Palsie, not only in her Members, but also in her tongue; she for some time can speak freely and readily enough, but after she has spoke long, or hastily, or eagerly, she is not able to speak a word, but becomes as mute as a Fish, nor can she recover the use of her voice under an hour or two (264,265).

Since Thomas Willis described “this sort of spurious Palsie” in 1672, the condition we now know as myasthenia gravis (MG) has provided many challenging, intriguing, and at times frustrating problems for clinicians, pathologists, and basic scientists. Associated thymic morbidity has been known for 90 years (38), and for the last five decades, the physiologic defect has been localized to the neuromuscular junction (NMJ) (94,151). Approximately 40 years ago, an autoimmune hypothesis for MG was proposed (229,230). Experimental autoimmune myasthenia gravis (EAMG) produced by immunization with acetylcholine receptor (AChR) protein was a landmark contribution (194). Evidence of an immune reaction against components of the NMJ (8,22) was subsequently demonstrated in the human disease, and in the vast majority of patients with MG, the disease has an autoimmune pathogenesis. Although the mechanisms leading to this autoimmune state remain to be fully elucidated, it is clear that the nicotinic AChR is the target antigen of this autoimmune disease. This chapter summarizes the clinical, physiologic, and pharmacologic aspects of MG and presents biochemical, pathologic, and immunologic findings that may lead to a better understanding of the pathogenic mechanisms in MG and related diseases of the neuromuscular junction.

THE NORMAL NEUROMUSCULAR UNIT

Advances in basic knowledge of the NMJ have contributed to the understanding and investigation of MG. A brief review of the normal anatomy, physiology, and pharmacology of this special interaction between nerve and muscle cells is necessary for an understanding of MG, in which transmission through the neuromuscular unit is impaired. As the axon passes distally from the motor neuron, it branches and gives rise to terminal nerve endings, which lie in a shallow primary groove in the muscle membranes. The ultrastructure of this region is shown in Fig. 51.1. The terminal axon is separated from the muscle cell membrane by an intersynaptic cleft 600 Å wide (75,105). Postsynaptically there are invaginations of the sarcolemmal membrane, forming secondary folds (75).

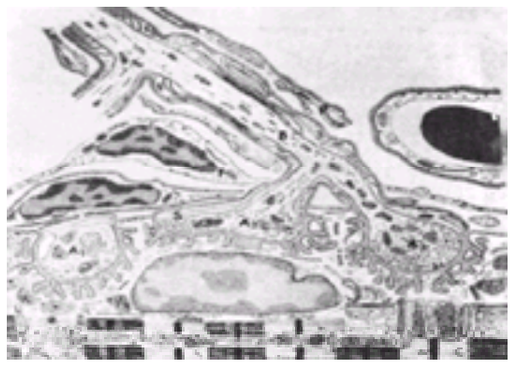

Figure 51.1. Normal neuromuscular junction. *B*, axon; A_1, A_2, A_3, axon terminals; *S*, Schwann cells; *P*, perineural cells; *F*, fibrocyte. (From Hubbard JI. Microphysiology of vertebrate neuromuscular transmission. *Physiol Rev* 1973;53:674, with permission.)

Acetylcholine (ACh) is synthesized by an enzyme, choline acetylase, in the cytoplasm of the terminal nerves and is packaged into vesicles, which tend to line up against an electron-dense area, known as the active zone, in the nerve terminal just opposite the secondary clefts in the sarcolemmal membrane. After exocytosis of the vesicles, some molecules of ACh are hydrolyzed by acetylcholinesterase; other ACh molecules traverse the intersynaptic cleft to the sarcolemmal membrane and act with the AChR. The electric organs of certain fish contain a high concentration of these AChR protein(s) and are used for many types of investigations (105,158). ACh is released from the terminal nerve both spontaneously and in response to a nerve impulse. The spontaneous release of ACh occurs constantly and gives rise to

miniature end-plate potentials (MEPPs), a phenomenon that has been shown to be quantal in nature (105). Each MEPP results from the release of ACh in a single vesicle; thus the frequency of MEPP is due to the frequency of spontaneous ACh release, whereas the amplitude of each MEPP reflects the amount of ACh released per vesicle. A nerve impulse triggers the release of larger amounts of ACh, approximately 100 to 200 quanta, which produce depolarization and subsequent contraction of the muscle fiber.

A variety of pharmacologic agents affect the NMJ (51). Three groups of agents deserve emphasis here: (a) the cholinesterase inhibitors, which decrease the breakdown of ACh and are used in the diagnosis and treatment of MG; (b) curare, which forms a competitive block of the AChR and can markedly potentiate the symptoms of MG; and (c) a-bungarotoxin (a-BTx), a protein that binds the AChR with extremely high affinity and has become a valuable tool for investigation of the NMJ (83). a-BTx is easily isolated from the venom of the elapid snake *Bungarus multicinctus* (41) and has been used to identify the presence of AChR biochemically and morphologically. The venom from another species (135), *Naja naja siamiensis*, forms a reversible reaction with AChR and is used to isolate AChR.

Biochemistry of the Acetylcholine Receptor

AChR has been purified from the electric organ of *Torpedo californica* in sufficient quantities to allow biochemical characterization. The monomeric form of *Torpedo* AChR is composed of five subunits: two a, one b, one g, and one d, with a combined molecular weight of 250,000 (117,238). The ACh-binding sites are on the a subunits (267). Recent investigations have suggested that the most likely arrangement of the subunits is abagd (238). The native receptor consists of a dimer with a disulfide bridge between the d subunits of each monomer (238). Electron microscopy in the plane normal to the membrane reveals that the subunits of each monomer form a disc with a central pit (238,267). The primary amino acid sequences of the a, b, g, and d subunit precursors of the *T. californica* AChR have been elucidated by cloning and sequencing complementary DNAs (cDNAs) for these polypeptides (47,48,180,181). Each subunit contains four putative transmembrane segments that are probably involved in forming the ionic channel (57,181).

The mammalian a subunits contain the four transmembrane segments and the regions proposed for ACh binding and major antibody-binding epitope(s), termed the *main immunogenic region* (MIR; (Fig. 51.2) (192). The AChR is a pentamer consisting of two a subunits, a single b and a single d subunit, and either a g or an e subunit. In humans, the g subunit is fetal and is replaced in infancy by an e subunit. The e subunit is necessary for normal development (178,219,269). The region on the a subunits near amino acids 192 and 193 has been proposed as the ACh-binding site (12,59,155) and is in close association with or overlapped by the a-BTx–binding site (59,87,161,179,249). ACh-binding is influenced by the d, g, or e subunits adjacent to the a subunits, resulting in different affinities of the two sites in each assembled AChR pentamer (3,172,231,238). The MIR is separate from the ACh-binding site and appears to be located in the region 67 to 76 of the polypeptide (130,238,249). The region 125 to 147 of the a subunit is exposed at the NMJ, and the synthetic peptide (125 to 147) is capable of inducing EAMG (127). The intracellular portions of the a subunits are important for binding to rapsyn, which is essential for the clustering of AChR on the postsynaptic folds of the NMJ (85,145,210). Neural factors including agrin and ACh also are required to facilitate clustering of AChR (196). Denervation results in dispersal of the clusters and increased turnover of the AChR (7).

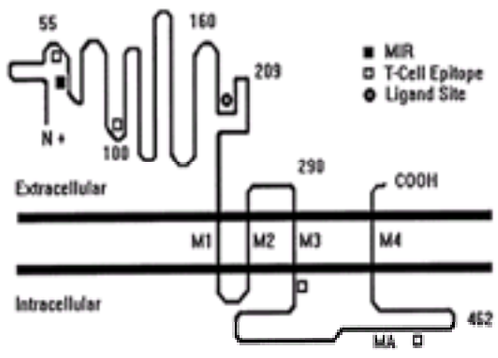

Figure 51.2. Schematic of the acetylcholine receptor (AChR) a subunit demonstrates extracellular and intracellular regions. The main immunogenic region (MIR) and ligand-binding sites are on the extracellular portion of the subunit. T-cell epitopes are more widely distributed (*open squares*). Four membrane-spanning domains are designated *M1, M2, M3*, and *M4*. The M2 domain is most critical for the ion channel formed by combination of the AChR subunits. MA is a potential fifth helix domain (58,88a,216,250).

The nucleotide sequence analysis of cloned DNAs has revealed the primary structure of the a subunit from both calf and human AChR. The mammalian a subunits show marked sequence homology with the *Torpedo* a subunits (179,180). Recent experiments using the translated products of AChR subunit–specific messenger RNAs (mRNAs) demonstrated that all four subunits are required to elicit a normal nicotinic response to ACh and confirmed that only the a subunit is necessary for a-BTx binding (161). The genes for the AChR subunits are located on three chromosomes in the mouse (a subunit on chromosome 17, b subunit on chromosome 11, and g and d subunit genes on chromosome 1) (98). In humans the a and d subunits are localized to chromosome 2, and the b subunit is on chromosome 17 (18,19). Our increased understanding of the structure and function of the AChR and the availability of pure mammalian AChR subunits are greatly enhancing knowledge of the pathogenesis and immunology of MG.

CLINICAL ASPECTS OF MYASTHENIA GRAVIS

Typical Features

MG is characterized by excessive fatigability and weakness that can be severe enough to produce total paralysis of the involved muscles (101,139,187). These symptoms usually affect muscle groups, but any individual skeletal muscle can be involved. This results in a variety of manifestations, including diplopia; ptosis; facial weakness; dysphonia; difficulty with chewing and swallowing; difficulty in breathing; inability to maintain support of the trunk, neck, or head; and weakness of the muscles of the extremities. The latter is more commonly proximal than distal. Symptoms are accentuated with the continued use of the involved muscle and improve after rest. Consequently many patients are least symptomatic in the morning and note increased severity of symptoms as the day progresses. Muscle wasting occurs in about 20% of patients, particularly in those with long-standing disease. The tendon reflexes and sensory responses are normal.

Fatigability also can be demonstrated electrophysiologically in many patients. Repetitive stimulation at low frequencies reduces the amplitude of the evoked potential and the amount of tension in the contracting muscles (Fig. 51.3) (118,199,203). The symptoms of MG lessen after the administration of cholinesterase inhibitors, and it is common practice to use either an intramuscular neostigmine (Prostigmin) or an intravenous edrophonium test (203,220) as an aid to the diagnosis. These tests are not without risk of bradycardia and syncope. Although uncommon, cardiac arrest can occur after the intravenous administration of edrophonium. In contrast, competitive neuromuscular blocking agents such as curare provoke and potentiate the weakness in this condition. Minute amounts of such agents can lead to life-threatening weakness (50).

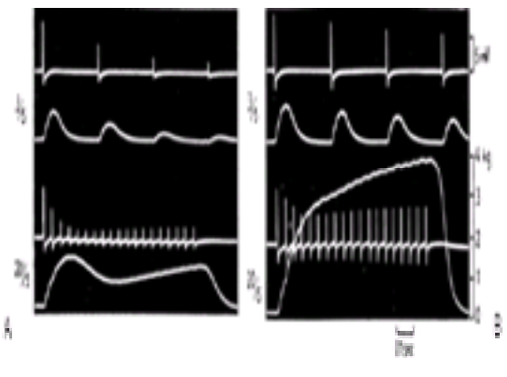

Figure 51.3. Decremental response to repetitive stimulation. **A:** Stimulation of a motor nerve first at 3 cps and later at 20 cps. **B:** Ten minutes after administration of neostigmine. (From Desmedt JE. The physiopathology of neuromuscular transmission and the trophic influence of motor innervation. *Am J Phys Med* 1959;38:248, with permission.)

Clinical Course

The peak ages of onset of MG are in the third and seventh decades. Female individuals predominate in the first group, and male individuals in the second; overall the incidence is higher in female individuals. Characteristically there is variability in both the distribution and the magnitude of involvement. Some patients have only mild involvement of a few muscles for a period of years; in others, the condition appears abruptly and in a period of weeks produces total incapacity. On the basis of clinical variants in the disease, Osserman (187) developed a classification that is a useful guide to prognosis and management. It is based on age and rapidity of onset, distribution of symptoms, and degree of involvement. The course may feature both exacerbations and remissions; however, the disease commonly progresses to severe weakness if the condition is left untreated. Since the discovery of anticholinesterase therapy and the improvement in life-support systems, the prognosis has been strikingly altered for MG patients (116,203). In general, the prognosis is best for those patients with disease restricted to the ocular muscles (29). Patients with disease involving bulbar and/or respiratory muscles have the poorest prognosis, because respiratory failure, pneumonia, or upper respiratory tract infections may develop.

Association with Autoimmune Diseases and Diseases with Immunologic Features

MG is documented in patients with systemic lupus erythematosus (SLE), thyroiditis, rheumatoid arthritis (RA), Sjögren syndrome, thyrotoxicosis, pernicious anemia, ulcerative colitis, and pemphigus (203,216). Rare cases of MG associated with multiple sclerosis are reported (140). The simultaneous occurrence of MG and any of these conditions may present extremely difficult management problems.

Ocular Myasthenia Gravis

MG may be restricted to the ocular muscles and not generalize (29). Although the patient may have major problems with diplopia, the disease has an excellent prognosis compared with that of generalized MG. Although this disorder is restricted to the ocular muscles, it can be resistant to standard treatments for MG. Only about two thirds of these patients have anti-AChR antibodies. Studies of extraocular muscles demonstrate the presence of both fetal and adult forms of mRNA for the AChR (143).

Neonatal Myasthenia Gravis

One of the pediatric forms of the disease, neonatal myasthenia, deserves special attention because it has a bearing on the pathogenic considerations discussed later. Approximately 15% of infants born of myasthenic mothers have a transitory form of the disease (160). Signs are usually present at birth but may appear at any time during the first 72 hours. Weakness of the extremities is usually symmetric and can be seen as limpness, reduced power, and absence of the Moro reflex. Weakness of the bulbar muscles can be recognized by feeble cry, inability to suck, expressionless face, and difficulty in swallowing. In severe cases, death due to respiratory failure has occurred (242). These abnormalities, which can last from only a few hours to as long as 7 weeks, respond to cholinesterase inhibitors. Recovery is complete, and the condition does not recur (242). Because of the transitory nature of neonatal MG, a circulating factor capable of crossing the placenta was implicated (159). It has now been demonstrated that in neonatal MG, there is a good correlation between decreasing anti-AChR antibody titer and improving clinical status (171).

Myasthenia Gravis Induced by Penicillamine

Autoimmune MG may develop in patients treated with penicillamine (13,244). A side effect of the drug is the induction of autoimmune diseases including not only MG, but also SLE, pemphigus vulgaris, Goodpasture syndrome, and polymyositis. MG induced by penicillamine is associated with anti-AChR antibody and, in some patients, antistriational antibody or antimuscle factor (AMF). This penicillamine-induced autoimmune MG is observed most frequently in patients with RA, and to a lesser extent in patients with progressive systemic sclerosis or Wilson disease. Clinically, the condition does respond to anticholinesterase medication and remits with discontinuation of the drug (268). Clinical improvement is associated with a marked decrease in anti-AChR antibody concentration.

SERONEGATIVE MYASTHENIA GRAVIS

Patients with clinical symptoms and signs but without detectable anti-AChR antibodies may account for 10% to 15% of individuals with MG. These patients have been described as having seronegative MG. They share characteristics of the other patients including increased sensitivity to curare and reduced AchRs at the NMJ, and their immunoglobulin can transfer disease to animals (36). The passive-transfer experiments suggest that immunoglobulin G (IgG) is more involved than is IgM. Studies by Burges et al. (36) showed that MEPPs are reduced, nerve-evoked ACh release is variable, and reduction in quantal ACh packaging can be observed (36). It is therorized that immunoglobulin may interfere with ACh packaging or release. Other studies have suggested a postsynaptic effect of the immunoglobulins (37,106). Seronegative MG may respond to plasma exchange and immunosuppressive therapy.

AUTOIMMUNE MYASTHENIC SYNDROME: LAMBERT-EATON MYASTHENIC SYNDROME

The Lambert-Eaton myasthenic syndrome (LEMS) was initially described as a paraneoplastic disorder in association with small cell carcinoma of the lung (122), but it also occurs with other neoplasms and in some patients with no tumor (65). Characteristic clinical and electrophysiologic features allow clear differentiation from typical MG (121). Weakness of ocular and bulbar musculature is rare, and is mild when present. Careful muscle testing may reveal a gradual improvement in strength on repetitive voluntary contraction. Correspondingly, repetitive electrical stimulation at frequencies greater than 10 cps increases the amplitude of the evoked potentials, whereas stimulation at 3 cps may result in a decreased response.

Electrophysiologic data indicate that the defect is due to reduced ACh released by the motor nerve (65). Freeze/fracture studies showed evidence of disruption and decreased numbers of active zones on the presynaptic membrane (80,174). Recent studies suggested that there are circulating immunoglobulins directed at the presynaptic membrane (80,81). These autoantibodies are often directed against the voltage-gated Ca^{++} channels (VGCCs) and prevent Ca^{++} entry into the nerve terminals (123,200,256). Recent evidence demonstrated depletion of presynaptic VGCCs in LEMS and the expression of neuronal class Ca^{++} channels on small-cell carcinoma cell lines (225). IgG from patients with LEMS can passively transfer the disease to the mouse and deplete the presynaptic membrane of active zones (80). Immunization of rats with cholinergic synaptosomes produces an animal model with features of LEMS (42). These findings are consistent with an autoimmune pathogenesis for LEMS (239). Treatment specific for the tumor consisting of chemotherapy and radiation therapy combined with pharmacologic and immunologic treatment may result in improved neurologic status (40).

MYASTHENIC SYNDROMES WITHOUT AN AUTOIMMUNE PATHOGENESIS

Drug-induced Myasthenic Syndromes

In certain patients, myasthenic symptoms develop after the administration of a variety of drugs including neomycin, streptomycin, kanamycin, colistin, polymycin, bacitracin, diphenylhydantoin, gentamicin, and trimethadione (268). Weakness and fatigue in such patients improve after treatment with cholinesterase inhibitors and usually resolve after discontinuation of the drug. This type of drug-induced MG is thought to be due to a direct effect of the drug agent on NMJ function (268).

Congenital Myasthenic Syndromes

Many forms of congenital myasthenia have thus far been identified and characterized (66,67,68,255). Four examples include (a) end-plate ACh esterase deficiency, (b) putative abnormality of the ACh-induced ion channel, (c) putative defect of ACh resynthesis or mobilization, and (d) congenital end-plate AChR deficiency (66,166). These diseases do not have features of autoimmune disease, are not associated with anti-AChR antibody, and generally do not respond to steroids or plasmapheresis.

Myasthenic Syndromes Associated with Denervating Diseases

A myasthenic syndrome has been described in a few patients with amyotrophic lateral sclerosis (ALS) and with the residua of poliomyelitis. In these individuals the involved muscles tire clinically and after repetitive nerve stimulation. Weakness and fatigue improve after the administration of cholinesterase inhibitors and are accentuated by curare. A potential autoimmune pathogenesis is under reconsideration as the cause of motor neuron disease in a subpopulation of patients (9). Rarely, patients appear to have features of both motor neuron disease and MG.

PATHOLOGIC CHARACTERISTICS

Thymus

About 80% of patients with MG have thymic hyperplasia, characterized by the presence of germinal centers in the medulla (Fig. 51.4) (39). In the past this was thought to represent a specific proliferation or lack of involution of thymic germinal centers, as no evidence of systemic lymphoid hyperplasia was observed. It is now believed that germinal center formation results from an intrathymic immunologic reaction (142,148).

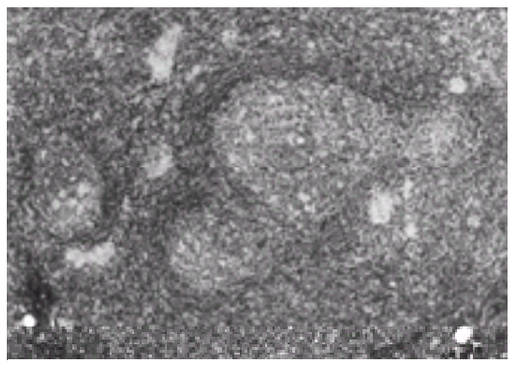

Figure 51.4. Thymic hyperplasia (×65).

Approximately 15% of myasthenic patients have a true tumor of the thymus. The thymoma associated with MG is usually well encapsulated and may show cyst formation and calcification. Microscopically, two cell types, lymphocytes and epithelial cells, are seen (Fig. 51.5). Either cell type may predominate in an individual tumor in different areas of the same tumor. Germinal centers also are seen in various areas of the thymoma. Spindle cell thymomas are virtually never seen in MG, with the possible exception of tumors that have been irradiated (216). Not all patients with MG have a hyperplastic thymus or thymoma. In a series of 35 myasthenics undergoing thymectomy, two were found to have normal thymuses histologically. In addition, involuted or atrophic thymuses without germinal centers were present in two patients (221). Germinal centers are associated with B cells that are activated (91).

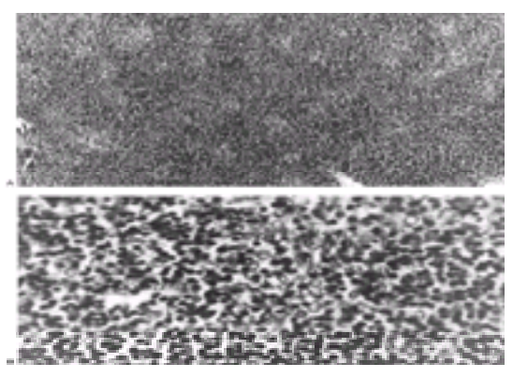

Figure 51.5. Thymoma [(A), ×50; (B), ×1,000].

Nerves

Microscopic examination of the terminal twigs of peripheral nerves, vitally stained with methylene blue, reveals two types of changes: (a) a dysplastic abnormality, characterized by marked elongation of the terminal nerve endings and by denervating processes; and (b) a dystrophic abnormality, with striking ramifications of the subterminal nerve fibers and disorganization of the terminal innervation pattern (216).

Neuromuscular Junction

Ultrastructural studies have shown that the density of synaptic vesicles in the nerve endings and the width of the synaptic cleft are normal. The major abnormality is a quantitative reduction in the subneural membrane area; the secondary folds of sarcolemmal membrane are reduced in number, and those that are present are shallow and widened (69,75,211,247). Radioiodinated a-BTx, in an autoradiographic study, showed less receptor binding in MG than in normal controls (74). In advanced cases associated with muscle wasting, the nerve endings are shrunken, and Schwann cell cytoplasm frequently extends into the cleft (75). Lymphocytes infiltrating the human NJM can be observed (150).

Muscle

The pathologic changes in skeletal muscle include focal abnormalities of the internal muscle structure (myopathic changes), which can occur with and without an inflammatory cell reaction. In some preparations, the cellular response may obscure a nearby necrotic fiber and thus appear as an isolated collection of inflammatory cells (38). Focal collections of lymphocytes in the absence of myopathic changes have been called *lymphorrhages* (38); however, serial-section studies have shown that such cellular accumulations usually lie in close proximity to a necrotic fiber (72). Reduction of fiber size (atrophy) is seen in myasthenic skeletal muscle, in one of two forms: (a) denervation atrophy, indistinguishable from that seen in diseases affecting the motor nerve; and (b) type II fiber atrophy, which is restricted to one histochemical type of muscle fiber and can accompany a variety of diseases having immunologic abnormalities as well as cachexia and disuse (72).

THE IMMUNOLOGY OF HUMAN MYASTHENIA GRAVIS

Antibody against Acetylcholine Receptor

Early attempts to demonstrate direct activity between myasthenic sera and the NMJ by immunofluorescence were unsuccessful (152). Subsequently, Almon et al. (8) reported that serum from five of 15 patients partially but significantly inhibited the binding between a-BTx and AChR from denervated muscle. An ultrastructural immunohistochemical technique that demonstrated a-BTx binding to the AChR was subsequently developed by Ringel et al. (214). Serum from 64 of 89 patients with MG blocked the binding of a-BTx to human extrajunctional receptor (137). Subsequently, radioimmunoassays were developed for detection of anti-AChR antibodies (137,162). Currently, antibodies specific for AChR are found in 90% of MG patients (185,253).

CURRENT STATUS OF THE ANTI-ACHR ANTIBODY ASSAY

Radioimmunoassay, immunoprecipitation, and enzyme-linked immunosorbent assay (ELISA) are used for the detection of anti-AChR antibodies. An internationally standardized procedure is not yet available, and methods as well as results differ in various laboratories. Therefore careful attention to laboratory standards and controls is necessary in interpreting results from these assays. A new and sensitive assay may demonstrate antibodies not detected by conventional methods in some patients thought to be seronegative by standard techniques (46,78,271). Other individuals who are seronegative for anti-AChR antibodies may have a distinct disease with an immune response directed at antigens other than the AChR and respond to immunologic therapies similar to those effective in MG (30,168).

RELATIONSHIP OF ANTI-ACHR ANTIBODY TO CLINICAL DISEASE

The majority of patients with a prolonged improvement have at least a 50% decrease in anti-AChR antibody concentration, regardless of the type of therapy (185). A decline in anti-AChR titer has been documented after thymectomy or steroid treatment (253,254). Disease severity and titer of anti-AChR antibody do not closely

correlate, but the antibody concentration is frequently high in MG patients with thymoma and relatively low in patients with localized ocular MG (183). Factors such as age, sex, and disease duration do not appear to influence the concentration of anti-AChR antibodies in MG patients when they are compared in groups.

Significant titers of anti-AChR antibody have been found in nonmyasthenic patients with SLE, Graves disease, or thymoma (84). Subsequently clinical MG develops in some of these patients, which suggests that a latent or subclinical form of the disease can exist. Significant titers of anti-AChR antibodies have been demonstrated in some patients with ALS and particularly in those treated with cobra venom (216).

Characteristics

The anti-AChR antibodies in MG patients are clearly polyclonal. IgM antibodies are produced early in the disease, and later there is a switch to IgG. Antibody to AChR is most prominent in the IgG1 and IgG3 subclasses, but can occur in all four subclasses (177,253). These antibodies are capable of passively transferring the disease to mice (245). Antibodies can be directed at epitopes on any of the AChR subunits, and some may cross-react with different subunits because there are shared epitopes (181). Although considerable diversity of antibody specificity exists, there is a predominant epitope on the a chain of AChR, referred to as the MIR (248,250). Monoclonal antibodies specific for this region can passively transfer the disease (199,234).

MECHANISM OF ACTION

Anti-AChR antibodies could change the function of AChR and/or structure of the NMJ, specifically the postsynaptic junctional folds, by at least three mechanisms: (a) blockade of the AChR, (b) modulation of the AChR, and (c) complement-mediated lysis of the postsynaptic folds.

Pharmacologic blockade of the AChR by specific antibody is the simplest explanation for the physiologic defect. Immunoglobulins capable of blocking the binding of a-BTx to mammalian NMJ were detected in the serum of 68% of MG patients (11), and Drachman (60), by using a rat muscle culture system, demonstrated that 90% of MG patients have serum immunoglobulins that produce significant blockade of a-BTx binding. Antibody that inhibits a-BTx binding to the receptor presumably also blocks ACh binding. This could result from binding to the ACh-combining site (163), steric hindrance of the ACh-combining site, or an allosteric shift of the AChR protein. Antibodies to the cholinergic binding site of AChR have been demonstrated in a large percentage of MG patients, but reactivity to other regions of the AChR is present in most individuals. The concept that anti-AChR antibodies directed at the a-BTxC binding site are the most direct and potent form for inducing disease is supported by experimental work with monoclonal antibodies. The transfer of monoclonal antibodies with this specificity against the a-BTxC binding site causes acute paralysis, but antibody directed at other epitopes does not (88). Anti-AChR antibodies from MG patients also can block the channel function of the AChR (266).

The second possible mechanism for anti-AChR action is modulation of the AChR after cross-linking by antibodies. Immunoglobulins from MG patients accelerate the rate of AChR degradation (*in vitro*). This effect was dependent on temperature and required energy, but was independent of complement (216). The accelerated degradation was produced by whole antibody and $F(ab')_2$ fragments but not Fab fragments, which implies that cross-linking of the AChR was required. The aggregates of AChR more readily undergo endocytosis after the cross-linkage (207). Although this series of studies demonstrated that antibody can modulate AChR, experimental data indicate that modulation by itself is insufficient to account for the development of EAMG in receptor-immunized mice (25).

The third mechanism for the action of anti-AChR antibody is activation of the complement pathway and damage to the postsynaptic folds (129). This is supported by a number of observations. Severe structural damage to the postsynaptic membrane, especially the junctional folds (69,211,212,247), has been demonstrated electron microscopically, and IgG, as well as C3 and C9, have been localized histochemically on the junctional folds in MG patients (Fig. 51.6) (69,211,247). Large intramembrane complexes in the junctional folds of MG patients, which are compatible with the presence of complement components (211), have been demonstrated by freeze/fractures. Interestingly, complement-mediated injury results in downregulation of muscle protein mRNAs, including those for AChR (124).

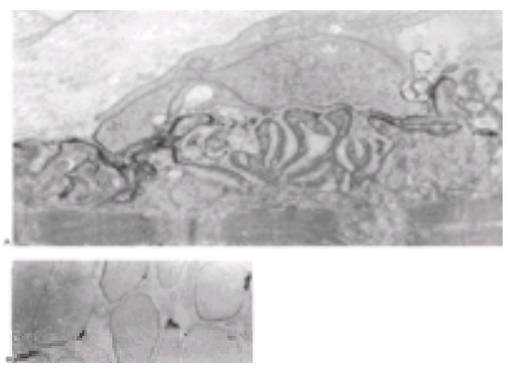

Figure 51.6. A: Localization of immunoglobulin G (IgG) at an end-plate by using peroxidase-labeled staphylococcal A protein. IgG deposits are dark and have a patchy distribution, occurring on some junctional folds but not on others and on debris in the synaptic space (×33,900) (Courtesy of A.G. Engel). **B:** Localization of C9 at the motor end plates by indirect immunoperoxidase method (×270) (Courtesy of A.G. Engel).

IN VITRO *PRODUCTION*

Peripheral blood lymphocytes (PBLs) from approximately 25% of MG patients produce anti-AChR when cultured *in vitro*. The production of AChR antibody is enhanced by culturing PBLs with mitogen or autologous irradiated thymus cell suspensions (93,176). This observation suggested that thymus from MG patients contains cell-associated antigen or helper T cells that facilitate specific antibody production. In addition, the percentage of B cells in sections of hyperplastic thymus from MG patients (2,202) was increased, and suspensions of thymus cells from MG patients contain increased numbers of B cells that spontaneously secrete immunoglobulins and anti-AChR antibody (132,139,263). In a study of cultured thymic tissue, 19 of 35 patients with MG spontaneously produced anti-AChR antibody. Antibody production correlates with each of the following factors: thymic hyperplasia, long duration of disease, and high serum anti-AChR antibody titer. *In vitro* antibody production was not detected in cultures from seven MG patients with thymoma. These findings suggest that in MG, the thymus is a site of accentuated B-cell activity (91,132).

Antimuscle Antibodies and Other Autoantibodies

Autoantibodies directed against human skeletal muscle were described by Strauss et al. (236). Subsequently, van der Geld et al. (251) found that g globulin from MG patients also reacted with a component of thymus. Immunofluorescence studies localized this reactivity to large epithelioid cells in fetal tissues. These thymic cells were shown to contain myofilamentous elements and sarcoplasmic features indistinguishable from skeletal muscle (237) and consequently became known as *myoid cells*. Antibodies from MG patients with thymoma may bind to titin (1,170) and or ryanodine receptor (232). These antimuscle protein antibodies are found in 30% of all MG patients and in up to 95% of MG patients with thymoma and can be detected by immunofluorescence or enzyme immunoassay (203). Antibodies to rapsyn, an intracellular muscle protein important in anchoring the AChRs in clusters can be found in some MG patients (5). The physiologic significance of these antibodies directed at muscle proteins is not known.

Antibodies to other tissues as well as to nuclei and nucleic acids are present in the serum of a significant proportion of MG patients. These include antithyroid and antiparietal cell antibodies, RF, antinuclear antibodies (ANAs), anti–single-stranded DNA antibodies, anti–double-stranded DNA antibodies, antithymocyte antibodies, and cold-reactive lymphocytotoxic antibodies (35,104). Antibodies directed against b-adrenergic receptors are found in MG patients (270). In some patients the presence of such autoantibodies is associated with another clinical disease, for example, RA or SLE; however, this is often not the case. Anti-presynaptic membrane receptor antibodies have been demonstrated in patients with MG (141).

HLA Association and Other Genetic Factors

Numerous studies demonstrated the association of human leukocyte antigen (HLA)-A1 and -B8 with MG in patients younger than 40 years (20,76,201,217). The highest association is with HLA-B8, which is characteristically found in young female patients with thymic hyperplasia and a high anti-AChR antibody titer (76,201). HLA-DRw3 also has been linked to MG (55). Recent study of Norwegian MG patients confirmed an increased frequency of HLA-B8, -DR3, and *DQA1*0501* (235). In a study of an Italian population, MG was associated with the DQB*502 antigen (14). In the Japanese population, in which there is an extremely low presence of HLA-B8, MG is associated with HLA-B12 and HLA-Bw35 (54). Japanese women with early-onset MG have an association with HLA-DRw53 and DQB (167). In Chinese patients the HLA-DRw9 and HLA-Bw46 antigens are associated with MG (97). Interestingly, HLA-B8 is not associated with penicillamine-induced MG. In this drug-induced MG,

the HLA-Bw35, -DR1 haplotype is the locus for susceptibility (55). The association of MG with particular HLA types and other autoimmune disease suggests that altered immunoregulatory mechanisms contribute to the pathogenesis of MG. This is confirmed by studies demonstrating altered disease severity in transgenic mice expressing different human class II molecules (240).

Other factors involved in the susceptibility to MG have included immunologic and nonimmunologic traits. Most of these associations are the results of a study in particular populations of MG patients. A number of investigators have found that polymorphisms in the region of tumor necrosis factor (TNF)-a genes are associated with MG (103). A study of markers in the CTLA-4 gene region has shown an association with MG (102).

T-Cell Studies

The numbers of blood lymphocytes and lymphocyte subpopulations have varied among reports. Although alterations in total PBLs and T cells were described by several groups, a number of investigators did not find such changes (31,104,216). Modest shifts in T-cell subsets identified by phenotype markers have been described (26,27,197). A more contemporary investigation of peripheral T-cell subsets revealed that MG patients have an increased percentage of $4B4^{+}$ T cells (helper/inducer subset), which could potentiate antibody production (165).

Cellular Immunity to Acetylcholine Receptor

Early studies of cell-mediated immunity to AChR were hampered by a number of factors. AChR is relatively insoluble, and detergents required for isolation are present in most preparations. Further, the crude extracts used for assay of antibodies largely consist of other components. Consequently, studies to date have largely used AChR isolated from fish. Lymphoproliferative responses to AChR have been studied by several investigators by using electric-organ AChR. Relatively low but significant stimulation indices (SIs) have been demonstrated in myasthenic patients as compared with controls (28,49,213). Recently, T-cell lines reactive to human AChR have been derived from the blood of MG patients (100,156,206). T-cell antigenic sites on the a chain of the AChR include amino acid sequences 48 to 67, 101 to 120, 304 to 322, and 419 to 437, which have been proposed as immunodominant epitopes (205). It is currently not known if these particular epitopes play a crucial role in the pathogenesis of MG. Abnormal and normal T-cell expansion and V gene utilization have been reported in MG lymphocytes and thymocytes (90). Dramatic results demonstrating loss of AChR from the NMJ and anti-AChR antibody production were recently obtained by injecting thymocytes from $DRW3^{+}$ MG patients into severe combined immunodeficiency syndrome (SCIDS) mice. Administration of anti-Vb5.1 antibody inhibited disease in these mice. The study implicates $CD4^{+}$ $Vb5.1^{+}$ T cells in the pathogenesis of MG in $DRW3^{+}$ individuals and suggests the potential for specific therapeutic interventions for this patient population (6).

However, many investigations strongly suggest immunologic heterogeneity of autoreactive T cells specific for the AChR in human MG (146,147,156,186,274). Transfer studies in the SCID mouse have established that CD4 cells are required and that CD8 cells may not be required for disease (259,260). Therefore, CD4 anti-AchR specificity is thought to be important for human MG (209). Establishing human T-cell lines and clones specific for AChR peptides and recombinant subunits is difficult and may limit our knowledge of the T-cell repertoire in MG. Clinical trials examining the efficacy of T cell–specific therapies will be of considerable interest but could potentially be limited by immunologic heterogeneity and epitope spreading (257).

TREATMENT

Myasthenic Crisis

Recent reviews provide useful guidelines for therapy; however, MG remains a challenging disease to manage (116,203). From time to time, an extensive muscle weakness develops in many patients with MG, including life-threatening paralysis of the respiratory muscles, which is referred to as a *crisis*. Frequently patients who are in this state are not undertreated but either are refractory to the cholinesterase inhibitors or are in cholinergic crisis. It cannot be overemphasized that in crisis, the proper management is maintenance of respiration by mechanical means, whether the weakness is due to undermedication (myasthenic crisis), a refractory state, or overmedication (cholinergic crisis) (187,203). Maintaining an airway is essential to the support of respiration. Early intubation and/or tracheostomy can be life saving. With intensive pulmonary care, it has become possible to carry virtually all patients through the crisis state. Some patients with respiratory failure can be managed for short periods with a nasotracheal tube. However, for patients with prolonged pulmonary complications, tracheostomy deserves consideration. It is easier to administer effective pulmonary care through a tracheostomy than through a nasotracheal tube (92).

Cholinesterase Inhibitors

Pyridostigmine bromide (Mestinon), neostigmine bromide (Prostigmin), and ambenonium chloride (Mytelase Chloride) are used. The degree of response to and side effects from these agents differs considerably among patients. The dose and time schedule for the administration of anticholinesterase medication must be adjusted in each individual. Potassium and ephedrine, alone or together, are commonly used as adjuncts to anticholinesterase therapy (216). Even with an optimal regimen, relief of 90% of symptoms is rarely obtained, and most patients receive far less benefit.

Excessive amounts of anticholinesterase drugs produce depolarization, which can superimpose weakness that in many cases is impossible to distinguish from that of the disease. On occasion the administration of a given dose of anticholinesterase can result in the improvement of one group of muscles and the simultaneous weakening of another group because of cholinergic side effects. Therefore an order of priority among functional muscle groups should be established. At highest priority are, obviously, the respiratory muscles, and second are the muscles used for swallowing.

Thymectomy

In 1939 the report by Blalock et al. (32) of dramatic improvement after thymectomy provided hope for an alleviative form of therapy. Recent evaluations of thymectomy reaffirmed its therapeutic potential in MG (45,62,169). Thymectomy by the transsternal approach allows the best visualization of gland and any ectopic thymic tissue. A combined cervicothoracic approach that produces lower postoperative morbidity and comparable clinical efficacy to transsternal thymectomy has been advocated by some thoracic surgeons (262).

In general, the indications for thymectomy (125,188) are (a) an operable mediastinal mass (presumably a thymoma), (b) poor medical control of the disease in patients with no contraindication to surgery, and (c) generalized MG in patients of either sex younger than 40 years who have had the disease for less than 5 years. The last group can be expected to receive the most benefit. Implicit in the use of thymectomy for the treatment of MG is commitment to highly individualized, meticulous management during the postoperative period. Some physicians have advocated the use of steroids before thymectomy for two reasons: to improve the clinical status of the patient preoperatively and to decrease the size of the thymus gland at the time of operation. The mechanisms by which thymectomy improves the clinical course of MG are not definitely known. Three factors that may alter the pathogenesis in MG after thymectomy are (a) removal of antigenic stimulus (73,110,173), (b) decline in thymic hormones or lymphokines (51), and (c) change in subsets of regulatory or autoreactive lymphocytes (31,48,82).

Corticosteroids

The use of corticosteroids in the treatment of MG was briefly investigated three decades ago and judged to exacerbate the disease (228). Von Reis and Matell (258) emphasized that after corticotropin is discontinued, the weakness is sometimes followed by a dramatic remission. Long-term treatment with high doses of prednisone is established as beneficial (108,203,261) but not without side effects (33,111,203).

When prednisone is started, it is preferable to have the patient off cholinesterase inhibitors. This is easily accomplished with patients in crises who are maintained on assisted respiration, or with patients with mild disease. Such patients may be started on approximately 1 mg/kg of prednisone, once every other day (104,261).

Patients taking cholinesterase inhibitors who are switched to corticosteroids present special and difficult problems of management. Some physicians gradually increase steroids (223). Others (71) first reduce the dose of cholinesterase inhibitors to the lowest amount that maintains respiration and swallowing, and then add corticosteroids in gradually increased amounts while reducing cholinesterase inhibitors. Other regimens including high-dose methylprednisolone have been effective (134). The simultaneous administration of steroids and cholinesterase inhibitors may result in unpredictable and life-threatening weakness and hence should be initiated on an inpatient basis.

Plasmapheresis

Plasmapheresis, either alone or in combination with immunosuppressive drugs, has been reported to be of therapeutic value in patients with MG (53,198,227). Similarly, exchange transfusion has been beneficial in the treatment of neonatal MG (58,193). The presumed mechanism is by reduction of the circulating antibodies against the AChR. Whereas plasmapheresis has proved to be a useful adjunct to therapy of acute, severe disease, it has not been effective as a long-term treatment. A favorable response to plasmapheresis suggests that the institution of immunosuppressive therapy will be beneficial (215); however, continued plasmapheresis does

not appear to act synergistically with immunosuppressive therapy such as azathioprine. Currently, plasmapheresis should be used for short-term treatment for relief of acute severe weakness unresponsive to anticholinesterase medication, thus benefiting the patient during the period before immunosuppressive therapy has become effective (96,227). Plasmapheresis also has been used preoperatively in patients undergoing thymectomy to improve postoperative respiratory function. One case report suggested that acquired antibody-negative MG may respond to plasmapheresis (159). Plasmapheresis must be administered with care in patients treated with anticholinesterase medication to avoid acute weakness precipitated by a rapid fluctuation in drug level. More selective forms of apheresis that remove IgG or anti-AChR antibodies alone are under development and will have to be compared with conventional plasmapheretic techniques (23,77,182,271).

Intravenous Immune Globulin Therapy

A recent review of intravenous immune globulin (IVIG) treatment compiled the results of the therapy in MG. Treatment of more than 60 patients is documented in the literature, with a 73% improvement rate. A significant decline in anti-AChR titer is documented after IVIG therapy (11). IVIG therapy is still under investigation. The immune globulin may be recommended as an adjunctive therapy that is beneficial in (a) reducing symptoms while other immunosuppressive treatments are initiated and (b) patients who are unable to undergo or are not responsive to plasmapheresis (11,52,115,149,275). Although it is generally well tolerated, there is the risk of anaphylaxis, leukopenia, and stroke with IVIG therapy.

Azathioprine and Cyclosporine

Results of therapy with azathioprine alone or in combination with steroid and/or thymectomy support the efficacy of this cytostatic drug. A long-term dosage in the range of 150 to 200 mg/day appears to be required for the maintenance of clinical improvement (157). Severe side effects including bone marrow suppression and systemic infection may be life threatening; therefore careful monitoring is required. Because the drug is teratogenic, it necessitates reliable birth control when administered to patients of childbearing age. Long-term therapy may be very effective, resulting in (a) decreased dosage of pyridostigmine and prednisone, (b) decreased number of hospitalizations, and (c) fewer instances of respiratory failure. The side effects of long-term therapy are not established. The possibility of oncogenesis exists with azathioprine therapy (147).

A double-blind, randomized, placebo-controlled trial of cyclosporine in MG documented that the drug is an effective treatment (243). Cyclosporine is not thought to be superior to prednisone and is limited by renal toxicity.

ANIMAL MODELS

Experimental Autoimmune Myasthenia Gravis

The major emphasis of this chapter is on MG as a human disease; however, significant contributions to the understanding of MG have emerged from studies in experimental animals, and considerable effort is still being devoted to this approach. The production of EAMG in rabbits by Patrick and Lindstrom (194) was a landmark contribution. This was accomplished by the administration of AChR from *Electrophorus electricus* in Freund's complete adjuvant. The animals developed weakness and electromyographic abnormalities consistent with neuromuscular dysfunction. Both of these parameters improved after the administration of ACh esterase inhibitors. EAMG was subsequently produced in rats (131,138), guinea pigs (136), monkeys (241), and mice (24,25,44). A variety of questions have been addressed and resolved. The sequential evaluation of physiologic and ultrastructural changes in the NMJ of rats documented that the postsynaptic region is the primary target of the autoimmune reaction. Massive destruction of this region occurs during the acute disease, and in chronic forms of the disease, the ultrastructural morphometric and electrophysiologic changes of the NMJ resemble the finding in human MG (70). Passive transfer of anti-AChR antibodies from rats with EAMG-induced signs of acute disease included invasion of the NMJ by mononuclear cells within 24 hours (70,138,204).

Animal investigations provide an approach for dissecting the immune response to AChR (191). Although some progress has been made, the results have not been as clear-cut as might have been anticipated. The presence of detergents in AChR preparations has complicated biologic experiments, and the presence of an immune response, either humoral or cellular, is not always associated with clinical disease; the latter is particularly true in mice. Lymphocyte proliferation and *in vitro* antibody production by lymphoid cells from Lewis rats immunized with *Torpedo* AChR were shown to be dependent on macrophages and T cells (56). B cells are not required as antigen-presenting cells (134). The proliferation of helper T (Th) cells was required for antibody production. The magnitude of the *in vitro* responses in this set of experiments was proportional to the total amount of circulating antibody *in vivo* and also to disease severity. Interestingly Th1 or Th2 help may generate EAMG (218). Obviously B cells are required, and the $CD5^+$ B cells associated with antibody secretion are increased (126). The demonstration that recombinant human AChR can induce EAMG in animals is a significant advance that may be particularly useful for evaluating immunotherapies (128).

The immune system of mice has been extensively characterized, and it would be expected that this would be an ideal species in which to study EAMG; however, the expression of disease varies considerably among different strains of mice and even among individual mice of a given strain (79,190,191). Variation in disease manifestation does not correlate with the immune response to AChR. Most studies of murine EAMG used *Torpedo* AChR, which shares some antigenic properties with mouse AChR; however, the shared epitopes may be less important in disease expression than the unshared ones. In addition, it was previously emphasized that many mice that appeared clinically normal after challenge with AChR clearly have NMJ dysfunction by electromyography (190). A small peptide of the AChR can stimulate T cells to provide help for a heterogeneous antibody response that results in impaired muscle function (246). Induction of EAMG in interleukin (IL)-4–deficient mice results in increased disease severity (112). IL-4 is not required for induction or progression of EAMG (17). Interferon-g (IFN-g) is important for EAMG (272). Deficiency of IFN-g reduces the incidence and severity of EAMG (273). Recent evidence implicates IL-12 in the induction of EAMG (164). Of particular interest are the investigations demonstrating that CD28–/– mice are less susceptible and CD40 ligand –/– mice are completely resistant to induction of EAMG (226).

The association between MG and major histocompatibility complex (MHC) gene products has stimulated the assessment of genetic factors that could account for variations of EAMG in mice (43). The data indicate that disease susceptibility and lymphocyte proliferation to AChR are influenced by genes within the MHC. Studies of lymphocyte proliferation in congenic and recombinant mice have mapped the response to a gene in the IA subregion. Further, experiments with allelic mutant animals indicate that the IA b chain is responsible for immune responsiveness (21,44). Obviously, extension of these studies using murine AChR and electrophysiologic techniques for evaluation would be of considerable interest.

The availability of the EAMG model has led to a better understanding of the nature of antibody responses and interactions with AChR (89), as well as regulation of the humoral responses (43). Recent studies demonstrated the therapeutic potential of a variety of treatments *in vitro* or *in vivo*, including (a) immunosuppressive drugs and IVIGs (61), (b) isogenic monoclonal anti-idiotype antibody (4), (c) Fab and Fv fragments of monoclonal anti-AChR antibody specific for the main immunogenic region (192,234,248), (d) suppressor-cell induction (153), (e) T-cell vaccination (109), (f) immunotoxins (16,119,154,184), (g) CTLA-4-Ig (154), (h) AchR and anti-AchR antibody feeding (15,99), and (i) immunizations with recombinant AchR or T-cell epitopes (113). Representative examples of the recent experimental immunotherapies are listed in Table 51.1, and of note several are effective during ongoing disease, which makes them potentially useful in human disease. A number of recent investigations have shown promise for peptide therapies in the EAMG model (10). These include cyclic peptide–blocking antibody specificity, AchR peptide sequences, and tandem peptides of T-cell epitopes (114,252).

Therapy	Effect
IVgG	Inhibits ongoing disease
IVgM (human)	Inhibits ongoing disease
CTLA-Ig	Inhibits ongoing disease
MIR specific Fv fragments	Inhibits ongoing disease
Recombinant AchR fragments	Inhibits ongoing disease and disease initiation
IL-2 Toxin	Inhibits ongoing disease
TCR specific epitope sequences	Augments ongoing disease but inhibits disease initiation
Oral anti-AchR	Inhibits disease initiation

IVgG, intravenous immunoglobulin G; MIR, main immunogenic region; AChR, acetylcholinase receptor; IL, interleukin; TCR, T-cell receptor.

TABLE 51.1. Experimental Treatments in the EAMG Model

FUTURE DIRECTIONS

MG represents a prototype of autoimmune disease in which antibodies against AChR lead to clinical manifestations. Recent discoveries reflect the value of basic and

clinical research. Nevertheless, important questions regarding the pathogenesis and treatment of the disease remain.

How Is the Autoimmune State Initiated and Perpetuated in MG?

Although the answer to this question is unknown, data from studies of the thymus demonstrate that AChR is expressed on the surface of certain thymus cell populations (73,110,173,195); these epithelial cells also are the source of immunoregulatory peptides (51). These cells in the thymus bearing AChR may be the primary targets of the autoimmune disease. Once initiated, autoimmunity to AChR in muscle could be secondary but responsible for the symptoms. The potential of MG being initiated by the molecular mimicry of AChR by antigens from specific pathogens is under investigation (222).

MG has two immunologically distinct forms, which are the early-onset disease and the late-onset disease. In the early-onset MG, thymic hyperplasia with prominent germinal centers harbors B cells secreting anti-AchR antibodies (91,132). Evidence also suggests that pathogenic T cells reside in the thymus (6). This form of MG, like many autoimmune disorders, is predominant in women and has a distinct HLA association. Other factors that may be common to autoimmune disease favoring expression in women remain to be found. In late-onset MG, there is an association with thymoma and a different HLA association. The tumor may be the source of muscle antigens. This form of MG is characterized by antibodies not only to AchR but also to titin (1,170). There is not a gender bias in the late-onset disease. Perhaps this form of the disease should be considered a paraneoplastic disorder.

Can the Immune Response Be Regulated?

It is now believed that an immune network regulates the immune response (107). Anti-idiotype antibodies interact with the antigen-specific, idiotype-bearing antibodies (120,189). Some antiidiotypes have an “internal image” of the antigen and, in some situations, are capable of initiating an immune response in the absence of antigen. MG provides an opportunity to evaluate and apply the concepts of the network hypothesis in a spontaneously occurring human disorder. Recently, in human MG, autoanti-idiotype antibodies have been described in some individuals (63,64). The interaction between idiotype and anti-idiotype does not appear to be restricted to immunoglobulin, and it now appears that the antiidiotypes may bind T-cell receptors as well. Both anti-idiotypic B and T lymphocytes have been found in MG (208). Anti-idiotype antibodies have been shown to interfere with antigen recognition by T cells (236) and, in some instances, may generate antigen-specific suppressor cells (88). Whether such mechanisms are operative in MG remains to be elucidated. Understanding of the immune response to AChR may provide new specific therapeutic approaches to the disease (34,175).

Can Therapy for MG Be Advanced?

At present we are fortunate to have effective therapies for MG, but they are not without substantial risks. Thymectomy in the early-onset patient may be curative or at least induce a remission of the disease in many patients. Administration of IVIG or plasmapheresis is particularly useful in acute crisis by respectively interfering with immunoglobulin reactivity or removing immunoglobulin with complement. Nonselective immunotherapies, particularly corticosteroids and azathioprine, help many other patients. Careful study of immunotherapies developed in the EAMG model is indicated, with the goal of finding safe and highly effective treatments. The design and conduct of clinical trials are not trivial. Special attention to patient population, treatment regimen, and outcome measures is required. Many therapies appear to be promising, based on efficacy in the EAMG model. It is our hope that over the next decade, we will have new and effective therapy for MG.

Finally, the reader of this chapter is encouraged to review the primary literature related to the AChR, the NMJ as well as MG, and the diseases of the NMJ. Only a few of the many excellent articles in these fields can be presented in this chapter.

Dedicated to the Memory of Dale McFarlin, M.D.

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52 AUTOIMMUNE BLISTERING MUCOCUTANEOUS DISEASES

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The autoimmune blistering mucocutaneous diseases are an interesting group of disorders for multidisciplinary investigation for several reasons. For clinicians, they are dramatic and potentially lethal dermatologic diseases that are therapeutically challenging. For clinical investigators, they provide unique opportunities to study organ-specific autoimmunity with a target tissue that is visually accessible and from which tissue can be easily sampled (1). For cell biologists, these diseases are defined by specific autoantibodies that have been used to identify structural proteins of the dermis and epidermis that are critical to the normal physiology of these tissues (2).

It is important to distinguish between pemphigus and pemphigoid (3). Historically, adult patients with acquired blistering diseases were all thought to have pemphigus. However, clinicians subsequently observed that a subset of elderly patients with “pemphigus” survived their disease, whereas middle-aged patients with skin and mucosal involvement inevitably died. The histopathologic studies of Lever revealed that patients with true pemphigus had intraepithelial blister formation, due to cell-to-cell detachment. Those elderly patients with a better prognosis had subepidermal blisters, which resulted from detachment of epidermis from the dermis (cell-to-substrate adhesion), and their disease was subsequently called *pemphigoid*. The similarity of the terms *pemphigus* and *pemphigoid* has caused confusion among physicians not thoroughly familiar with immunodermatology. This matter has been further complicated by the recent identification of a dermolytic blistering disease with autoantibodies against a collagen molecule that can mimic pemphigoid but was named *epidermolysis bullosa acquisita.* This chapter discusses diseases affecting epithelial cell-to-cell adhesion; disorders affecting epithelial cell-to-substrate adhesion; and vesiculobullous diseases, some of which are characterized by lysis within the dermis.

AUTOIMMUNE DISEASES AFFECTING THE DISORDERS OF CELL-TO-CELL ADHESION

The term pemphigus refers to three autoimmune syndromes that have in common the following characteristics: (a) loss of epidermal cell/cell adhesion, manifested clinically by blistering and erosions of epithelial surfaces; (b) autoantibodies of the immunoglobulin G (IgG) isotype bound *in vivo* around the plasma membranes of affected epithelium and detected in the serum (each form of pemphigus is characterized by specific autoantibodies directed against normal epithelial structural proteins); and (c) circulating autoantibodies that are pathogenic and reproduce the clinical features of the disease with precision *in vivo* (4). These three major forms of pemphigus, termed *pemphigus vulgaris, pemphigus foliaceus,* and *paraneoplastic pemphigus* (PNP), have distinguishing clinical, histologic, and immunologic features. Pemphigus foliaceus can be further subdivided into four subsets that have identical immunopathologic features but arise in unique circumstances (see later).

Pemphigus Vulgaris

CLINICAL FEATURES

Lesions of pemphigus vulgaris typically first develop in the oropharynx and subsequently affect the cornified epithelium of the upper trunk, head and neck, and intertriginous areas such as the axillae and groin. Lesions are restricted to stratified squamous epithelia, and internal organs other than the esophagus are not affected. The upper airway can be affected only to the level of the true vocal cords; below this, the airway is surfaced by respiratory epithelium, which is not affected. Small, flaccid bullae that break give rise to large painful denuded areas that do not heal without specific therapeutic intervention. Before the introduction of oral corticosteroids in the 1950s, the disease had a 50% mortality at 2 years and a 100% mortality by 5 years after onset. Death resulted from debilitation, electrolyte and protein loss through the skin, poor oral intake, and sepsis. Now mortality is estimated at about 5% and almost invariably results from complications of immunosuppressive therapy and not from the disease itself.

Cell dysadhesion affects all layers of the epidermis, and the blister forms just above the least differentiated, lowest layer of epidermal cells, the basal cells. Microscopic examination of an early lesion reveals loss of adhesion of the apposed plasma membranes of epidermal cells between the focal adhesion organelles, the desmosomes (also called *macula adherens*). Subsequently, the desmosomes also detach, and the detached cells assume a rounded morphology, called *acantholysis*. The basal epithelial cells remain tightly attached to the basement membrane of the dermis, for cell/substrate adhesion is not affected (32). Infiltration of blisters with inflammatory cells is highly variable.

EPIDEMIOLOGY AND IMMUNOGENETICS

Pemphigus vulgaris is the most common form of pemphigus in North America and Europe. It has been long suspected that a genetic predisposition exists, based on observations that this disease occurs more frequently in Ashkenazi Jews (5). In Jews of eastern European origin, the allele associated with predisposition to the disease is a rare human leukocyte antigen (HLA)-DR4 haplotype termed DRB1*0402 (6). It is interesting that this allele varies in only one amino acid from the gene associated with risk for development of rheumatoid arthritis, DRB1*0401. In other populations, a different DR allele is associated with risk for development of pemphigus vulgaris, DQB1*0503 (7). These data suggest that specific alterations in the hypervariable region of the b1 chain of HLA class II genes may confer susceptibility to pemphigus vulgaris. Given the immunogenetic predisposition, there surely must be a “second hit” that induces autoantibody production, the nature of which is unknown.

PATHOBIOLOGY

The antigen specificities of the three variants of pemphigus are summarized in Table 52.1. Pemphigus vulgaris sera immunoprecipitate and immunoblot a 130-kd glycoprotein that is complexed to an 85-kd protein called *plakoglobin*, a common cytoplasmic plaque protein. Amagai et al. cloned the gene, and sequence analysis showed the antigen to be a member of a family of adhesion glycoproteins called cadherins (calcium-dependent adhesion molecules) that play a critical role in homophilic cell/cell adhesion. The pemphigus vulgaris antigen is a newly defined cadherin, desmoglein 3 (8), with about 65% homology to desmoglein I, which is the pemphigus foliaceus antigen (9).

Disease	Antigen
Pemphigus vulgaris	Desmoflein 3
Pemphigus foliaceus	Desmoglein 1
Paraneoplastic pemphigus	Plakins, desmoglein 3, 1
Bullous pemphigoid	230-kd bullous pemphigoid antigen, 180-kd bullous pemphigoid antigen
Herpes gestationis	180- and 230-kd bullous pemphigoid antigen
Cicatricial pemphigoid	Bullous pemphigoid antigens, laminins 5, 6, others
Epidermolysis bullosa acquisita	Type VII collagen
Linear IgA bullous disease	Ectodomain of 180 kd, bullous pemphigoid antigen
Dermatitis herpetiformis	Tissue transglutaminase?

TABLE 52.1. Autoantigens in Bullous Diseases

There is convincing proof that the autoantibodies are pathogenic in all forms of pemphigus. *In vitro*, the addition of pemphigus serum or purified IgG fractions to skin organ cultures induces acantholytic changes that closely mimic pemphigus lesions. When neonatal mice are injected parenterally with human pemphigus autoantibodies, they develop cutaneous lesions of pemphigus in 18 to 72 hours (10). Therefore pemphigus is one of the handful of autoimmune diseases whose pathophysiology can be reproduced *in vivo* by passive transfer of human autoantibody alone.

Historically, there has been considerable debate about the importance of inflammatory events that occur after autoantibody binding to the epithelial cell surface. For example, the role of complement in the development of pemphigus lesions has been controversial. The data indicate that complement activation does occur *in vivo* after binding of pemphigus IgG to the epithelial cell surface and that this might augment acantholysis (11); but if enough autoantibody attaches to the cell surface, the cells will lose their adhesion in the absence of any activation of the complement system.

Early experiments suggested that pemphigus autoantibody might activate a cell-surface proteinase, such as plasminogen activator (PA) (12). The hypothesis suggests that localized increases in the activity of PA would generate plasmin and lead to the cleavage of cell-adhesion molecules in addition to the desmogleins and produce a fragile and disrupted epidermis. Enhanced proteolytic enzyme activity was observed in the conditioned medium of normal human explant cultures incubated with pemphigus IgG; the conditioned medium induced acantholysis in fresh explants, even those depleted of all immunoglobulin. Pepstatin A, an inhibitor of carboxyl-type proteinases, or high concentrations of soybean trypsin inhibitor, an inhibitor of serine-type proteinases, prevented pemphigus IgG induced acantholysis in organ culture. However, *in vivo* studies have solidly refuted this concept and showed that acantholysis can occur in the absence of increased PA activity. In mice that were pretreated with high-dose corticosteroids that effectively blocked PA activation, passively transferred pemphigus antibodies still produced blistering lesions. In PA-type I knock-out mice, the ability of pemphigus IgG to induce skin blistering also was undiminished.

In pemphigus vulgaris, antibodies impair the adhesive function of desmoglein 3 and cause acantholysis in the oral cavity, where this molecule seems to be of primary importance in maintaining cell attachment. This simple model for acantholysis was initially greeted with some skepticism, for there is considerable redundancy in epidermal cellular adhesion molecules, and it seemed unlikely that by impairing the function of just a single molecule (desmoglein 3), the integrity of the epithelium could be disrupted. This hypothesis that desmoglein 3 has such unique contributions to adhesion primarily in oral epithelium is supported by new data. Recently, a desmoglein 3 “knock-out” mouse was produced (13). These mice lacked desmoglein 3 expression in skin and mucous membranes, but acantholysis occurred only in the mouth, and in a limited fashion in areas of trauma such as the snout and paws. The skin was not affected, although hair abnormalities occurred later in life. As pemphigus vulgaris progresses, many but not all patients will develop cutaneous disease. That skin involvement occurs only in some pemphigus vulgaris patients is explained by the “desmoglein compensation hypothesis.” The key to this hypothesis is the desmogleins (pemphigus antigens) are key adhesion molecules that keep cells attached to each other. In some areas of the body, both desmogleins 1 and 3 are present, and both must be impaired by autoantibody to cause cell detachment. In some areas, only one desmoglein may be present at some level in the skin or mucous membrane, and there only one desmoglein must be damaged to cause cell detachment. The appearance of skin involvement in pemphigus seems to be caused by a “spread” of the autoantibody response and development of antibodies against both desmoglein 3 and desmoglein 1 (14). If both these desmogleins are inhibited by autoantibody, not just oral but also cutaneous blistering occurs (15). This may represent an example of “epitope spreading,” a phenomenon seen in autoimmunity, in which early in the disease course, patients make antibodies against one protein, but as the disease evolves, they then make additional antibodies against molecules that are similar in structure, or even unlike each other in structure, but are physically closely associated in a tissue.

In contrast, as explained later, in pemphigus foliaceus, blistering occurs only in the most superficial layer of the epidermis. These patients have antibodies that are directed against desmoglein 1. This desmoglein is expressed mostly in the upper levels of the epidermis, and antibodies binding to it cause cell detachment (blistering) only where it is primarily expressed. Desmoglein 1 is present in oral epithelium, but oral lesions “never” occur because there is coexpression of desmoglein 3 in the superficial oral epithelium, and the desmoglein 3 is able to keep the cells from detaching, even if desmoglein 1 is knocked out by the antibody (16). In skin, desmoglein 3 also is expressed in lower levels of the epidermis, so that knocking out desmoglein 1 does not cause blistering there, but just in the upper levels of the skin where desmoglein 1 is the only key adhesion molecule present.

THERAPY

The primary target for therapeutic intervention is the Ig-producing hematopoietic system, not the affected end organ, the skin. Topical corticosteroid therapy alone does not control this autoimmune disease because of its systemic nature; as long as there is sufficient autoantibody binding in the epidermis, lesions will be induced. Patients with pemphigus vulgaris should first be treated with oral corticosteroids (17). The disease will be controlled with reasonable doses in approximately half of such cases. As one would anticipate, based on the known pathophysiology, drugs that are effective at reducing autoantibody synthesis are useful clinically in controlling the disease. Immunosuppressive drugs used in the treatment of pemphigus, in order of their effectiveness, are cyclophosphamide (Cytoxan), azathioprine (Imuran), mycophenolate mofetil (CellCept) (18), high-dose intravenous immunoglobulin (IVIG), and cyclosporine (19). A coordinated regimen of short-term plasmapheresis combined with long-term cyclophosphamide therapy can produce dramatic improvement in even the most refractory or aggressive cases of pemphigus.

Pemphigus Foliaceus

CLINICAL FEATURES

In contrast to pemphigus vulgaris, mucosal lesions in pemphigus foliaceus are extraordinarily rare, and acantholysis occurs high in the epidermis, through the granular cell layer. Intact, flaccid blister formation is unusual, but widespread scaling and superficial denuding of the skin are common. Pemphigus foliaceus is a chronic, debilitating disease that only rarely results in death, possibly because of the superficial nature of the lesions and lack of mucosal involvement.

EPIDEMIOLOGY AND IMMUNOGENETICS

Several variants of pemphigus foliaceus are recognized. In pemphigus erythematosus, the features of pemphigus foliaceus coexist with clinical and immunologic features of lupus erythematosus.

Brazilian pemphigus (endemic pemphigus foliaceus/fogo selvagem) is immunologically identical to pemphigus foliaceus and is differentiated by its unique epidemiology (20). Its endemic occurrence in rural areas of South America suggests that an arthropod vector may play a role in the transmission of the disease, but direct proof of this is lacking. One study of Brazilian pemphigus involved 485 cases in 201 families. In 187 (93%) of the 201 families, the fogo selvagem occurred in genetically related members, whereas in 14 (6.9%) families, the disease occurred in genetically unrelated household members. There are increased frequencies of HLA-D1, DQ1, and DR4, and decreased frequencies of HLA-DR3, DQ2, and DR7 (21). Other data suggest that the HLA-DR7, DQw2 haplotype may confer resistance to the development of pemphigus foliaceus (22).

Drugs also can induce pemphigus, with most cases being identical to pemphigus foliaceus, whereas a few resemble pemphigus vulgaris (23). The agents that induce pemphigus are most often drugs with highly reactive sulfhydryl groups such as d-penicillamine and captopril (24). These drugs can directly induce acantholysis *in vitro* in the absence of autoantibody or, more commonly, may initiate autoantibody-mediated disease *in vivo* that persists even after withdrawal of the implicated drug.

PATHOBIOLOGY

Pemphigus foliaceus sera recognize a 165-kd transmembrane glycoprotein, desmoglein I (25). Desmoglein I is a cadherin and differs from “e” cadherins (epithelial cadherins found in simple epithelia) primarily by the presence of a very long cytoplasmic domain (26). This domain may mediate its interaction with plakoglobin and other intracellular proteins that link the cell surface with the keratin cytoskeleton of the cell. When IgG from patients with pemphigus foliaceus is injected parenterally into mice, the characteristic lesions of pemphigus foliaceus are reproduced precisely. In contrast to the results with IgG from humans with pemphigus vulgaris, intraepidermal blistering developed in the superficial granular cell layer, precisely replicating the distinctive changes of human pemphigus foliaceus (27). In endemic pemphigus foliaceus, the major autoantibody activity is found in the IgG4 subclass, which is thought to be functionally monovalent and incapable of activating complement (28). When the IgG4 fraction of serum from patients with pemphigus foliaceus was separated from other immunoglobulin subclasses and passively transferred into neonatal mice, characteristic lesions were induced (99). Monovalent $F(ab')_2$ fragments of the IgG4 autoantibodies also induced lesions *in vivo*. There was no detectable complement activation in the lesions of animals treated with intact IgG4 or the monovalent $F(ab')_2$ fragments. This finding is remarkable, for it is further evidence that antibodies binding to the cadherin protein, desmoglein I, can downregulate its function to the point of inducing blister formation *in vivo*, with lesions

confined to the precise layer in the epidermis where desmoglein 3 is not coexpressed.

TREATMENT

The treatment of pemphigus foliaceus is similar to that of pemphigus vulgaris, but in most patients, less aggressive therapy is required for control. In contrast to pemphigus vulgaris, drugs such as oral gold and antimalarial agents may be more useful than in pemphigus vulgaris.

Paraneoplastic Pemphigus

Paraneoplastic pemphigus is a newly described variant (29). To date, all patients have a known or occult neoplasm, most commonly, non-Hodgkin lymphomas, thymomas, and hematologic malignancies, especially chronic lymphocytic leukemia and giant follicular hyperplasia (Castleman tumor) (30). Patients have painful oral erosions that prove refractory to standard therapies and develop a polymorphous cutaneous eruption with episodes of blistering and healing. The mucosal and cutaneous blisters show a characteristic combination of suprabasilar acantholysis similar to those of pemphigus vulgaris, and keratinocyte necrosis that may resemble erythema multiforme or a lichenoid inflammatory eruption. All patients have tissue-bound and circulating autoantibodies that bind to all epithelia *in vitro* and have a common and unique immunologic specificity.

PATHOBIOLOGY

In PNP, the complex humoral immune response is still in the process of being completely defined. Current data indicate that a complex of epithelial antigens is recognized by autoantibodies in PNP. Almost all patients have antibodies against desmoglein 3, and a majority also have antibodies against desmoglein 1. Passive transfer of these autoantibodies into mice reproduces lesions only in skin and esophagus, and it has been shown that the desmoglein antibodies are specifically responsible for the induction of blistering by passive transfer (31). The concomitant presence of antibodies against plaque proteins of the desmosome distinguishes PNP from other forms of pemphigus. The antigen complex of targeted desmosomal plaque proteins consists of desmoplakin I and desmoplakin II (intracellular plaque proteins of desmosomes) (32); the 230-kd bullous pemphigoid antigen and plectin (plaque proteins of the hemidesmosomes); two other desmosomal plaque proteins, envoplakin (33) and periplakin (34); and an as-yet-uncharacterized 170-kd antigen. The autoantibodies of paraneoplastic pemphigus are most distinctive because they bind to the cell surface of all epithelia, not just stratified squamous epithelia. This is due to the wide distribution of desmoplakins in all epithelia and some nondesmosomal structures. The implications of autoantibodies with such a broad specificity are not clear; however, there is good evidence that these autoantibodies may be responsible for the induction of pulmonary epithelial lesions in patients. In about one third of patients with PNP, late onset of hypoxia and shortness of breath heralds the onset of a progressive disease of respiratory epithelium, eventuating in the syndrome of bronchiolitis obliterans, with death due to respiratory failure. Bronchoscopy of patients with PNP who have dyspnea and hypoxia reveals laryngeal, tracheal, and bronchial erosions with histologic signs of cell/cell dysadhesion and deposition of autoantibodies in the respiratory epithelium. These findings suggest that the pemphigus autoantibodies were the cause of the respiratory tract erosions (35). The multiorgan effects of these unique autoantibodies are being studied. It appears that PNP is a devastating illness, with autoantibody-induced damage to numerous epithelia.

THERAPY

No known effective therapy for this syndrome exists. In those individuals who have an associated benign neoplasm (thymoma, Castleman tumor), complete excision of the tumor results in slow improvement and often complete resolution of the syndrome. In most patients with malignant tumors, the disease becomes worse despite therapy with corticosteroids, immunosuppressives, and plasmapheresis. Death often results from debilitation, sepsis, respiratory failure, or gastrointestinal hemorrhage. Treatment of the underlying malignant neoplasm, or reduction of tumor burden alone, does not reduce the activity of the autoimmune disease, once it is initiated.

AUTOIMMUNE DISEASES AFFECTING THE DERMAL/EPIDERMAL JUNCTION: DISORDERS OF EPITHELIAL CELL/SUBSTRATE ADHESION

The term pemphigoid applies to mucocutaneous diseases that are characterized clinically by the presence of large, tense blisters, by subepidermal blister formation, and by the deposition of IgG and complement components along the basement membrane zone (BMZ) of affected skin or mucous membrane (36). Three major forms exist: (a) bullous pemphigoid, (b) gestational pemphigoid (herpes gestationis), and (c) mucous membrane pemphigoid. These diseases have clinical, histologic, and immunopathologic similarities, but the exact relationship of one to another is not fully defined.

Bullous Pemphigoid

The incidence of bullous pemphigoid is not certain but is estimated to be 1 per 100,000 per year, making the disease much more common than pemphigus vulgaris. Bullous pemphigoid occurs most frequently after age 60 years, but it may rarely occur in infancy or childhood. Blistering lesions in bullous pemphigoid are most often generalized, but in 15% to 30% of patients, the bullae may be localized, often on the pretibial area. Mucosal lesions are usually transient, trivial, and heal without scarring; when they are present, they are usually restricted to the mouth. The lack of mucosal involvement is the major criterion for distinguishing bullous pemphigoid from mucous membrane pemphigoid. In the latter, mucosal epithelia are primarily affected, scarring is prominent, and cutaneous lesions are transient and trivial. No racial or geographic predilection is recognized in bullous pemphigoid. In contrast to pemphigus, there is no increased incidence in the Jewish population, and there is no known immunogenetic association.

PATHOBIOLOGY

In bullous pemphigoid, autoantibodies are found along the dermal/epidermal junction in a fine linear distribution. The most frequently detected autoantibody is of the IgG4 subclass, and there is significant and sometimes more intense deposition of complement components that colocalize with IgG deposition (37). Occasionally IgE anti-BMZ antibodies are detected. The histology of a blister shows the accumulation of eosinophils and a few neutrophils along the dermal-epidermal junction. Blistering occurs between the epidermis and the dermis, and the roof of the blisters is formed by full-thickness epidermis. There is also a variable dermal infiltrate consisting primarily of eosinophils. Therefore the diagnosis of the pemphigoid is established by the clinical finding of tense blisters, subepidermal blister formation by light microscopy, and direct and indirect immunofluorescence that shows *in vivo* bound and circulating autoantibodies against the BMZ. Serum antibodies are detectable in 50% to 70% of patients with the disease. By indirect immunofluorescence, these antibodies bind to the BMZ of stratified squamous epithelia from numerous species.

Two unrelated proteins are recognized autoantibodies in bullous pemphigoid. Patients produce an immune response to (a) a 230-kd antigen (BP 230 antigen or BP Ag 1), which is a plaque protein of the epidermal basal cell hemidesmosome and has approximately 30% structural homology to desmoplakin 1; and (b) to a 180-kd protein (BP 180 antigen or BP Ag 2) (38), which spans the plasma membrane of the basal cell. It is a transmembrane protein with type II orientation, with its carboxyl-terminus in the extracellular environment. The extracellular domains of the antigen have several collagen-like repeats that probably interact with matrix proteins of the underlying dermal surface, and for this reason, it is also referred to as collagen type XVII. The intracellular portion is embedded in the hemidesmosomal plaque, and almost certainly mediates adhesion between extracellular proteins and the cytoskeleton of the basal domain of the epithelial cell. Patients with bullous pemphigoid often produce antibodies against both the BP 230 and the BP 180 antigens (69), even though there is no significant homology between these two molecules.

It is now established that the autoantibodies in patients with bullous pemphigoid are pathogenic. Curiously, antibodies against the BPAG1 (39) have not been shown to be pathogenic in any *in vivo* system tested, but antibodies against the BPAG2 were capable of inducing blistering *in vivo*. Passive transfer of human BP autoantibodies into neonatal mice, using techniques so successful in the study of pemphigus, failed to cause disease. The cause of this failure was that human anti-BPAG2 (BP 180) autoantibodies preferentially recognize an epitope of the ectodomain of the BP 180 antigen, and that in this key region, the murine BP 180 was not homologous to the human antigen. To overcome this problem, Liu et al. modified the traditional neonatal mouse model by first producing antibodies against the key epitope of the murine BP 180 domain by immunization of rabbits with a fusion protein. Then they infused polyclonal rabbit antimurine BPAG2 antibodies. The injected mice developed subepidermal blistering disease that closely mimicked bullous pemphigoid. Histologic examination of the skin of these mice revealed findings typical of pemphigoid, with subepidermal blistering, and a polymorphonuclear cell infiltration along the BMZ and blister cavity. Direct immunofluorescence examination of these mice revealed linear deposition of rabbit IgG and murine C3 along the epidermal BMZ. This study clearly demonstrated that anti–BP 180 antibodies bind the extracellular domain of the BP 180 antigen and initiate a cascade of events, including the activation of complement, attraction of polymorphonuclear cells to the BMZ, release of proteolytic enzymes, and finally subepidermal blistering. The complex cascade of inflammatory events (40) that occurs subsequent to autoantibody binding has been dissected, and the requisite participation of mast cells, eosinophils, neutrophils (41), complement activation, and potentially other proinflammatory events is apparently required for the formation of blisters *in vivo*. In contrast to pemphigus, in which antibody binding alone to the relevant antigen(s) can cause the characteristic tissue injury (acantholysis), in pemphigoid, lesions do not occur in the absence of a sequential series of inflammatory events.

TREATMENT

Pemphigoid has a very low mortality rate, so treatment can be less aggressive than that for other autoimmune blistering disorders such as pemphigus. Some mildly

affected individuals respond to high-potency topical steroids, but often the disease progresses to the point at which disfiguring or debilitating blisters arise on a large portion of the body. Oral corticosteroids are the drug of choice (such as prednisone at a starting dose of 0.5 mg/kg/day), and this frequently produces complete clearing of the lesions. The corticosteroids can then be slowly tapered over the next 6 months. Approximately half of the patients treated in this manner either go into remission or require a very low dose of alternate-day steroids to control the disease. Severely affected individuals can be treated with one of the following agents, listed in order of effectiveness: azathioprine (2 to 3 mg/kg/day), cyclophosphamide (1 to 2 mg/kg/day), dapsone (2 to 3 mg/kg/day), a combination of tetracycline and niacinamide (1,500 2,000 mg of each/day), and other agents such as methotrexate and cyclosporine. Mortality from bullous pemphigoid is rare; sepsis from cutaneous lesions is similarly rare; and the prognosis is good.

Gestational Pemphigoid (Herpes Gestationis)

Herpes gestationis is a disease of pregnancy. Most commonly, women in the late second or third trimester develop lesions that are clinically and histologically typical of bullous pemphigoid (42). These lesions begin in the periumbilical area and then spread peripherally. The disorder is usually intensely pruritic. Most commonly, the disease worsens until the child is delivered, and then resolves. It may flare for a short time in the postpartum period but then remits spontaneously in most affected individuals. The disease can be reactivated by subsequent pregnancies, and in general, in subsequent pregnancies, the disease starts earlier, and the eruption is more severe. The disease can be reproduced by stimulation with oral estrogen. It also has been associated with choriocarcinoma and hydatidiform molar pregnancies.

PATHOBIOLOGY

All patients with herpes gestationis have intense complement deposition along the BMZ of perilesional skin There is very weak IgG deposition, and circulating in the serum is a very low titer of avidly complement-fixing anti-BMZ antibody. Before this antibody was recognized as an IgG autoantibody, it was named the *herpes gestationis factor*. This antibody is specific for the BP 180 hemidesmosomal protein (43). Approximately 90% of individuals have reactivity solely with the BP 180 antigen, and about 10% of patients react with both the BP 180 and BP 230 antigens, similar to patients with bullous pemphigoid.

Although there is significant evidence that herpes gestationis is a form of bullous pemphigoid that occurs during pregnancy, there appears to exist a genetically determined predisposition for the development of this disease. In contrast to bullous pemphigoid, 60% to 85% of patients with herpes gestationis are HLA-DR3 positive, but more strikingly, approximately 45% have the combination of HLA-DR3 and DR4, which is present in only 3% of unaffected women.

What is curious about this disorder is the very specific induction of the autoimmune phenomenon by estrogens, either endogenous from the fetal tissues or exogenous from oral contraceptives. How this autoimmune disease is invoked in a genetically susceptible individual by these steroids is a subject of much speculation, but scarce hard data.

Mucous Membrane Pemphigoid

Mucous membrane pemphigoid is predominantly a disease of the elderly, with a peak incidence between the ages of 60 and 80 years. Juvenile cases are exceedingly rare. Lesions can arise on any mucosal surface covered by stratified squamous epithelium, including the nasopharynx and oropharynx, conjunctiva, esophagus, larynx, urethra, and anus. Morbidity and mortality are due to the scarring produced by recurrent lesions. Scarring is a major criterion for distinguishing mucous membrane pemphigoid from bullous pemphigoid. Moreover, in bullous pemphigoid, cornified epithelium of skin is the primary tissue involved, whereas in mucous membrane pemphigoid, mucosal epithelium is primarily affected. Therefore the two diseases are distinguished primarily by clinical criteria.

Clinically, lesions are smooth-bordered erosions with distinct margins, and only rarely are intact blisters observed. The gingivae are commonly involved, and mucous membrane pemphigoid is one cause of “desquamative gingivitis.” Other causes of desquamative gingivitis include erosive oral lichen planus and pemphigus vulgaris. About 10% to 15% of patients with either epidermolysis bullosa acquisita or linear IgA dermatitis may have scarring mucosal lesions that closely mimic the clinical phenotype of mucous membrane pemphigoid.

The conjunctival epithelium is affected in about 80% of patients with mucous membrane pemphigoid and is cause for considerable concern. Signs of active disease include a violaceous inflammatory infiltrate, superficial fine fibrotic bands, shrinkage of the conjunctival fornices, and entropion. Lesions of the larynx, esophagus, and genital mucosae consist of smooth-bordered erosions, with eventual stricture formation. Skin lesions occur in fewer than 20% of patients and are usually transient. When present, they consist of small intact blisters or erosions, usually in the head and neck area.

The diagnosis is established by three criteria: (a) scarring blisters and/or erosions, (b) subepithelial blistering as seen on histologic examination, and (c) direct immunofluorescence of perilesional epithelium showing IgG and complement components along the BMZ. In mucosal pemphigoid, indirect immunofluorescence for circulating antibodies is highly unreliable, and the findings are negative in the majority of cases. (This is a sharp contrast to the reliability of indirect immunofluorescence in pemphigus vulgaris and the presence of circulating antibodies in 50% to 70% of patients with bullous pemphigoid.)

PATHOBIOLOGY

It is presumed but not proved that mucous membrane pemphigoid is an autoimmune disease. This presumption is based on the invariable presence of immunoglobulins and complement components at the basement membrane of affected mucosae. There are no clear indications of what may precipitate the autoimmune disease, although, as in pemphigus, there are some reports of d-penicillamine producing the autoimmune phenomenon. There is a reported immunogenetic predisposition to the development of ocular pemphigoid associated with the HLA-B12 haplotype, but this has not been confirmed.

Studies to define the antigen that is identified by mucous membrane pemphigoid autoantibodies have been hampered somewhat by the absence of high-titer circulating autoantibody. Although some investigators showed that the sera from most patients with mucous membrane pemphigoid recognize the BP 230 or the BP 180 antigen or both, others found other antigens to be bound by circulating autoantibodies. It is now becoming apparent that the clinical disease is caused by multiple autoantibody-antigen interactions. The common link is that all of the implicated antigens are part of the keratinocyte's anchoring filament complex. This is a series of linked proteins that extend from the hemidesmosomal plaque, through the lamina lucida, and into the upper dermis. Proteins in this complex that have been targets of autoantibodies in individual cases of mucous membrane pemphigoid include the bullous pemphigoid antigens 1 and 2 (BP 230 and BP 180) (44), laminins 5 (45) and 6 (46,47), type VII collagen, and others. It has been found that different antigens were recognized by IgA antibodies as opposed to those of the IgG isotype from the same patient.

Indirect immunoelectron microscopic studies of mucous membrane pemphigoid suggest that the autoantibodies preferentially deposit in the lower lamina lucida, in contrast to the distribution in bullous pemphigoid, in which they are found in the upper lamina lucida. This suggests that the autoantibodies of some patients with mucous membrane pemphigoid have different antigenic specificities from those of the autoantibodies of patients with bullous pemphigoid (48).

THERAPY

Treatment is dictated by the organs involved and the anticipated morbidity. Once the disease develops, it is progressive, and spontaneous remission is rare. This disease is not highly responsive to steroids. Therefore, for example, if only the oropharynx or nasopharynx is affected, the anticipated morbidity is minimal, and treatment should be limited to topical steroids, intralesional steroid injections, or occasional short bursts of oral corticosteroids. These maneuvers provide palliation but do not halt progression of the disease. If only the gingivae are involved, topical therapy can be delivered by the construction of a flexible dental tray in which a topical steroid is applied with occlusion.

If the eyes, esophagus, or larynx is involved, the anticipated morbidity includes blindness and asphyxiation, and aggressive systemic therapy is warranted. Systemic steroids alone do not adequately control progression, and only an occasional patient responds well to dapsone therapy. Of the various immunosuppressive agents available, cyclophosphamide is the most effective. It is administered for a period of 18 to 24 months (at a dose of 2 mg/kg/day, until the white blood cell count is depressed to 3,000 4,000/µL). About three fourths of those treated with this regimen tolerate the drug, and at the end of the treatment period, most of those patients have complete clinical remissions that persist after all drugs are discontinued. To date, this cyclophosphamide regimen is the only treatment that provides the potential for a “cure” of this disease. Azathioprine is an alternative for patients who cannot tolerate cyclophosphamide, but it is not as effective.

DERMOLYTIC AUTOIMMUNE DISEASES

Epidermolysis Bullosa Acquisita

Epidermolysis bullosa acquisita is a disease of adult onset, originally described as trauma-induced blisters, predominantly on extensor surfaces, and healing with milia and scars. The fact that epidermolysis bullosa acquisita can mimic other vesiculobullous diseases, especially bullous pemphigoid, leads to diagnostic difficulties. It has

been found that a bullous pemphigoid like clinical presentation may occur in up to 50% of patients with epidermolysis bullosa acquisita. Neither histopathology nor routine direct immunofluorescence studies necessarily differentiate epidermolysis bullosa acquisita from bullous pemphigoid, because similar linear deposition of IgG occurs in both diseases, and histologic differentiation is not always reliable. Definitive diagnostic differentiation can be accomplished by direct or indirect immunoelectron microscopy, by indirect immunofluorescence on split skin, and by immunoprecipitation or immunoblotting. In epidermolysis bullosa acquisita, the immune deposits are in the lamina densa and in the sublamina densa area, whereas in bullous pemphigoid, they reside in the lamina lucida and the hemidesmosome complex. The antigen recognized by epidermolysis bullosa acquisita antibodies is type VII procollagen (49), a major component of the anchoring fibrils of the dermis. This antigen specificity can be established by immunoblotting or immunoprecipitation when circulating antibodies are detectable (50).

Dermatitis Herpetiformis

Dermatitis herpetiformis is most often seen in young adults with intensely pruritic, burning papules and vesicles distributed in a highly characteristic symmetric pattern over extensor surfaces of the elbows and knees, as well as the sacrum and scalp. Intact blisters are rarely observed, for the patients excoriate them vigorously in the earliest stages of formation. Small, 2- to 5-mm excoriations in a characteristic distribution are the most consistent clinical feature of the disease. Before the discovery of diaminodiphenylsulfone (dapsone) as an effective therapy for this disease, the most common cause of mortality in dermatitis herpetiformis was suicide prompted by intractable pruritus and complicated by skeptical physician reaction, for the pruritus is wildly out of proportion to the apparent severity of the lesions. Essentially all patients with dermatitis herpetiformis have an associated gluten-sensitive enteropathy, which is asymptomatic in most of them, and also have a distinctive genetic predisposition, with almost constant presence of the HLA-AI and B8 (75% to 90%), and DR3, DQw2 haplotype (95% to 100% of patients) (51). Also associated with dermatitis herpetiformis is an increased risk of intestinal lymphoma. Dermatitis herpetiformis may be confused with vesicular pemphigoid in the rare instances in which large blisters are present, but lesions in the latter disease usually lack the characteristic extensor symmetry of dermatitis herpetiformis. The two diseases can easily be distinguished by direct immunofluorescence, which in dermatitis herpetiformis uniformly shows granular deposits of IgA along the BMZ and in the upper papillary dermis (52), with accentuation within the dermal papillary tips, and circulating autoantibodies against the BMZ are usually not detectable.

There has been much speculation regarding the antigenic specificity of the dermal deposited IgA autoantibody and the role of gluten-sensitive enteropathy in this disease, but little is really known. Speculations include cross-reactivity between IgA antigluten antibodies and some dermal structure and trapping of intestine-derived IgA immune complexes in the skin, but no convincing pathogenetic mechanism has yet been proven. The tissue transglutaminase has been recently found to be the autoantigen for T lymphocytes and also the antigen for the antiendomysial antibodies (53) in patients with celiac disease and dermatitis herpetiformis (54).

THERAPY

Diaminodiphenylsulfone, in a usual oral dose of 50 to 150 milligrams per day, is a highly specific and effective therapy for dermatitis herpetiformis. Dapsone provides dramatic relief from pruritus in 24 to 48 hours. If dapsone cannot be tolerated by an individual because of congenital glucose-6-phosphate dehydrogenase (G6PD) deficiency (devastating hemolysis could result) or sulfone allergy, sulfapyridine can be obtained on a compassionate basis and will provide adequate symptomatic relief.

The complete and long-term elimination of gluten in any form from the diet allows affected patients to reduce or eliminate their dependence on dapsone (55). Compliance with such a regimen in our culture is usually impossible.

Linear IgA Bullous Dermatosis

The term *linear IgA bullous dermatosis* is applied to a clinically heterogeneous group of disorders that have in common the linear deposition of IgA, with or without complement components, along the dermal/epidermal junction. Patients with linear IgA deposits include those with clinical features of chronic bullous dermatosis of childhood, those with mixed clinical features of dermatitis herpetiformis and bullous pemphigoid, those with disorders resembling classic dermatitis herpetiformis, and a small number (~10% to 15%) who have the clinical features of mucous membrane pemphigoid. Patients with linear IgA bullous dermatosis differ from those with classic dermatitis herpetiformis by the absence of jejunal enteropathy; a less predictable therapeutic response to sulfones; and a different immunogenetic background, with no increase in the incidence of the HLA-B8, DR3, DQw2 histocompatibility antigens. Most patients with linear IgA bullous dermatosis are younger than those affected with other bullous diseases. Immunoelectron microscopy has confirmed that IgA dermatosis is heterogeneous. Deposition of IgA *in situ* has been observed in many different locations, including the hemidesmosome and upper lamina lucida, the lower lamina lucida, and beneath the lamina densa.

These findings suggest that individual cases of linear IgA dermatosis may represent unique syndromes or variants of other known syndromes such as epidermolysis bullosa acquisita and bullous pemphigoid, mediated by IgA rather than IgG isotypes. For example, in patients with linear IgA disease in whom epidermal deposition is found, many of these cases will recognize a 97-kd lamina lucida. Recent studies have shown that this antigen is homologous with a portion of the BP 180 antigen (56), but whether this is a processed form of the antigen or a product of alternate splicing is not known (57). In drug-induced linear IgA dermatosis, vancomycin is the leading implicated drug, but those patients have exclusively dermal deposition of autoantibody (58,59), suggesting that type VII collagen may be the target antigen. There is uncertainty about the nature of this syndrome, as the diagnosis is based on a single morphologic criterion (linear IgA deposition) rather than on a specific clinical presentation or a single antigenic specificity.

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53 IMMUNE FUNCTION IN OCULAR INFLAMMATORY DISEASE

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OCULAR IMMUNE DISEASE

Studies designed to understand the immunologic basis of ocular inflammatory disorders have been largely unsuccessful. This shortfall may be due to the inherent complexity of the disease processes. However, the failure to make significant advances in the understanding of ocular inflammatory disease may rest on the lack of clinically relevant model systems. The literature is replete with hundreds of references to an “alphabet soup” of uveitis models [experimental autoimmune uveitis (EAU), endotoxin-induced uveitis (EIU), experimental autoimmune anterior uveitis (EAAU), anterior chamber–associated immune deviation (ACAID), EIEIO, etc.] produced mostly in rodents. Although it is not clear how these models relate to human disease, this has not suppressed enthusiasm to explore even their most arcane features. This has led to a somewhat confusing literature in which paradigms are established based on perceptions and review articles rather than on experimental evidence. In this chapter, we attempt to make sense of the available experimental knowledge in the context of clinical eye disease.

INFLAMMATION LEADS TO DYSFUNCTION

The host response to pathogens involves complex inflammatory responses and immune reactions. Although these are central to host defense and vital to clearing infections, they are often associated with nonspecific injury to nearby tissue. These localized reactions do successfully contain pathogens before they spread to other areas, but they are often accompanied by nonspecific injury to surrounding tissue. Most organ systems can tolerate these responses without permanent consequences; however, there are sites that limit the spread of inflammation because it can threaten organ function. The most prominent examples of these are the eye, brain, and reproductive organs (testis, ovary), where even minor bouts of inflammation can have long-term consequences for the survival of the organism. In these organs, immune responses either do not proceed, or proceed in a manner different from those in other areas; thus they are called “immunologically privileged.” Here a functioning immune response can be the culprit that leads to disease.

Although the concept of immune privilege has been around for more than 125 years (1), only recently have we begun to understand its molecular basis. Defined as places where allogeneic or xenogeneic (1,2 and 3) tissue grafts have prolonged survival, it was originally suggested that these sites were ignored by the immune system. The prototype organ for the study of immune privilege is the eye, and most information accumulated concerning immune privilege pertains to this organ. Because even relatively minor inflammation can cause damage, dampening immune reaction protects the visual axis (i.e., vision).

HOW DO WE KNOW THE EYE IS “PRIVILEGED”?

The eye's unique relationship with the immune response was discovered serendipitously during studies with allogeneic tumor cell transplants (1,2 and 3). These investigations were designed to study tissue rejection, and the investigators chose the eye as a model for ease of observation. It was found that allogeneic tumors transplanted to the eye actually survived longer compared with those in other sites. Clinically, this finding led to the development orthotopic allogeneic cornea grafts. The vast majority of these grafts are accepted without rejection and without the use of immunosuppressive medications that are typically prescribed for all other allogeneic tissue transplants (e.g., heart and lung). These clinical and experimental observations served to define the unique nature of the eye and led to the concept of immune privilege. Because of the initial observations with grafts to the eye, many studies have demonstrated that the eye goes to great lengths to prevent the expression of immunity. Two general models could explain these findings: either the eye does not generate an immune response (passive model), or the eye actively suppresses immune reactions (active model). The blood/ocular barrier serves as a passive suppressor by limiting access of immune-response effector cells. However, were this the only mechanism of ocular immune privilege, the eye would be abnormally susceptible to damage from infectious pathogens (which is not the case). Recent studies have identified active mechanisms that prevent the expression of the immune response. Present in the eye are molecules that kill inflammatory cells (FasL), suppress the immune response [transforming growth factor (TGF)-b], inhibit complement, suppress natural killer (NK)-cell activation, and prevent the expression of systemic immunity (immune deviation). These mechanisms (active and passive) work together to dampen immune responses and protect vision.

THE MECHANISMS OF IMMUNE PRIVILEGE

Blood–Ocular Barrier

The anterior chamber (AC) and the retina possess a barrier to the movement of cells, as well as to the transport of macromolecules. Local alterations in the barrier are likely pivotal events in the development of inflammatory disease in the eye. It has been suggested that the blood–ocular barrier allows selected migration of leukocytes into the eye. Although there is currently no evidence that leukocytes normally traffic through the organ, the presence of resident macrophages, mast cells, and dendritic cells in the uveal tract suggests that they must be permitted in by some mechanism. Whether there are eye-specific homing molecules similar to those found in the skin and lymph nodes is unknown. Under normal circumstances, the entry of leukocytes into the retina is restricted. Migration of mononuclear cells through the retinal vessels is an early sign of inflammation and may signal the breakdown of immune privilege.

The transport of macromolecules into the eye is regulated differently depending on the compartment of the eye. In the retina, the blood–ocular barrier exists in tight junctions formed between adjacent endothelial cells. This excludes plasma-derived proteins such as antibody and albumin. Tight junctions in the retinal pigment epithelium also limit diffusion of these molecules into the retina. In contrast, the vessels of the uveal tract are permeable to macromolecules. In the AC of the eye, the blood–aqueous barrier normally limits protein in aqueous humor to less than 1% of the plasma concentration. Thus levels of Ig are quite low in the eye.

If the blood–aqueous and blood–retinal barriers serve to limit access of immune effector cells to the eye, the lack of a robust lymphatic drainage system likewise limits

access of antigen to the immune system. The eye has minimal, if any, lymphatic drainage. Although antigen is thought to exit the eye via the “uveoscleral” outflow pathway, the anatomic basis of this route is unknown. After AC injection of virus or protein antigen, antigens do arrive in the cervical lymph nodes (4), but this is likely due to leakage from the injection site. More important, there is currently no evidence to show that this pathway has any relationship to immune privilege. Materials in the AC do escape via the trabecular meshwork and Schlemm's canal. Schlemm's canal empties directly into the venous circulation to deliver antigen to the spleen and liver. This pathway contributes to immune privilege, partially through the induction of immune tolerance [i.e., the anterior chamber–associated immune deviation (ACAID) response, later].

Sequestration of antigens by the blood–ocular barrier has been postulated to prevent access of ocular protein to the immune system. More important, these barriers are thought to prevent deletion of self-reactive lymphocytes, such that later exposure can result in autoimmune disease. Induction of ocular disease by immunization with retinal proteins (5) has argued for this; however, observations of drainage of antigens and apoptotic cells (6,7 and 8) from the eye to the vascular system and spleen suggest a conundrum. To confuse the issue further, it was recently observed that the immune system was not made tolerant to a foreign protein expressed in the retina of transgenic mice (9). This suggests that, under certain conditions, sequestration may indeed play a role. It is safe to conclude that the role of organ antigen sequestration in the development of tolerance and autoimmunity awaits further study.

Fas/FasL

Recent studies have revealed that the parenchymal cells of the eye constitutively express FasL. FasL is a protein belonging to the tumor necrosis factor (TNF)/nerve growth factor (NGF) family (10). FasL was initially thought to be expressed on activated T cells, but recent studies have demonstrated that it is expressed constitutively in the eye (11), the testis (12), and a number of other places during neonatal and adult life (13). FasL induces apoptotic cell death in cells expressing the Fas receptor. Fas is a protein of the TNF/NGF-receptor family (10) expressed on cells of the lymphoid system, as well as at other sites in the body such as liver and heart.

Figure 53.1 shows areas of FasL expression in the eye, and demonstrates how it is strategically placed wherever there is an opportunity for interaction between the eye and the outside world. It is a sentinel at the connections of the blood–ocular barrier (11). In the cornea, FasL is expressed on the endothelium and epithelium, where it can control inflammatory cells entering from the conjunctiva or the AC. It is a major reason for the success of corneal transplant in mouse models, and possibly humans (see disease mechanisms, later) (14). FasL is expressed on the retinal pigment epithelial (RPE) cells that compose the outer most layer of the retina, and it is prominently expressed on the photoreceptors (rods and cones), where it may play a vital protective role to vision. Interestingly, FasL expression on the RPE cells also controls neovascularization associated with age-related macular degeneration (15). FasL on the iris and ciliary body can contact and kill cells entering from the vessels prominent in this tissue.

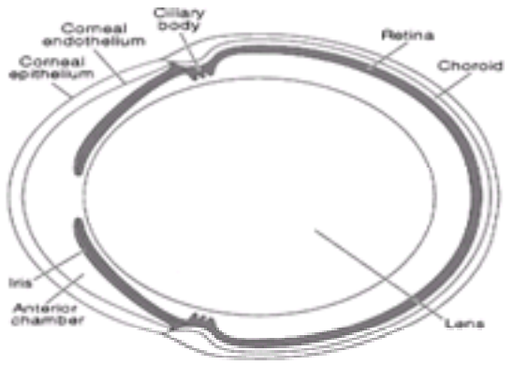

Figure 53.1. Fas ligand (FasL) expression. Schematic drawing of a mouse eye showing localization of FasL (FasL tissues in red). FasL is expressed on the corneal endothelium and epithelium, iris, ciliary body, and throughout the retina, forming a protective barrier (From Griffith TS, Brunner T, Fletcher SM, et al. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 1995;270:1189–1192, with permission.) (See Color Figure 53.1.)

Little is known about the modulation of FasL function during inflammation or eye disease, but it is clearly important in controlling the spread of inflammation throughout the eye. This is demonstrated in Figure 53.2, in which HSV-1 infection of the AC of normal mice results in minimal damage to ocular structure. In sharp contrast, infection of FasL-defective mice (*gld*) results in significant pathology and destruction of ocular structures. This observation suggests the centrality of FasL to immune privilege. Interestingly, experimental animals defective in Fas and/or FasL do not seem to develop spontaneous inflammation. However, challenge of these mice with an infectious agent leads to massive inflammation (Figure 53.2 and ref. 11). Thus it appears that homeostasis for the eye is exclusion of inflammatory cells (blood–ocular barrier), the presence of suppressive cytokines (TGF-b, see later), and sentinel FasL to control induced inflammation. FasL is the only mediator of immune privilege for which reduced functional expression has been shown to increase inflammation.

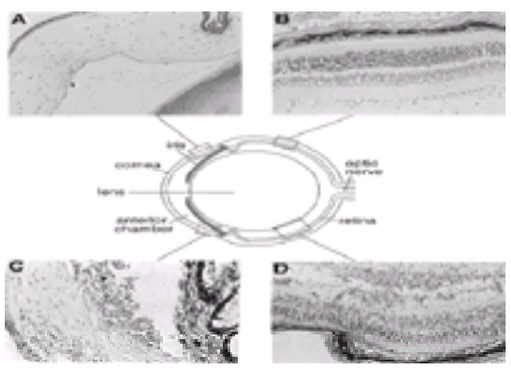

Figure 53.2. The consequences of not expressing functional FasL in the eye. B6 (**A, B**), B6-*gld* (**C, D**) mice were injected in the anterior chamber of the eye with 2.5 × 10^4 herpes simplex virus (HSV)-1 (KOS) as described (From Griffith TS, Brunner T, Fletcher SM, et al. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 1995;270:1189–1192, with permission.) Eyes were removed 10 days after infection, processed for paraffin sections, and stained with hematoxylin and eosin (H&E). *Boxes* on the schematic drawing of the eye (**center**) denote the area of detail for the H&E stain (× 200). (See Color Figure 53.2.)

Immunosuppressive Cytokines and Neuropeptides

Several soluble factors have been implicated in the maintenance of immune privilege (6). These include TGF-b, neuropeptides, and glucocorticoids that are made by resident ocular cells or by the innervating nervous system. Each compound has a unique immunosuppressive function that can prevent certain aspects of the immune response. TGF-b seems to be the most abundant cytokine (6) in aqueous fluids. It is derived from the ciliary epithelium, although most is latent, with only a certain level active in this constitutive pool. Because TGF-b is converted from latent to active form by low pH and/or protease activity, it must be presumed that this conversion takes place during an inflammatory response. TGF-b has well-known antiproliferative qualities, prevents macrophage activation, and is a potent inhibitor of T-cell and B-cell activation. It most likely functions in this capacity in the eye. There are three homologues of TGF-b (TGF-b1, -b2, and -b3). TGF-b2 is the family member in aqueous humor. Antigen handling by adherent cells (macrophages/dendritic cells) *in vitro* is altered in the presence of TGF-b (or ocular fluids containing TGF-b), such that injection of these cells into mice induces immune deviation by direct antigen presentation to T cells (7). Whether this actually takes place *in vivo* is not known, but has been inferred (16). Other evidence suggests that TGF-b–treated adherent cells must undergo Fas-mediated apoptosis after transfer to induce ACAID (17).

Vasoactive intestinal peptide (VIP), a-melanocyte stimulating hormone (a-MSH), calcitonin gene–related peptide (CGRP), and glucocorticoids also are present in the eye. These are most likely derived from the nerves. Constitutive concentrations of these molecules (picomolar to nanomolar range) have been shown to inhibit immune responses *in vitro*. Although their function in the eye is not well defined, the well-known antiinflammatory properties of these molecules suggest that they may play a role in the maintenance of immune privilege.

Limited Expression of Major Histocompatibility Complex Molecules

Immune responses involving T cells require recognition of foreign antigen in the context of major histocompatibility complex (MHC) molecules. This is called MHC restriction and applies to the interaction between the T cells and the antigen-presenting cells. Sites that display abundant MHC molecules, such as the spleen, lymph nodes, and skin, are very effective at inducing immune responses. Thus, organs such as the eye can maintain their “privilege” by limiting the availability of these molecules. Restricted expression of class II in the eye limits the induction of local T-cell responses such as delayed-type hypersensitivity (DTH). Cells that express MHC class II molecules occupy the tissue plane between the epithelial layers of the iris and ciliary body, and they line the uveal tract (18). These cells are bone marrow derived, dendritic, and bear the surface phenotype of splenic, skin, and airway dendritic cells (18,19). They are very efficient antigen-presenting cells when removed from the ocular environment and function to remove molecules or pathogens during aqueous outflow. They have been reported to capture foreign antigens and migrate to the spleen, where they induce immune deviation (ACAID) (7). Activation of effector T cells that enter the eye during inflammatory responses may involve these cells, and this may explain why the uvea is the frequent target of immunogenic inflammation.

The eye has been reported to be devoid of MHC classic class I (Ia) molecules (HLA-A, B, C) (9). The absence of class I would restrict the activity of cytotoxic T cells, which are responsible for antiviral and antitumor immunity. Recently, however, transcripts for these molecules have been identified (20). In addition, cultured ocular cells and fresh human donor corneal buttons have been reported to express class I (21). Nonclassic class I (Ib) molecules (e.g., Qa-2) have been reported to be present in the eye (22), but their function is not established. Further experimental evidence is needed to demonstrate the role of MHC class I (or lack of it) in the eye.

Complement Regulatory Proteins

Several complement regulatory proteins are associated with ocular tissue (23). These include MCP (membrane cofactor protein, CD46), DAF (decay-accelerating factor), and CD59, which regulate the function of complement. Their differential distribution in ocular tissue suggests that they may protect cells in the eye from destruction by complement-activating events. However, further studies will be required to determine a specific role for these molecules in the immune privilege.

Natural Killer Cell Inhibitors

A recently identified NK-cell inhibitory factor also may contribute to immune privilege (24). According to current theories, NK cells recognize and lyse cells that are deficient in MHC class I molecules (25). If the eye does not express MHC class I molecules (or expresses them minimally), the presence of this NK-inhibitory factor might be required to limit spontaneous intraocular inflammation resulting from NK-cell activity. It has yet to be shown that ocular tissue is damaged by NK cells when the NK inhibitory factor is missing.

Immune Regulation

ACAID describes the pattern of systemic responses when certain antigens are introduced to the immune system through the AC of the eye. It is a prototype immune-deviation response that may be an adaptation of the immune system to prevent destruction of the visual axis in the face of vigorous systemic cell-mediated immunity. The pattern of responses seen after AC antigen injection is distinctive. It involves activated antibody responses, and inhibited DTH responses. Recent studies (7,8) have revealed that this state of DTH “tolerance” is not simply the failure to prime an immune response, but the inhibition of T-helper 1 (Th1) cells by the preferential activation of Th2 cells. What sets this it apart from some other types of immune deviation is that there is also a $CD8^+$ cell response that actively downregulates DTH (26,27); however, these cells are not well characterized. ACAID has been induced to a variety of antigens (7,8,28) and has been associated with corneal graft survival (29), although this is not absolute (30).

Apoptosis of lymphoid cells induced by FasL is a prerequisite for the induction of ACAID to all antigens tested thus far (8). A second prerequisite is that the dying cells must be capable of producing interleukin (IL)-10 (31). Although it is not clear why this happens, the induction of the cell-death pathways in these cells initiates production of IL-10, which may lead to the DNA damage as observed after UV irradiation of some cells (32). Dying and dead apoptotic cells are washed from the eye, enter the blood, and are taken up by phagocytic cells (macrophages/dendritic cells) in the spleen. Because the dead cells contain IL-10 and are associated with antigen acquired in the eye, when they present to the immune system, there is a preferential activation of Th2-type $CD4^+$ cells (antibody production). These Th2 cells then regulate Th1 function (DTH) through cytokines such as IL-10. Apoptosis of T cells in the eye also has another effect. After cell death, T-cell proteins (most notably TCR a-chains) are released into the serum (33) and are presented in the class I pathway to $CD8^+$ cells in the spleen. The $CD8^+$ cells regulate by acting as either killer cells deletion of $CD4^+$ cells involved in DTH, or as TGF-b secreting regulatory cells.

Anterior Chamber–Associated Immune Deviation and Visible Light

An interesting twist to the ACAID model involves the capacity of the eye to trap light. It was observed that ACAID was regulated by visible light (28). Whereas mice reared under normal conditions (12 hours light/12 hours dark) develop ACAID when antigen is presented via the anterior chamber, mice dark reared or dark adapted for a short time are immune. This effect of light is an ocular event not dependent on circadian rhythms, but linked to the length of time the eye is exposed to light or dark. Incident light of 500 to 510 nm at a minimal lux of 2 was found to be responsible. Subsequent studies (34) revealed that light controlled the level of substance P–induced and FasL-induced apoptosis. Thus dark-adapted animals have high levels of substance P (which is proinflammatory), which leads to reduced levels of FasL-induced death. Because apoptosis of the lymphoid cells is a requirement for ACAID, the lack of cell death leads to the absence of ACAID. In addition, dark-adaptation also prevents the contralateral spread via the central nervous system of herpes simplex virus (HSV)-1 from the AC of one eye to the retina of the other (26). Whether the mechanism has a practical application to clinical disease is still an open question.

FasL and Tranforming Growth Factor-b

Besides the noted apoptotic (hence antiinflammatory) functions of FasL, it also has a proinflammatory function (35). This was observed after expression of FasL on pancreatic islet cells in transgenic mice (36). It was observed that these mice had a granulocytic response that accelerated rejection of this tissue. Thus FasL expression did not confer immune privilege on a nonprivileged tissue, but augmented the immune response. The proinflammatory effect was mediated by engagement of Fas on neutrophils by FasL. Interestingly, in the presence of TGF-b, FasL loses its proinflammatory function; thus ocular FasL effectively kills infiltrating lymphocytes, and TGF-b inhibits neutrophil activation, showing for the first time that two components of immune privilege can work together to prohibit inflammation.

IMMUNE DEFENSE MECHANISMS

Although the eye is known for its ability to resist immune and inflammatory responses, it also has the capacity to resist infection. Like other surface tissues, the eye is constantly exposed to microorganisms and is very capable of thwarting most of these. Much of the eyes' natural resistance relies on the properties of the lids, tears, conjunctiva, and cornea. These physiologic and anatomic mechanisms lie outside the immune-privileged environment attributed to the internal milieu. Once an invader has passed these external defenses, the eye is not very effective at preventing destructive effects of infectious agents because of immune privilege, which inhibits inflammatory responses.

Nonimmunologic

The eyelids protect the eye from trauma and screen out particulate substances by the blink reflex. Additionally, the tear film washes away debris from the ocular surface. The tear film coats and lubricates the eye surface and physically prevents the attachment of microbes.

Immunologic

The tear film contains lysozyme, b-lysin, lactoferrin, and antibody. Lysozyme is a cationic, low-molecular-weight enzyme that attacks the mucopeptide in bacteria cell walls. It is thought that tear lysozyme is produced by the lacrimal gland and is in much higher concentration in tears than it is in serum. Although all Ig classes have been detected in tears, the major type in tears is IgA. Tears contain the secretory dimeric form of the molecule. Tear Ig is produced by plasma cells of the lacrimal gland. During inflammatory conditions, there is a proportional increase in tear Ig that mimics the appearance of these proteins in serum. This is likely due to transport of antibodies into the tears.

Lactoferrin and b-lysin are synthesized by the lacrimal gland and are present in human tears. b-Lysin causes lysis of bacterial cell walls. Lactoferrin was believed to function in a nonspecific manner through its iron-binding capacity, but it may directly affect certain bacterial strains. Lactoferrin can inhibit complement activation by blocking C3 convertase. This suggests that it might play a dual role as an agent of defenses and a mediator of immune privilege.

Conjunctiva

The conjunctiva forms a natural barrier to the invasion of the ocular surface by foreign material. It contains a very high number of mast cells and leukocytes, which seem to increase with age. This suggests that exposure to inflammatory substances throughout life affects the populations of cells present. The conjunctival epithelium with its underlying blood vessels and lymphatics is similar to the epidermal/dermal junction of skin. Thus there is drainage of these areas for exposure to the immune system in regional lymph nodes. Additionally, the vascularity of the area may contribute to the passage of antimicrobial substances from the blood.

Other, less-defined mechanisms present in the conjunctiva contribute to immune protection. Epithelial cell turnover and cool temperature may serve a protective function. Additionally the conjunctiva is resistant to certain viruses, but susceptible to bacterial infection (e.g., gonococcus). This is mediated by unknown “nonspecific factors” present in this area. Thus anatomic considerations, secretions, and normal flora may all contribute to the protection of the eye from invasion. Interestingly, these all lie outside the blood–ocular barrier and the immune-privileged environment.

Cornea

The cornea is a unique tissue forming a barrier between the outside world and the inner structures of the eye. The corneal epithelium lies outside the immune-privileged area, but the endothelium is an important component. The avascularity of the cornea likely contributes to immune privilege rather than to immune protection. The central cornea is usually devoid of cells that would participate in immune responses, although the limbus (the tissue at the junction of the cornea and the conjunctiva) contains numerous MHC class II–positive dendritic cells. These cells can be attracted to the central cornea to inflammatory stimuli. They likely enter in response to infection by phagocytose invaders. They then exit to the draining lymph nodes to initiate immune responses.

The cornea also contains uncharacterized “nonspecific” inhibitors of bacterial and viral replication. There also is a natural resistance of the cornea to the adherence of bacteria and viruses to the surface. This serves to prevent invasion and colonization of the ocular surface. Infection of the cornea can result in the outpouring of inflammatory cells from the conjunctival vessels to counter the pathogen.

THE SPECTRUM OF OCULAR INFLAMMATORY DISEASE

Ocular inflammatory disease is quite prevalent. In the last major survey (37), approximately 1.2 million Americans carried the diagnosis of uveitis (the major category of ocular inflammatory disease), and about 45,000 new cases were reported that year. Ocular inflammatory disease has a high morbidity. When infectious causes such as *Oncocerca volvulus* (river blindness endemic in portions of Africa) and trachoma are included, ocular inflammatory disease ranks with cataract and glaucoma as one of the three major causes of vision loss worldwide.

There is no single, encompassing scheme for classifying ocular inflammatory disease. Individual anatomic structures can be subject to inflammatory disease through multiple mechanisms (i.e., infection, autoimmunity, or a mixture of the two), so a simple anatomic classification is incomplete. Classification by appearance also is problematic, because we know that some identical-appearing diseases have very different mechanisms. Conversely, incomplete knowledge of the pathogenesis of even the most common forms of ocular inflammatory disease makes classification by disease mechanism applicable to only a small fraction of diseases. Therefore because no classification scheme is ideal, we present an outline of each to highlight the full range of ocular inflammatory disease.

ANATOMIC CLASSIFICATION

Conjunctival and Scleral

Most conjunctival inflammatory diseases are infectious. Common “pink eye” is due to viral conjunctivitis and is frequently caused by one of several strains of adenovirus. Other infectious causes of conjunctivitis include herpes viruses, *Chlamydia trachomatis,* and *Neisseria gonorrheae.* Ocular cicatricial pemphigoid (also called mucous membrane pemphigoid) is an immune-mediated conjunctival disease. Conjunctival biopsies reveal C3, IgG, IgM, and IgA localized to the basement membrane zone, which has led to the suggestion that this disease is caused by cytotoxic hypersensitivity. There is a weak association of HLA-DR4 with this condition (38). Inflammation of the sclera, or scleritis, is a frequent feature of collagen/vascular diseases such as rheumatoid arthritis and systemic lupus erythematosus. This inflammation can be necrotizing, with spontaneous rupture of the globe. The pathobiology of scleritis is unknown.

Anterior Uveitis

Anterior uveitis also is known as iritis or iridocyclitis. Patients usually have a red eye (because of dilation of the ciliary vessels serving the iris), photophobia, and pain. Anterior uveitis is characterized by a visible inflammatory infiltrate in the AC. Normally, the aqueous humor filling this space is completely acellular, with low protein content. In anterior uveitis, the observed inflammatory reaction ranges from a few cells in the AC to frank layering of white blood cells. Increased protein concentration in the AC (due to compromise of the blood–aqueous barrier) is manifest as *flare*, or a scattering of oblique light observed with the slit lamp. When inflammation is severe enough to cause layering of white blood cells, the term *hypopyon* is used. The clinical sequelae of chronic anterior uveitis include inflammation of the cornea as well as scarring of the iris to the cornea (anterior synechiae) and lens capsule (posterior synechiae). Changes in eye pressure (either high pressures associated with glaucoma, or low pressures associated with hypotony) and cataract also are observed. Even nearly asymptomatic levels of chronic inflammation can lead to these vision-threatening sequelae. Leakage of as-yet-unidentified mediators also can cause a focal swelling of the retina [cystoid macular edema (CME)] that can significantly reduce vision.

Among the diseases that cause anterior uveitis are the HLA-B27–linked diseases, such as ankylosing spondylitis and Reiter disease. Approximately one third of all anterior uveitis patients are HLA-B27 positive, and about one third of HLA-B27–positive patients will have anterior uveitis. In children, juvenile rheumatoid arthritis (JRA) is a common cause of anterior uveitis. Upwards of 30% of patients in the pauciarticular (fewer than five joints), antinuclear antibody (ANA) positive, rheumatoid factor (RF)-negative subgroup of JRA patients will show a chronic anterior uveitis. Other diseases frequently associated with anterior uveitis are Behçet's disease, sarcoidosis, herpetic uveitis, and several idiopathic conditions (such as Fuch's heterochromic iridocyclitis, and granulomatocyclitic crisis).

Intermediate Uveitis

Intermediate uveitis refers to inflammation of the vitreous cavity. Isolated intermediate uveitis is the least common form of uveitis. Patients typically have complaints of “floaters” and blurred vision. The floaters are shadows cast by the white blood cells, whereas the blurred vision is frequently due to accompanying CME. Late sequelae of intermediate uveitis include retinal detachments from traction exerted by the vitreous on the retina, neovascularization of the peripheral retina, and chronic CME. The most common intermediate uveitis is pars planitis, a bilateral condition of unknown cause, affecting mostly younger patients. About 5% of intermediate uveitis is thought to be due to multiple sclerosis; however, the pathogenic link between the two conditions is unknown.

Posterior Uveitis

Posterior uveitis encompasses inflammation of both the neurosensory retina and the underlying choroid. Posterior uveitis is usually accompanied by some degree of vitritis. Patients have complaints of distorted or blurred vision, or loss of visual field. Although some diseases represent a pure retinitis [i.e., the cytomegalovirus (CMV) retinitis seen in immunocompromised patients], most posterior uveitis represents a chorioretinitis, with continuous inflammation noted in both the retina and the underlying choroid. The most common forms of posterior uveitis are infectious, including reactivation toxoplasmosis, CMV retinitis, acute retinal necrosis syndrome caused by herpesviruses, and a focal chorioretinitis called presumed ocular histoplasmosis disease. Other diseases significantly associated with posterior uveitis are sarcoidosis and Behçet's disease. Sequelae of posterior uveitis include retinal detachments, chronic CME, loss of choroid and retina leading to scotoma, retinal vasculitis, and subretinal neovascularization.

Panuveitis

Panuveitis refers to simultaneous inflammation of the anterior, intermediate, and posterior compartments. When caused by a bacterial infection, this is referred to as bacterial endophthalmitis. Although many agents can cause panuveitis, this is seen most commonly with sarcoidosis, syphilis, toxoplasmosis, and Behçet's disease.

OCULAR DISEASE CAN BE CLASSIFIED BY CLINICAL APPEARANCE

Our brief excursion into the anatomic variants of uveitis makes it clear that many diseases can have similar presentations, and one disease can have protean manifestations. Certain groups of diseases, however, have very similar presentations. The most common distinction applied by clinicians is granulomatous versus nongranulomatous disease. Although precise identification of a granuloma can be made only histologically, granulomas can be observed clinically as aggregations of cells visible on the corneal endothelium, iris, and conjunctiva (Fig. 53.3). Granulomatous causes of uveitis include sarcoidosis, syphilis, tuberculosis, toxoplasmosis, as

well as some idiopathic eye diseases such as sympathetic ophthalmia and Vogt-Koyanagi-Harada disease. Most other uveitides are considered nongranulomatous, including the HLA-B27 anterior uveitides, Behçet's disease, and most of the herpesvirus-caused diseases.

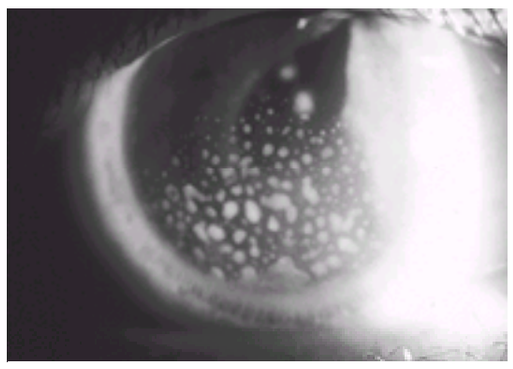

Figure 53.3. Typical appearance of granulomatous anterior uveitis on the cornea of a 46-year-old African-American woman with sarcoidosis. The corneal endothelium is covered in dense keratic precipitates, composed of epithelioid cells in small noncaseating granulomas. (See Color Figure 53.3.)

OCULAR DISEASE CAN BE CLASSIFIED BY MECHANISM

The causes of many forms of uveitis, including Behçet's disease, sarcoidosis, and idiopathic anterior uveitis, are unknown. Of the known mechanisms, some are clearly infectious, a few are primarily immune-mediated, and many appear to be mixed.

Infectious Disease

CMV retinitis is an example of an infectious uveitis. This virus is nearly ubiquitous in the American population, and generally does not cause significant primary ocular disease. However, in an immunocompromised host (particularly with $CD4^+$ cell count less than 50), this virus will reactivate and cause an aggressive, necrotizing retinitis, with a very characteristic clinical appearance. Evidence that CMV is causative of this condition includes histologic identification of CMV inclusions in postmortem specimens, recovery of viral DNA by polymerase chain reaction (PCR) from actively infected patients, and successful treatment with antiviral therapy effective against CMV (ganciclovir and foscarnate). Another form of infectious uveitis is acute retinal necrosis syndrome. This is a fulminant retinochoroiditis caused by reactivation of herpes simplex or varicella zoster virus. Herpetic keratopathy is frequently associated with an anterior uveitis, and herpes simplex virus can be recovered from the anterior chamber in these cases. This suggests that this condition is infectious. The most common form of posterior uveitis in the United States is ocular toxoplasmosis, caused by the intracellular parasite *Toxoplasma gondii.* Reactivation of latent cysts in the retina leads to local necrosis, whereas the accompanying inflammatory reaction leads to a severe vitritis and vasculitis. Other uveitides may be infectious, with unknown pathogens. Whipple's disease, for example, can cause an aggressive panuveitis; only in the last several years has the pathogenic organism for this condition been identified as *Tropheryma whippelii* (39).

Autoimmune Disease

The extent to which uveitis is autoimmune is unknown. A few conditions clearly have an autoimmune basis. Ocular cicatricial pemphigoid, for example, is a chronic inflammatory disease of the conjunctiva that leads to scarring and foreshortening of the conjunctiva. Its pathogenesis includes the deposition of antigen/antibody complexes in the conjunctiva. Lens-induced uveitis occurs after trauma that leads to lens proteins being released from the lens capsule into the aqueous and vitreous. Because lens proteins are sequestered in the privileged space of the eye during development, these proteins are highly immunogenic. The combination of a compromised blood–ocular barrier and large antigen load overcomes active immune privilege, and leads to a fulminant inflammatory reaction to these proteins, causing a severe uveitis. Surgical removal of the lens fragments is curative, allowing a return to baseline immunologic privilege.

Direct evidence of autoimmunity is suspected, but not proven, for many other diseases. Sympathetic ophthalmia is a rare chronic granulomatous inflammation caused by injury. It follows injury with loss of uvea to the fellow eye. It is hypothesized that priming with previously sequestered uveal antigens causes autoimmune disease in the uninjured eye. Removal of the injured eye early in the disease limits the extent of damage to the fellow eye. Cancer-associated retinopathy is a rare paraneoplastic condition associated with certain tumors, particularly small-cell lung cancer. Autoantibodies to retina-specific proteins, particularly recoverin, are found in most patients with this disorder. It has been hypothesized that these antibodies are pathogenic, although definitive demonstration is difficult. Other diseases, such as ankylosing spondylitis, have a very strong HLA association, frequently taken as a hallmark of autoimmune disease. The relative risk of ankylosing spondylitis, given an HLA-B27 haplotype, is ~100. Birdshot chorioretinopathy, an uncommon posterior uveitis, has one of the highest known HLA associations, with a relative risk of disease of greater than 150 for the HLA A-29 haplotype in some populations (40). A list of uveitic diseases with significant HLA associations is shown in Table 53.1.

Disease	Antigen	Relative Ris
Acute anterior uveitis	HLA-B27	10
	HLA-B8	5
Ankylosing spondylitis	HLA-B27	100
Behçet's disease[a]	HLA-B51	4–6
Birdshot choroidopathy	HLA-A29	50–200
Mucous membrane pemphigoid	HLA-B12	3–4
Reiter syndrome	HLA-B27	40
Rheumatoid arthritis	HLA-DR4	11
Sympathetic ophthalmia	HLA-A11	4
Vogt-Koyanagi-Harada disease	HLA-DR4	15

HLA, human leukocyte antigen.
[a] In Asian populations.
Adapted from Nussenblatt RB, Whitcup SM, Palestine AG. Uveitis: fundamentals and clinical practice. St. Louis: Mosby, 1996:26.

TABLE 53.1. HLA Associations of Uveitic Disease

Indirect supporting evidence for an autoimmune cause for uveitis comes from animal models of uveitic disease. Because the eye is a privileged site, immune tolerance to native eye proteins frequently fails to develop. One can generate uveitis by immunizing animals (in adjuvant) to a number of eye-specific proteins. These include arrestin [retinal S antigen causing experimental autoimmune uveitis, or EAU (41)], interphotoreceptor retinoid-binding protein (IRBP) (5), recoverin, rhodopsin, or melanin-associated proteins [causing experimental autoimmune anterior uveitis, or EAAU (42)]. In these models, adoptive transfer of $CD4^+$ T cells can transfer disease, suggesting that these cells are sufficient to cause uveitis. Antibodies to these same retinal proteins have been detected in a variety of uveitic conditions. Although strong priming with proteins in adjuvant is sufficient to cause autoimmune uveitis in rodent models, it is unclear that this level of priming occurs in human disease. For example, panretinal photocoagulation is routinely carried out in the treatment of diabetic retinopathy. Despite abrogation of the blood–retinal barrier (by laser-induced anastomosis of the retina and RPE), and release of retina-specific proteins from damaged cells, neither ipsilateral nor contralateral uveitis is commonly observed. Retinal damage secondary to uveitis also will expose these proteins to the immune system. This results in detectable antibody titers but no known disease pathology directed to the retina. Thus it is unclear if the detected self-reactive antibodies represent a primary disease-inducing agent, or are merely indicators of ongoing ocular damage from other causes. These models do demonstrate, however, that autoimmune targeting of previously sequestered antigens is sufficient to induce uveitis. Unfortunately, the clinical appearance of the rodent “diseases” does not resemble most of the human ocular diseases d escribed earlier.

Unknown Disease Mechanisms

Many of the more common causes of uveitis have unknown mechanisms. There are no models at present to account for the most common form of uveitis: non–HLA-B27 idiopathic anterior uveitis. Although injection of endotoxin into the AC of rodents produces a transient anterior uveitis mimicking clinical disease [endotoxin-induced uveitis, or EIU (43)], an infectious trigger for idiopathic anterior uveitis has not been identified. Sarcoidosis is a very common form of uveitis, particularly among African-American patients. Although patients with sarcoidosis demonstrate widespread granulomas and cutaneous anergy, it is not clear that this

disease is primarily immune mediated rather than infectious. Some of the epidemiology of sarcoidosis suggests the latter (44).

FINDING THE LINK: OCULAR IMMUNOLOGY AND CLINICAL UVEITIS

From the preceding sections, it should be clear that (a) much is understood about the unique nature of the eye with respect to immune privilege, and (b) much is understood about the natural history and differential diagnoses of uveitis. Unfortunately, very little is understood about the connection between these, and useful answers to questions such as, “Why does uveitis appear and behave clinically the way it does?” do not exist. Fortunately, from a clinical perspective, the ability to create substantial systemic immunosuppression with agents ranging from prednisone to cyclophosphamide has allowed treatment of many of these conditions without precise knowledge of their pathogenesis.

Plausible molecular mechanisms have been elucidated for a handful of clinical conditions. In this section, we discuss animal models of two conditions for which good molecular models exist for the pathogenic mechanisms.

Corneal Transplantation and Fas/Fas Ligand

One clinical consequence of ocular immune privilege is the ready acceptance of corneal allografts. Approximately 40,000 corneal transplants [or penetrating keratoplasties (PKPs)] are performed in the United States annually. Donor corneas are routinely screened for viral disease, but are not HLA matched, as a large prospective study showed no increased risk of rejection in non–HLA-matched donors (45). Postoperative systemic immunosuppression is generally not given, and topical therapy is often limited to prednisolone eye drops. When it occurs, graft rejection is usually seen several weeks to several months after surgery. It is readily observed clinically by an encroaching line (called the Khodadoust line) of immune complex deposition (Fig. 53.4). The overall rejection rate of corneal grafts is only about 10%. This remarkable success has been linked to ocular immune privilege.

Figure 53.4. Allotypic corneal graft rejection. The *white line* in the graft represents an infiltrate of host lymphocytes. Very subtle rejection can be readily seen because of the transparency of the cornea. Despite routine use of non–human leukocyte antigen (HLA) matched donors and minimal immunosuppressive medication, corneal allografts are rejected in fewer than 10% of cases. (See Color Figure 53.4.)

As described in the first section of this chapter, the eye is protected from invading inflammatory cells by the activity of the Fas/FasL proteins (11). In this model, invading lymphocytes (which are Fas^{+}) are killed by FasL expressed on the corneal endothelium. This model makes the strong prediction that corneas lacking FasL should be rejected at higher rates than are wild type. Similarly, lymphocytes lacking Fas should cause increased rates of rejection of wild-type corneas. Mouse mutants exist for both Fas (the *lpr* mutation) and FasL (the *gld* mutation). Corneal grafts of *gld* or *lpr* corneas onto wild-type hosts are rejected with much higher frequency (nearly 100%) than are wild-type corneas. Conversely, *lpr* and *gld* host mice reject wild-type corneas that are accepted by wild-type hosts. The dependence of ACAID on Fas/FasL-induced apoptosis (8) and the association ACAID with graft acceptance also lends support for the role of Fas/FasL in corneal graft success. Experiments demonstrating the identical expression of FasL on human corneas suggest that the same mechanisms may be operative in humans (14).

Herpetic Stromal Keratitis

Much uveitic disease appears to have both infectious and immune-mediated features. A mechanistic link between the two has now been identified for one ocular inflammatory disease. Herpetic stromal keratitis (HSK) is a T-cell–mediated disease causing clouding of the corneal stroma after HSV-1 infection (Fig. 53.5). Although disease only occurs in patients with a history of epithelial herpes infection, attempts to culture the virus from the inflamed stroma have been unsuccessful. The disease can be adoptively via T cells from an affected animal, but is not clear that these cells are specific for the virus. Similarly, viral antigens are rarely recovered from diseased eyes. The question then is “How does the herpesvirus trigger this autoimmune condition?”

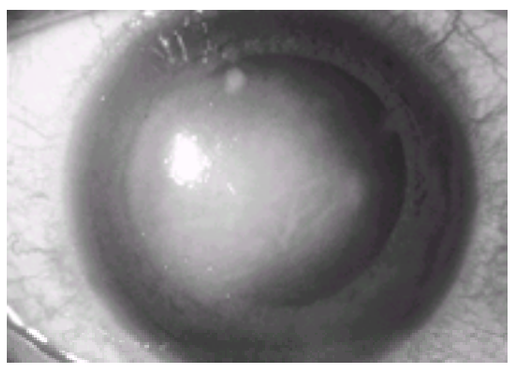

Figure 53.5. Herpetic stromal keratitis. After corneal infection with herpes simplex type 1 (HSV-1), a subset of patients subsequently develops a chronic, autoimmune keratitis. Herpesvirus is not recovered from these lesions. Murine models of this condition suggest that molecular mimicry between a corneal antigen and an HSV-1 coat protein causes the autoimmune keratitis. HSV-1 mutants lacking this antigen cannot trigger stromal keratitis, and mice made tolerant to this antigen are likewise protected from this disease. (See Color Figure 53.5.)

Recently Avery et al. (46) used a fortuitous observation to demonstrate that autoreactive T cells directed against a molecular mimic likely underlie HSK. The group noted that allotypic variation in IgH genes (Ig heavy-chain genes) determines susceptibility to experimental HSK. Mouse strains carrying the IgH^{d} or IgH^{e} alleles are susceptible to stromal keratitis after HSV-1 infection, whereas congenic strains carrying IgH^{b} allele are not. A peptide sequence within the IgH^{b} protein is stimulatory to T cells from mice with HSK (i.e., mice carrying IgH^{d} or IgH^{e}) (46). This suggested that IgH^{b} mice are protected from HSK because of tolerance to a peptide shared between IgH^{b} and the cornea. Autoreactive T-cell clones to this peptide are eliminated in IgH^{b} mice, but not in IgH^{d} or IgH^{e} mice. Remarkably, there is a nearly identical peptide on a coat protein of the HSV-1 virus. Mutant HSV-1 lacking this peptide is unable to produce HSK (47). The proposed mechanism of HSK is as follows: (a) Immune privilege in the eye prevents tolerance to intrinsic corneal antigens by limiting exposure to these antigens during thymic selection; (b) Infection with HSV-1 induces T-cell clones that also recognize the intrinsic corneal antigens; because of the broken immune privilege created by the infection, these cells can then attack the cornea; (c) IgH^{b} mice are protected from HSK because of the fortuitous presence of the immunogenic peptide in the IgH allele. This allows developmental deletion of the autoreactive T-cell clones.

This mechanism may be much more general for antigens whose identity is hidden from thymic selection because of immune privilege. Identification of uveitic conditions with this mechanism awaits identification of the stimulatory peptides underlying their pathogenesis. However, diseases such as presumed ocular histoplasmosis syndrome, serpiginous choroidopathy, and others are certainly candidates.

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54 AUTOIMMUNE HEPATOBILIARY DISEASES

Stephen P. James, M.D.

The liver has the largest mass of any internal organ of the body, and it carries out a wide variety of critical and complex metabolic functions. In addition, the liver plays several roles in the normal function of the immune system. First, it synthesizes most complement components (see Chapter 26). Second, the liver synthesizes most coagulation factors, which may interact with certain components of the immune system and thereby play a role in immunologic reactions. In addition to these metabolic functions, the liver is a major organ of the reticuloendothelial system in the body, and thus plays an important role in removal of particulate materials and antigen/antibody complexes, particularly those originating in the gastrointestinal tract. Last, in some species, the hepatobiliary system plays a major role in immunoglobulin (Ig)A responses in the gut. In rodents, biliary excretion via a specific secretory component–mediated pathway is a major route for transporting secretory IgA into the intestinal lumen, and probably for clearance of IgA immune complexes. However, in humans, biliary excretion of IgA is only a minor pathway for secretion of secretory IgA.

The following sections review the clinical, laboratory, and immunologic aspects of hepatobiliary diseases that are thought to have primarily an immunologic basis. Immune-mediated injury also is a critical component of other important liver diseases such as viral hepatitis, but these are not reviewed here. As might be guessed from the discussion of the role of the liver in normal immune responses, severe liver disease may secondarily alter immune function. Particularly for the diseases of unknown cause, such as primary biliary cirrhosis (PBC) and sclerosing cholangitis, it is important to try to distinguish the immunologic features of the disease that are important in pathogenesis from those that are secondary effects of liver disease. Two other interesting aspects of liver disease, graft-versus-host disease and liver transplantation, are included in Chapter 83 and Chapter 84.

AUTOIMMUNE CHRONIC ACTIVE HEPATITIS

Introduction

HISTORICAL FEATURES

Autoimmune hepatitis is an uncommon form of chronic hepatitis of unknown etiology, predominantly affecting women. The features of the disease were first clearly described by Waldenström in 1950 (1) in young women with cirrhosis, plasma cell infiltration of the liver, and marked hypergammaglobulinemia. In 1955 the lupus erythematosus (LE) cell phenomenon was demonstrated in patients with this disease (2), and because of the finding of frequent multisystem disease in these patients (3,4), the term *lupoid hepatitis* was subsequently applied to the disorder (5). However, it is now clear that autoimmune hepatitis is distinctly different from systemic lupus erythematosus, and this term should not be used.

Since the original descriptions of the syndrome, the current understanding of the disease has been repeatedly modified by the discovery of hepatitis viruses and the advent of specific diagnostic tests for viral hepatitis. Earlier descriptions of the syndrome are complicated by the inclusion of patients with viral hepatitis. Further clarification of the clinical syndrome has occurred with the realization that many drugs may produce a syndrome resembling autoimmune hepatitis. Of particular importance was the demonstration that untreated patients with severe symptomatic disease had a high likelihood of progression to cirrhosis and death, and that this poor course can be significantly altered by immunosuppressive treatment. A discussion of autoimmune hepatitis must be now tempered with the realization that there may be forms of hepatitis for which there are as-yet-undiscovered viral or environmental etiologies.

IMMUNOLOGIC HIGHLIGHTS

The characteristic immunologic features of autoimmune hepatitis include chronic liver disease with infiltration of the liver by lymphoid cells associated with hepatocyte necrosis, the presence of serum autoantibodies, and absence of known viral or toxic etiologies. Type I autoimmune hepatitis is characterized by high titers of antinuclear antibodies (ANAs) and/or smooth muscle antibodies (SMAs). Type II is characterized by absence of ANA and presence of anti–liver-kidney microsomal (anti-LKM-1) antibodies. The disease is associated with specific human leukocyte antigen (HLA) serotypes and has a favorable response to therapy with corticosteroids with or without azathioprine.

Epidemiology

GENETICS

A number of features of autoimmune chronic active hepatitis suggest the importance of genetic factors (6). Autoimmune hepatitis typically is diagnosed in women, with a sex ratio of about 8:1, and has two peaks of incidence: in young women aged 10 to 30 years, and in women aged 50 to 70 years. There is a definite preponderance of cases in individuals of northern European descent. The disease is rare in southern Europeans, Africans, and Asians. Familial occurrence is distinctly unusual (7), but there are reports of multiple cases in siblings, parents, and grandparents. There is an increased incidence in family members of circulating autoantibodies, hypergammaglobulinemia, and occult liver disease (8,9). Multiple studies have confirmed that there is a significantly increased frequency of HLA-B8 in autoimmune hepatitis (10,11), and also an increase in other alleles in linkage disequilibrium with HLA-B8, including HLA-A1 and -Cw7 (12), and HLA-DR3 (13). More recently it was found that these HLA antigen associations occur in young women with the “classic” form of autoimmune hepatitis with a high frequency of autoimmune phenomena, whereas older women diagnosed as having autoimmune hepatitis have a lower frequency of these HLA types. These earlier serologic studies have been validated at the allelic level with significant associations of HLA DRB1*0301 and DRB1*0401 with autoimmune hepatitis (14). Further studies have examined the possible associations with other HLA region genes, such as association of type 1 autoimmune hepatitis with a variant allele of tumor necrosis factor (TNF)-a (15).

Mackay (6) and others (16) described a significant association of the Gm allotype ax with autoimmune hepatitis; in particular, the increased risk of this allotype was confined to patients with HLA-B8. These findings led to the suggestion (6) that the association of autoimmune hepatitis with these genetic factors may represent a multifactorial process at the molecular level: an increased tendency for certain antigens to be recognized in the context of particular major histocompatibility complex (MHC) genes, a particular tendency of certain immunoglobulin genes to code for antibodies reactive with antigens important in the disease, and finally, the importance of female sex in conferring a higher tendency to autoimmune phenomena.

ENVIRONMENTAL

Compared with other liver diseases, autoimmune hepatitis is rare. The prevalence in northern European and North American white populations is estimated to be between 50 and 200 cases per million. The disease is infrequent in Africa and Asia; however, the high prevalence of viral hepatitis in these locations may obscure the diagnosis. The search for specific environmental triggers has provided no conclusive associations. Reported associations with measles, Epstein-Barr virus (EBV), and various drugs has not been reproducible.

Clinical Presentation

The clinical presentation is very heterogeneous, and none of the manifestations is pathognomonic for autoimmune hepatitis (Table 54.1) (17). Some patients are discovered with very mild, asymptomatic disease from screening blood studies. Patients with clinically severe disease may have easy fatigability, jaundice, dark urine, abdominal pain, anorexia, myalgias, delayed menarche, and amenorrhea. Superimposed symptoms of advanced liver disease may occur late in the disease or sometimes be the presenting manifestations. Occasionally patients have an acute or subfulminant course. Abnormal physical findings include hepatomegaly, jaundice, splenomegaly, spider nevi, and cushingoid features. Likewise late in the disease, other physical abnormalities typical of advanced decompensated liver disease may be found. A significant proportion of patients have no physical abnormality.

Etiology	Unknown
Epidemiology	Predominantly young women, northern European descent; second peak of incidence in older women
Genetics	Strong HLA-B8, -DR3 -DR4 association with type I; familial cases uncommon
Symptoms	Fatigue, jaundice, abdominal pain, myalgias, delayed menarche, amenorrhea
Physical findings	Hepatomegaly, splenomegaly, spider nevi, cushingoid features
Laboratory	High transaminases, hypergammaglobulinemia Type I: high-titer ANA, anti-SMA Type II: anti-liver-kidney microsomal (LKM-1) antibodies
Associated syndromes	Thyroiditis, arthritis, Sjogren syndrome, multiple others
Liver histology	Piecemeal necrosis; may be associated with bridging necrosis fibrosis, and cirrhosis
Prognosis	Untreated severe cases have high mortality
Treatment	High response rate to corticosteroids; may be combined with azathioprine

SMA, smooth muscle antibodies; HLA, human leukocyte antigen; ANA, antinuclear antibody

TABLE 54.1. Typical Clinical Features of Autoimmune Chronic Active Hepatitis

Associated Autoimmune Syndromes

Approximately half of patients with autoimmune hepatitis have other syndromes of possible autoimmune nature (3,4,17,18). The most common of these are thyroiditis, rheumatoid arthritis, Sjögren syndrome, and other connective tissue disorders. Additional associations include thrombocytopenic purpura, pernicious anemia, myasthenia gravis, iritis, urticaria, pyoderma gangrenosum, peripheral neuropathy, Coomb-positive hemolytic anemia, membranoproliferative glomerulonephritis, cryoglobulinemia, pericarditis, and fibrosing alveolitis. The relationship of these associations to the underlying liver disease is uncertain.

Laboratory Abnormalities

Common laboratory abnormalities include elevation of serum aminotransferase levels and hypergammaglobulinemia. Hyperbilirubinemia is commonly found, but the laboratory findings of cholestasis are much less prominent than in other diseases such as PBC. Results from tests of copper metabolism are usually only slightly abnormal. Abnormal prothrombin time reflects hepatic synthetic dysfunction.

Pathologic Findings

The major histologic finding is “piecemeal necrosis,” in which hepatocytes in the periportal region undergo necrosis in association with disruption of the limiting plate of the portal tract and infiltrating lymphoid cells (Fig. 54.1). The degree of hepatocyte necrosis is highly variable, from minimal lesions to severe lesions in which necrosis may extend to central veins (bridging necrosis) or to adjacent lobules (multilobular necrosis). These pathologic changes are not pathognomonic and may be found in viral hepatitis. Cirrhosis, with or without active inflammation, may be present.

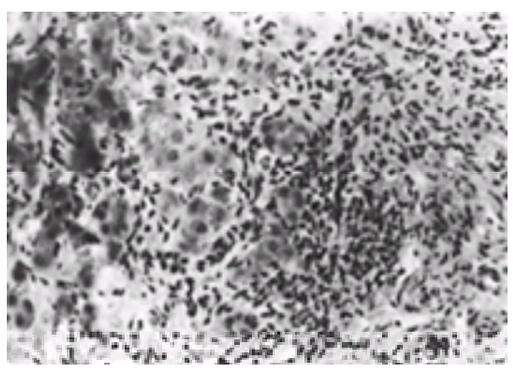

Figure 54.1. Idiopathic autoimmune chronic active hepatitis. Piecemeal necrosis with portal infiltrate and lymphocytes surrounding hepatocytes.

Treatment and Prognosis

Patients with severe autoimmune chronic active hepatitis, who served as controls in therapeutic trials, have a high probability of progressing to cirrhosis and have a high mortality (19). In patients with moderate disease activity, significant morbidity and mortality also occur. Primary hepatocellular carcinoma has been reported, but appears to be rare in autoimmune hepatitis.

Controlled clinical trials have shown that corticosteroid drugs, alone or in combination with azathioprine, significantly improve symptoms and survival in severe autoimmune chronic hepatitis (17). These beneficial effects are often achieved at the expense of significant drug-induced side effects, however. The indications for treatment of patients with mild or moderately active disease are less certain. The indications and regimens for treatment are described in detail elsewhere (17). Remission of disease is achieved in the majority of treated patients. Some patients have long-lasting remissions, whereas others have relapses requiring retreatment. Patients with end-stage autoimmune hepatitis may be candidates for liver transplantation, but the liver disease may recur after transplantation (20). It is not now certain whether underlying autoimmune phenomena, a transmissible agent, or graft rejection is the cause of the recurrent hepatitis.

Immunopathogenesis

IMMUNOPATHOLOGY

Immunohistochemical staining shows the presence of numerous plasma cells, and, in contrast to chronic viral hepatitis, a lower proportion of T cells have the CD8 phenotype (21,22,23 and 24). IgG can be demonstrated by indirect immunofluorescence on hepatocyte membranes (25). In some studies, the deposited IgG has a linear pattern, whereas other authors found both linear and granular patterns.

AUTOANTIBODIES

Type 1 Autoimmune Hepatitis (Typical or Classic Autoimmune Hepatitis)

The frequency with which specific serologic abnormalities are found in typical autoimmune hepatitis varies in the literature and depends in part on arbitrary diagnostic criteria. The typical case of classic lupoid hepatitis is associated with high titers of ANAs in serum, which is the immunologic hallmark of type 1 autoimmune hepatitis. Classic autoimmune hepatitis (CAH) does not appear to be associated with any one specific pattern of nuclear reactivity, and there are no clinical correlates with different patterns of reactivity. The frequency of antibodies to double-stranded DNA, the hallmark of active lupus, appears to be low in CAH. ANA positivity can be found in other forms of chronic liver disease.

By generally accepted criteria, typical or type I autoimmune hepatitis is by definition associated with the presence of another non–organ-specific autoantibody, anti-SMA. The antigen usually recognized by anti-SMA is actin, a major cytoskeletal component of cells. However, in many cases, it appears that antigens other than actin are the targets of the antibody, and these have yet to be identified. Although the presence of this antibody is necessary for diagnosis, its presence has no diagnostic specificity, because anti-SMA is frequently found in many forms of chronic liver injury.

Antimitochondrial antibodies (AMAs) also can be found in a minority of patients with CAH. Sometimes, patients with this antibody, which is typically found in high titers in patients with PBC, have a syndrome that truly has features that overlap CAH and PBC (13). However, one distinguishing feature is that this syndrome behaves clinically like CAH in that the response to corticosteroids is good, whereas in PBC, the evidence for good responses to corticosteroids is more controversial.

Type 2 (Anti–Liver-Kidney Microsome) Autoimmune Hepatitis

Type 2 autoimmune hepatitis is a relatively infrequent syndrome that has distinctive clinical and laboratory features. The age at onset is typically younger than 15 years, and it has been found primarily in European countries. The disease is more often associated with other autoimmune syndromes such as insulin-dependent diabetes, autoimmune thyroid disease, and vitiligo. The presentation may be acute and have a rapidly progressive course to cirrhosis. Usually, non–organ-specific autoantibodies such as ANAs and SMAs are absent, but patients characteristically have antibodies to LKMs (anti-LKM) (26). The target antigen was identified by Manns et al. (27) as cytochrome P-450 db1 (now termed P-450 IID6). Two other types of antimicrosomal antibodies have been found. In patients with tienilic acid–induced hepatitis, antibodies against cytochrome P-450-8 were found, and in patients with chronic hepatitis D virus infection, a third type of anti-LKM (anti–LKM-3) was found.

The relationship of type 2 autoimmune hepatitis to chronic hepatitis C is an interesting problem. About one third of patients with type 2 autoimmune hepatitis have anti–hepatitis C antibodies, usually in low titer, which initially raised the suspicion that these were false-positive reactions associated with autoimmunity. That the majority of such patients respond to treatment with prednisone, and that some patients with the syndrome treated with interferon (IFN)-g appear to deteriorate, tend to support this argument. However, some patients have viral RNA in their circulation, indicating that they do indeed have hepatitis C virus infection. In such patients, whether the viral infection is the trigger of the apparent autoimmune disease or whether hepatitis C virus infection is the etiology of the syndrome is unclear. It has been suggested that type 2 autoimmune hepatitis be divided into two subgroups, type 2a and type 2b, on the basis of the absence or presence of hepatitis C virus markers, respectively, because it seems likely that, at least in some patients, hepatitis C virus infection is important in the pathogenesis of the syndrome.

Soluble Liver Antigen (SLA)

A subgroup (about 10% of CAH patients) has a novel pattern of autoantibody to soluble liver antigens. Manns et al. (28) identified patients who had no history of blood exposure and clinical features of CAH but had no ANAs, SMAs, or LKM antibodies. These patients were found to have serum antibodies that reacted with cytosolic fractions of hepatocytes [soluble liver antigens (SLAs)]. Subsequent studies revealed that the antigens recognized are cytokeratins, which are not specific for liver cells but are expressed at high levels in hepatocytes. Clinically the small number of patients found to date with this syndrome is similar to that of patients with type I CAH, in that they are predominantly female and respond well to immunosuppressive treatment.

Other Autoantibodies

In early experimental studies on the pathogenesis of autoimmune hepatitis, it was found that immunization with crude extracts of liver membranes was capable of inducing hepatitis in rabbits (29). These preparations were designated *liver-specific proteins* (LSPs), although it is clear now that these preparations contained numerous components, many of which are not liver specific. These studies gained widespread attention because it was shown that patients had both antibodies directed against LSP and evidence of lymphocyte reactivity to LSP, providing evidence for autoimmune mechanisms in patients with the disease. More recently it was shown that LSP contains at least one hepatocyte-specific membrane protein, the hepatic asialoglycoprotein receptor (ASGP-R) (30). Anti-ASGP-R has been detected using human-specific recombinant proteins, and it appears that these antibodies can be found in at least some patients with a wide array of acute and chronic liver diseases (31), suggesting that the autoreactivity to this protein is a secondary consequence of liver injury. Furthermore, the prevalence of anti-ASGP-R is similar in the different subgroups of patients with CAH. Titers of anti-ASGP-R are much higher in active CAH, consistent with either a primary pathogenic role or a secondary epiphenomenon. Antineutrophil cytoplasmic antibodies (ANCAs) also are found in some patients, with the perinuclear pattern (p-ANCA) found in patients with ulcerative colitis.

Cryptogenic Autoimmune Hepatitis and Cirrhosis

Although recent sophisticated methods for diagnosis of viral hepatitis, including improved serologic and polymerase chain reaction (PCR) methods, have shown that many patients with “cryptogenic” cirrhosis have liver disease caused by viruses, there remain subgroups of patients in whom no etiology or autoantibodies can be identified. Some of these patients, although lacking autoantibodies such as ANAs, SMAs, LKM antibodies, and SLAs, have clinical features that otherwise resemble those of patients with CAH, including hypergammaglobulinemia, typical liver biopsy features, and favorable responses to corticosteroids (32). Although generally indistinguishable from patients with typical CAH, they tend to present at an older age and are more likely to have cirrhosis at the time of presentation. It has been suggested that these patients may have had autoantibodies early in the course of their disease that had been lost.

COMPLEMENT SYSTEM

Because of the possibility that antibody-mediated tissue injury might be responsible for tissue injury in autoimmune hepatitis, a number of studies have focused on the complement system in these patients. In contrast to patients with PBC and chronic hepatitis B, however, patients with autoimmune hepatitis do not have abnormal catabolism of complement or increased levels of cleavage products of C3 (33,34). C3 and C4 may be low in patients with autoimmune hepatitis, but in view of lack of other evidence of activation of the complement system, these findings may reflect decreased hepatic synthesis of complement components. Properdin and factor B levels are normal.

IMMUNE COMPLEXES

Materials reactive in different immune-complex tests have been detected in the sera of patients with autoimmune hepatitis (35). The frequency of positive results appears to be lower than that in viral hepatitis. Positive results are obtained in many other liver diseases, and to date, the nature of the materials reacting in these tests and whether there are any disease-specific components of these complexes remain undetermined.

LYMPHOCYTE SUBPOPULATIONS AND RESPONSES

The percentage of circulating lymphocytes may be diminished in autoimmune hepatitis, but this appears to be a nonspecific effect of liver disease. The proportion of T cells in the circulation may be normal or diminished (36). The CD4/CD8 ratio is variable but on the average higher than that in controls (37). The infiltrates within the liver are predominantly T cells, but in autoimmune hepatitis, there are more B cells and plasma cells than in viral hepatitis. In contrast to viral hepatitis, in autoimmune hepatitis, liver T cells demonstrate a higher CD4/CD8 ratio. Lymphocytes with the phenotypes of natural killer (NK) cells are rare in the liver. HLA-DR–positive lymphocytes have been reported to be present in the liver. By lymphocyte stimulation tests and migration inhibition tests, patients with this syndrome have been demonstrated to have sensitivity to LSP antigen preparations (38,39). These responses are not specific for autoimmune hepatitis, and whereas they suggest that certain membrane antigens may serve as common effector targets in different liver diseases, these findings also are consistent with secondary epiphenomena.

LYMPHOCYTE CYTOTOXICITY

Many studies of lymphocyte-mediated cytotoxicity have been carried out. By measuring the loss of adherent viable autologous hepatocytes from microwells, a number of studies have shown that peripheral blood lymphocytes mediate “cytotoxicity” against autologous hepatocytes in patients with autoimmune hepatitis (40).

Interestingly, this reaction was blocked by addition of LSP or aggregated IgG to the test systems, and was mediated by non-T cells (41). Thus it has been suggested that antibody-dependent cell-mediated cytotoxicity (ADCC) may play a role in hepatocyte necrosis in autoimmune hepatitis, presumably mediated through NK cells. There was much less T cell–mediated "cytotoxicity" in comparison with that in patients with viral hepatitis, as determined by this test system. As mentioned earlier, the majority of lymphocytes infiltrating the liver in autoimmune hepatitis are T cells, and so it remains to be proved that ADCC plays an effector role in liver damage in this disease.

IMMUNOREGULATORY FUNCTION

Because of the prominent role of autoimmune phenomena and marked hypergammaglobulinemia in some patients, a number of investigators examined the possibility that alterations in the function of immunoregulatory T cells are important in the pathogenesis of this syndrome. Using methods in which T cells are preactivated with concanavalin A (Con A), it was demonstrated that patients have diminished suppression of proliferative responses (39). Similarly, suppression of pokeweed mitogen–stimulated immunoglobulin synthesis *in vitro* is diminished. Suppressor function improved after treatment of patients with corticosteroid therapy (42,43 and 44). However, these *in vitro* abnormalities of polyclonally activated cells are not specific for autoimmune hepatitis, being found in other liver diseases described in this chapter and in nonliver diseases. Using production of migration inhibition factor (MIF) in response to LSP as an assay of specific lymphocyte function, it was found that patients with autoimmune chronic active hepatitis lacked cells that suppress this response, suggesting a possible mechanism whereby abnormal function of suppressor cells might contribute to specific immune damage to hepatocytes (39). However, the importance of antibodies to LSP remains to be proved in this disease. Nonetheless, alteration of the function of immunoregulatory T cells, particularly diminished suppressor function, may contribute secondarily to the development of autoimmune phenomena.

PRIMARY BILIARY CIRRHOSIS

Introduction

HISTORICAL FEATURES

The first descriptions of what was probably PBC were published in the nineteenth century (45), but the recognition of PBC as a distinct clinical entity did not occur until the appearance of reviews on the syndrome in the twentieth century (46). The most commonly used name for the syndrome is a misnomer, in that many patients do not have cirrhosis early in the course of the disease, which may have a very mild, indolent, subclinical course. Important historical developments in PBC include the introduction of cholestyramine for treatment of pruritus and hypercholesterolemia, the description of the characteristic antimitochondrial antibody (47), the discovery of high levels of hepatic copper, and the recognition that the syndrome often has an asymptomatic, indolent course. Large treatment trials studied the efficacy of azathioprine, D-penicillamine, and ursodeoxycholic acid. End-stage PBC is an important indication for hepatic allograft transplantation, which offers the hope for long-term survival to patients with severe progressive liver disease.

IMMUNOLOGIC HIGHLIGHTS

PBC is a chronic disease of unknown etiology, affecting primarily middle-aged women, and characterized pathologically by inflammation and necrosis of intrahepatic bile ducts (Table 54.2). The destruction of intrahepatic bile ducts causes chronic cholestasis, with its multiple clinical manifestations, and may progress to biliary cirrhosis and death. The immunologic hallmark of the disease is the presence of high titers of serum AMAs in most patients with the disease. Because of the histologic features of the disease, the occurrence of autoantibodies, and the frequent association with systemic autoimmune diseases, abnormal immune mechanisms may play a primary role in the pathogenesis, although this hypothesis remains to be proved.

Etiology	Unknown
Epidemiology	Predominantly middle-aged women
Genetics	No known HLA association; familial cases rare
Symptoms	Pruritus, fatigue, arthralgias, dry eyes, hyperpigmentation
Physical findings	Hepatomegaly, splenomegaly, hyperpigmentation, excoriations, xanthelasma
Laboratory	Increased alkaline phosphatase, bilirubin, cholesterol; antimitochondrial antibodies usually present in high titer
Liver biopsy	Early: nonsuppurative destructive cholangitis; late: biliary cirrhosis
Cholangiography	Normal in early disease; nonspecific changes due to cirrhosis late in disease
Prognosis	Mean survival, 11 years; asymptomatic patients may have normal survival
Treatment	Supportive; cholestyramine, fat-soluble vitamins; ursodeoxycholic acid; liver transplantation for advanced disease

HLA, human leukocyte antigen.

TABLE 54.2. Typical Features of Primary Biliary Cirrhosis

Epidemiology

GENETICS

Multiple familial occurrence of PBC is rare, having been reported in sisters (48), in mothers and daughters (49,50), in brothers (51), and in twins (52). PBC has been associated with an increased incidence of HLA DR8 (53). Family members of patients with PBC have been reported to have an increased incidence of immunoglobulin abnormalities (54) including autoantibodies, and abnormalities of suppressor cell function (55), suggesting the possibility that hereditary immunologic characteristics may predispose to the disease. To date, genomewide searches for linkage have not been conducted in PBC, in part because familial occurrence is uncommon.

ENVIRONMENTAL

The epidemiologic features of PBC have been studied best in Great Britain, where the point prevalence is estimated to be 2.3 to 14.4 per 100,000 (56,57 and 58). The variation in prevalence may be due to the more frequent diagnosis of asymptomatic patients in urban areas. Although clustering of cases was reported in one report (56), this was not confirmed by another study (58). The distribution of the disease is worldwide and occurs in all races. The diagnosis is made most commonly in the fifth and sixth decades, but the age at diagnosis varies widely from the early twenties to the ninth decade. Approximately 90% of patients are female. There is no evidence to implicate infectious or toxic factors in etiology, although it is known that certain drug reactions may cause chronic cholestasis that resembles PBC.

Clinical Presentation

The clinical features of PBC have been reviewed in detail (59,60,61 and 62). Typically, the symptoms have an insidious onset and include pruritus, fatigue, increased skin pigmentation, arthralgias, dryness of the mouth and eyes, and Raynaud phenomenon. Jaundice or gastrointestinal bleeding may be presenting complaints. Common physical findings include hepatomegaly, splenomegaly, skin hyperpigmentation due to melanin deposition, excoriations, xanthomata, xanthelasma, and spider telangiectasia. Late in the course of the disease, deep jaundice, petechiae and purpura, and signs of hepatic encephalopathy occur because of advanced liver disease. The symptoms and signs of some of the associated syndromes (see later) may be the presenting manifestations. In asymptomatic patients, it is not unusual for the physical findings to be entirely normal (60,63,64). Patients have been described with AMAs and histologic features of PBC on liver biopsy, but without symptoms of liver disease or abnormalities of liver enzymes (65). These patients appear to represent an early phase in the spectrum of the disease, because some may eventually develop more significant manifestations of PBC.

Associated Autoimmune Syndromes

PBC has been associated with a number of autoimmune syndromes (Table 54.2) (3,66 and 66a). Keratoconjunctivitis sicca is probably the most commonly associated syndrome. It is often asymptomatic or present in a very mild form and is a secondary sicca syndrome (usually anti-SSA, SSB negative) (67,68). Several forms of arthritis are frequently found. Most often patients have a seronegative nondestructive arthritis affecting small joints, and less often there is evidence of joint destruction (69,70). Rheumatoid arthritis has been associated with PBC but may not be more common than the fortuitous association of two diseases. Scleroderma, either as the complete syndrome or as the CREST (calcinosis, Raynaud phenomenon, esophageal involvement, sclerodactyly, and telangiectasia) variant, also is associated with PBC (71,72). Evidence of hypothyroidism is common, and autoimmune thyroiditis may be the presenting manifestation of PBC (73,74 and 75). Other associations have

been reported uncommonly, and may represent fortuitous associations. They include systemic lupus erythematosus (76), dermatomyositis (61), gluten-sensitive enteropathy (77), bullous pemphigoid (78), pyoderma gangrenosum, lichen planus (79,80), cutaneous vasculitis with membranous glomerulonephritis (81), polymyalgia rheumatica (82), hemolytic anemia (83), interstitial lung disease (84), and myasthenia gravis (3).

LABORATORY

Common laboratory abnormalities include elevation of the serum alkaline phosphatase and g-glutamyl transpeptidase. Total bilirubin levels are normal early in the disease, and increase progressively with advancing disease. Mild to moderate elevations of serum aminotransferases are found. Hypercholesterolemia and lipoprotein abnormalities are frequent. Urine copper excretion is increased because of reduced biliary copper excretion in cholestatic liver disease, and serum ceruloplasmin is elevated. Abnormalities found with advanced liver disease, such as hypoalbuminemia and prolonged prothrombin time, occur with hepatocellular decompensation. Cholangiography shows normal biliary ducts, except when cirrhosis is present, which may cause nonspecific irregularity and tortuosity of small intrahepatic bile ducts (85). Serum total IgM is often elevated (see later). AMAs are present, usually in high titer, in more than 90% of patients, and is the only relatively specific diagnostic test other than liver biopsy.

PATHOLOGIC FINDINGS

The liver histopathologic abnormalities in PBC are relatively specific and have been classified in four stages (86), but these may overlap (Fig. 54.2). The earliest and most specific lesions identified (stage 1) show chronic inflammation and necrosis of intrahepatic bile ducts, which are infiltrated with lymphocytes and may be surrounded with granulomas. In stage 2, bile ductules proliferate, mononuclear infiltration of portal areas is prominent, and some portal fibrosis may be evident. In stage 3, there is a reduction in the portal inflammatory infiltrate, bile ducts are absent from portal triads, and there is an increase in portal fibrosis. Stage 4 is characterized by biliary cirrhosis with a paucity of bile ducts and increased hepatic copper. Thus the histopathologic features of PBC consist of a chronic inflammatory process that leads to destruction and disappearance of intrahepatic bile ducts and progressive portal fibrosis, leading to biliary cirrhosis. Hepatocellular necrosis is not a prominent feature, although in occasional cases piecemeal necrosis surrounding portal tracts may resemble autoimmune chronic active hepatitis; in some cases, there is a clinical syndrome with features of both chronic active hepatitis and PBC (autoimmune cholangiopathy).

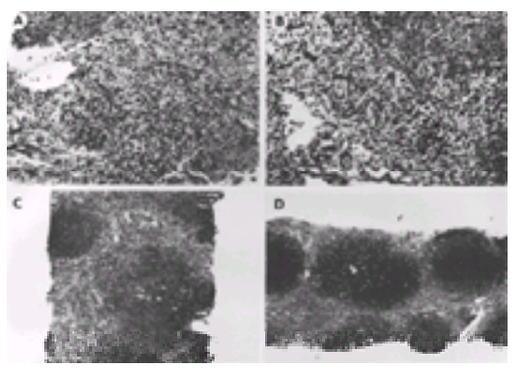

Figure 54.2. Primary biliary cirrhosis. **A:** Stage 1, showing dense lymphocytic infiltrate around a bile duct. **B:** Stage 2, showing portal inflammation and proliferation of bile ductules. **C:** Stage 3, showing extensive portal fibrosis. **D:** Stage 4, showing cirrhosis. (Reproduced from James SP, et al. Primary biliary cirrhosis: a model autoimmune disease. *Ann Intern Med* 1983;99:500, with permission.)

TREATMENT AND PROGNOSIS

The treatment of PBC includes supportive care, such as cholestyramine for pruritus and fat-soluble vitamins for nutritional deficiencies, and antiinflammatory and immunosuppressive drugs directed at the underlying medical disorder. Corticosteroids have been shown to cause improvement in biochemical abnormalities in some patients, but these drugs are generally regarded as contraindicated for long-term treatment of PBC because they greatly exacerbate the metabolic bone disease to which these patients are prone (87). Although previous studies found no benefit of treatment with azathioprine (88,89), more recently, treatment with this drug was shown to prolong survival; however, this beneficial effect of the drug appears to be marginal (90). Although initially treatment with D-penicillamine appeared to increase survival (91), long periods of follow-up indicate that it causes no significant increase in survival, and it is associated with many potentially serious side effects (92,93 and 94).

A small study of chlorambucil indicated that treatment with this drug is associated with decreased intrahepatic inflammation and improvement in some biochemical abnormalities, but the long-term usefulness of this drug is unknown (95). Cyclosporine has been used in a small number of patients (96), and its use also is associated with improvement in some clinical measurements of disease activity; however, significant renal toxicity developed in patients, possibly because the drug is metabolized in the liver, and its biologic half-life may be significantly prolonged in liver disease. Levamisole was studied in a small preliminary trial, but was without benefit (97). A controlled trial suggested a beneficial effect of treatment with colchicine, but the benefit appears to be marginal (98). The single most effective therapy is ursodeoxycholic acid, which has been shown in multiple trials to improve symptoms, serum biochemical abnormalities, and, in one study, prolong survival (99). The mechanism of improvement caused by this drug is unknown.

For patients with advanced and deteriorating liver disease, the best available treatment is hepatic transplantation. The survival rate for patients with PBC receiving hepatic allografts is excellent. Recurrence of PBC in hepatic allografts has been reported in approximately 10% of patients, and may be difficult to distinguish from chronic allograft rejection (100). However, the recurrent disease appears to be indolent, possibly as a consequence of immunosuppression.

The prognosis for PBC is highly variable. Overall mean survival time is about 12 years (60), in contrast to earlier studies of primarily symptomatic patients in whom the average survival time is only about 6 years (59). Survival in patients who are asymptomatic at the time of diagnosis is very much better (59,64). The presence of granulomas, which are found in early disease and correlate inversely with fibrosis, is a good prognostic sign (101), whereas increasing serum bilirubin and onset of symptoms and signs of portal hypertension are poor prognostic signs (102). The complications of PBC are many, including symptoms and signs associated with chronic cholestasis such as pruritus, steatorrhea, hyperlipidemia, metabolic bone disease, and those complications related to cirrhosis and portal hypertension. These are reviewed in detail elsewhere (62).

Immunopathogenesis

IMMUNOPATHOLOGY

Immunofluorescence studies have shown that plasma cells in the portal infiltrates stain predominantly for IgM (103). Deposition of IgM and complement has been observed in portal areas (104). There is increased expression of HLA-DR antigens (53) and intracellular adhesion molecule-1 (ICAM-1) on bile duct epithelial cells (105). The portal infiltrates are composed primarily of T cells, which have a higher proportion of $CD4^+$ cells than in viral hepatitis, but $CD8^+$ T cells may be found in close proximity to some bile ducts (106,107,108 and 109). The differential diagnosis of the hepatic histologic lesions includes secondary biliary obstruction, sclerosing cholangitis, drug-induced cholestasis, pericholangitis associated with bowel disease, sarcoidosis, chronic active hepatitis, graft-versus-host disease (110), and allograft rejection (111,112).

SERUM IMMUNOGLOBULINS

Polyclonal elevation of serum immunoglobulins is found in most patients with PBC as the disease progresses (61,113), although immunoglobulin levels may be normal early in the disease. There is frequently a disproportionate elevation of IgM (114), a feature that distinguishes PBC from other chronic hepatic diseases. One explanation of the striking IgM elevation found in some PBC patients is that low-molecular-weight (monomeric) IgM may give a falsely elevated IgM level, as determined by radial immunodiffusion (114,115). The presence of monomeric IgM is not, however, specific for PBC. Elevation of serum total and secretory IgA also is found in PBC (116,117), but this finding also is common in other liver diseases such as alcoholic liver disease. A case has been reported with PBC and selective IgA deficiency, indicating that IgA is not required as an immune effector mechanism for the pathogenesis of PBC (118).

The elevated immunoglobulin levels in PBC are usually due to polyclonal increases in immunoglobulins, but production of oligoclonal antibodies is occasionally found,

and in some cases, these monoclonal antibodies have been shown to have specificity for mitochondrial antigens.

AUTOANTIBODIES

The discovery of AMAs in the serum of patients with PBC (47) was one of the first observations that suggested that altered immunity may play a role in the pathogenesis of the disease, and it also provided an important diagnostic test. As detected by the standard indirect immunofluorescence method using whole cells rich in mitochondria, the antibodies are found in more than 90% of patients with PBC. When performed this way, reactivity with mitochondria is found in other liver diseases and in rheumatologic diseases (119), although usually in lower titers than those in PBC patients. AMAs are not species or organ specific. Although predominantly of the IgG class, they also may be IgM or IgA. A number of the mitochondrial antigens have been cloned and identified. The dominant antigen is the E2 subunit of pyruvate dehydrogenase, and the other antigens are related component mitochondrial 2-oxo-acid dehydrogenase enzymes (120). Interestingly, AMAs inhibit the function of pyruvate dehydrogenase, but the significance of this abnormality is uncertain. The cloning of the AMA antigens has led to more sensitive and specific enzyme-linked immunosorbent assay (ELISA) for PBC. Interestingly, antigens cross-reactive with the E2 subunit of pyruvate dehydrogenase are expressed on the apical surface of biliary epithelial cells of patients with PBC, even in AMA-negative patients.

Many other autoantibodies have been described in PBC, but these are not specific for this disease. SMAs are found in the serum of 20% to 50% of patients (121,122), but titers are usually lower than those in patients with diseases characterized by prominent hepatocellular necrosis, such as CAH. Antibodies to cytoskeleton components, which contain actin to which SMAs bind, also are found in PBC (123). In one study, the majority of patients with PBC had serum antibodies that reacted with bile ducts (103); however, this reactivity is not specific for PBC and may be due to the presence of SMAs. Antiasialoglycoprotein antibodies are found in patients with PBC, in common with other chronic liver diseases. ANAs are found in about one third to one half of patients. The presence of the multiple nuclear dot pattern correlated with the presence of sicca syndrome (124), and the presence of anticentromere antibodies correlated with the presence of sclerodactyly (125). The pattern of autoantibody reactivity in PBC differs from that of primary Sjögren syndrome in that the latter, but not the former, have reactivity to small ribonucleoproteins by radioimmunoassay (RIA) (126). Other autoantibodies commonly found in PBC include rheumatoid factor (about two thirds of patients) (127), antithyroid antibodies (75), and anti–native DNA (128), and antiribosomal antibodies (129).

Whatever the significance of autoantibodies in PBC, it is unlikely that such antibodies play a primary role in the pathogenesis of the bile duct lesions. First, the titer of the autoantibodies does not correlate with activity of disease, and patients with otherwise typical PBC may not have detectable AMAs. Second, none of the autoantibodies described to date is entirely disease specific, each having been found in a variety of other hepatic and nonliver diseases. Third, immunization of animals with purified autoantigens, such as mitochondrial antigens, does not produce liver disease, although it does lead to production of AMAs (130). Fourth, patients with AMA-negative PBC have similar clinical and pathologic abnormalities as do AMA-positive patients. It is possible nonetheless that such antibodies may play a secondary role in tissue injury. The pathogenic mechanism by which autoantibodies are formed in PBC is no better understood in this disease than it is in other autoimmune states. It is possible that their occurrence relates to a fundamental abnormality in immune regulation.

IMMUNE COMPLEXES

The discovery of the important role of circulating immune complexes in other human diseases led to a search for such complexes in liver diseases. Circulating immune complex–like materials have been reported in PBC in several studies. Patients have serum substances reactive in the Raji cell (RIA) for immune complexes (131,132 and 133). A correlation was found between the presence of immune complex–like material and the severity of portal inflammation and the presence of autoimmune syndromes. In addition, materials reactive in the iodine 125–Clq binding assay for immune complexes (134) have been found. Many patients with PBC have been shown to have cryoprecipitable material in serum, which, although not containing complement components, is able to activate complement, suggesting the presence of antigen-antibody complexes in the cryoprecipitates (135). One report found evidence of antigens derived from bile in circulating immune complexes (136), and another report found evidence that they contained AMAs (137). Patients with PBC have a defect in Kupffer cell–mediated clearance of C3b-coated immune complexes (137) in the liver. The cause of this abnormality is unknown, but it may account for the accumulation in serum of immune complex–like materials that are normally cleared by the liver. In contrast to these findings, in another carefully conducted study, using several well-defined methods, little evidence for circulating immune complexes was found in patients with PBC (138) when fresh serum specimens were used.

Part of the difficulty in defining the presence of circulating immune complexes in PBC may be the abnormal properties of the IgM, which has been shown to have the capacity to fix complement in the apparent absence of antigen binding, and to be more readily cryoprecipitable (139). Other arguments against an immune complex–mediated pathogenesis of PBC are the absence of typical histopathologic features of immune complex deposition in the liver, and the low frequency of associated immune complex–mediated syndromes such as vasculitis and glomerulonephritis.

COMPLEMENT ABNORMALITIES

Because antibody-mediated tissue injury may play a primary or secondary role in tissue injury in PBC, a number of studies have focused on the function of the complement system in this disease. The investigation of serum complement in liver disease is complicated by the fact that a number of complement components are synthesized in the liver, and therefore altered levels of complement components may be secondary to the liver disease itself. Studies of complement turnover using ^{125}I-labeled purified C3 showed that patients with PBC have an increased fractional catabolic rate of C3, as well as evidence of an increased extravascular to intravascular pool (140). Similarly, the fractional catabolic rate of C1q is increased in patients with PBC. These findings are not simply due to the presence of liver disease, because patients with hepatitis B surface antigen (HbsAg)-negative chronic active hepatitis do not have these abnormalities. Patient sera have been shown to contain elevated levels of C3, decreased levels of C4, and circulating conversion products of C3 (141). More recently, activation of the classic complement pathway was demonstrated by the finding of C1r, C1s, and C1 inactivator complexes in serum of patients, but no evidence of activation of the alternative pathway was found (142). The mechanisms by which the classic complement pathway are activated in PBC are unknown; these findings are compatible with the presence of antigen-antibody complexes that might be formed in the disease, but might also be due to abnormal properties of serum proteins, such as that referred to earlier for serum IgM.

LYMPHOCYTE POPULATIONS IN PERIPHERAL BLOOD

Patients with PBC often have a decrease in the absolute number of total circulating lymphocytes, which is a nonspecific feature of liver disease. The proportion of T cells, determined by rosetting or with the monoclonal antibody anti-CD3, is either normal or decreased. The proportion of B cells is normal. Subpopulations of T cells defined with the monoclonal antibodies CD4 and CD8 are highly variable. In some studies, no differences were found compared with controls, whereas in others, the proportion of $CD4^+$ cells is diminished (143,144). In one study, the proportion of $CD4^+$ cells was diminished in patients with less advanced disease, whereas the proportion of $CD8^+$ cells was diminished in patients with more advanced disease (143). Because these monoclonal antibodies define heterogeneous populations of cells, it is not clear what implications these findings have for the pathogenesis of PBC; they may in part represent secondary changes due to liver disease.

STUDIES OF LYMPHOCYTE FUNCTION

More than half of patients with PBC have diminished or absent delayed-type skin-test reactions to a variety of antigens, including purified protein derivative (PPD), keyhole limpet hemocyanin (KLH), and dinitrochlorobenzene (DNCB) (145). The mechanism of skin-test anergy is unknown, but is probably a secondary effect of liver disease, because anergy correlates with stage of disease and is found in other liver diseases. Many patients have diminished *in vitro* lymphocyte proliferative responses to mitogens (145). The diminished response may at least in part be due to serum inhibitory factors, because extensively washed lymphocytes have normal responses, and patient sera inhibit the responses of normal lymphocytes. The nature of this inhibitory factor is unknown. It is of interest that inhibitory substances have been isolated from normal liver, and it is possible that inhibitory substances are released secondary to liver injury. High concentrations of bile acids, cholesterol, and lipoproteins, which are found in liver disease, might also contribute to diminished lymphocyte responses.

Antigen-specific lymphocyte responses have also been examined. Lymphocytes from patients have been shown to produce MIF in response to the liver membrane preparation LSP (38), and in response to protein preparations from human bile (146). These responses, however, are not specific for PBC. Again, this type of reactivity might represent secondary sensitization due to liver damage.

It is worthwhile to note that pathologic lesions closely resembling those of PBC are found in patients with chronic graft-versus-host disease (110,147,148) and in chronic liver allograft rejection (111,112). Furthermore, in a mouse model of chronic graft-versus-host disease, similar biliary lesions also are noted (149). These findings suggest that immunologic mechanisms similar to those in graft rejection may play a role in PBC. Attempts have been made to determine whether patients have cell-mediated cytotoxicity against liver target cells. In a number of different experimental systems, patients' cells showed increased cytotoxicity against xenogeneic liver target cells, but normal cytotoxicity against LSP-coated red cells (150) and against the PLC/PRF/5 hepatoma cell line. Because of the design of these studies, it is likely that, in each instance, the cytotoxic reaction observed was not mediated by specific T-cell mechanisms, but by non–antigen-specific (NK) cytotoxic mechanisms. Nevertheless, PBC patients' cells have diminished NK cytotoxicity against typical NK target cells. This decrease in function does not appear to be due to a decrease in the number of circulating cells having phenotypic markers of NK cells, but rather to a decrease in their functional activity. Interestingly, in studies of ADCC, it was found

that patients' cells have normal activity, indicating that this activity is not affected by the disease process.

Because of the autoimmune associations of this disease, a number of studies have examined the question of whether immunoregulatory cell function is abnormal in PBC (151). Although the proportion of $CD8^+$ lymphocytes is usually normal (143), T cells from patients have a diminished ability to suppress pokeweed mitogen–stimulated immunoglobulin synthesis (152). In addition, Con A–activated lymphocytes from patients have a diminished capacity to mediate suppression of proliferative responses or pokeweed mitogen–stimulated immunoglobulin synthesis. Interestingly, healthy relatives of patients with PBC have a similar abnormality of suppressor cell function (55), suggesting that this may be a genetic marker of disease predisposition. This abnormality, which has been identified in other diseases, may play a role in hypergammaglobulinemia and the production of autoantibodies, as well as in autoreactive T cell–mediated cytotoxicity.

Patients with PBC have a diminished autologous mixed lymphocyte reaction (153), which also has been found in a variety of other autoimmune diseases. Autoreactive cells have the ability not only to provide help for, but also to suppress immunoglobulin synthesis *in vitro* (154). Thus autoreactive cells may have important immunoregulatory functions. Whether this defect is intrinsic, or secondary to liver disease, has not yet been determined, but it may play a role in the altered immunoregulatory function mentioned earlier.

To summarize the immunologic abnormalities of PBC (Table 54.3), the disease is characterized by the presence of non–organ-specific autoantibodies in serum, evidence of activation of the classic complement pathway, and alteration of the function of immunoregulatory T cells. As yet there is no definitive evidence of immunologically mediated damage to the primary tissue site of injury: the intrahepatic bile ducts. It remains an important unanswered question as to whether the damage to bile ducts is a primary autoimmune event, or whether the inflammatory response is secondary to damage to bile ducts that occurs by some other mechanism. It is likely that there is an underlying genetic predisposition, as well as important hormonal effects, which interact with the immune system by mechanisms that are not yet understood.

Humoral immune abnormalities
1. Hypergammaglobulinemia, particularly IgM
2. Autoantibodies
a. AMA
b. Nonspecific: SMA, LMA, anti-LSP, ANA, RF, antithyroid antibodies
3. Immune complex–like materials in serum
4. Increased complement catabolism
5. Decreased Kupffer cell–mediated clearance of C3b-containing immune complexes
Cellular immune abnormalities
1. Skin test anergy (nonspecific)
2. Diminished T-cell proliferative responses
3. Diminished NK-cell activity
4. Abnormal function of immunoregulatory T cells
a. Diminished autologous MLR
b. Decreased suppressor T-cell function
5. Morphologic: infiltration of liver with $CD4^+$ cells, plasma cells, and $CD8^+$ T cells in close proximity to damaged bile ducts

IgM, immunoglobulin M; SMA, smooth muscle antibody; ANA, antinuclear antibody; RF, rheumatoid factor; NK, natural killer; AMA, antimitochondrial antibody; LMA, liver membrane autoantibody; LSP, liver-specific protein; MLR, mixed lymphocyte reaction.

TABLE 54.3. Immunologic Abnormalities in Primary Biliary Cirrhosis

PRIMARY SCLEROSING CHOLANGITIS

Introduction

HISTORICAL FEATURES

Primary sclerosing cholangitis (PSC) is a disease of unknown etiology that is characterized by inflammation and fibrosis of both intrahepatic and extrahepatic bile ducts and the common association with inflammatory bowel disease (IBD). PSC apparently was not recognized prior to the twentieth century. The term seems to have originated with Castleman and Towne (155), and was used later by Schwartz and Dale (156). The first large review by Warren et al. (157) suggested diagnostic criteria that excluded other causes of biliary strictures. The most important historic aspect of this disease was the advent of endoscopic retrograde cholangiography in the 1970s, which led to a marked improvement in the ability to diagnose the disease, which in turn led to the realization that what was thought to be a rare disease is relatively common. More recently, clinical trials for the medical treatment of sclerosing cholangitis have resulted in a clearer definition of the clinical features and natural history of the disease (158,159 and 160). Finally, the other important historic development was the recognition that hepatic transplantation could salvage patients from inexorable progression of this form of liver disease, which frequently leads to biliary cirrhosis and death from hepatic complications or cholangiocarcinoma (161,162).

IMMUNOLOGIC HIGHLIGHTS

The pathognomonic feature of PSC is the characteristic inflammatory destruction of intrahepatic and extrahepatic bile ducts, which leads to progressive fibrosis, strictures, and cirrhosis. The strong association with IBD (50% to 75% of patients), particularly ulcerative colitis, suggests that overlapping immunologic mechanisms are important, including both genetic and environmental factors. No specific immunologic features of the disease can be distinguished from those of IBD, other than the presence of concurrent liver disease. Thus the immune features are similar to those of ulcerative colitis, with a high prevalence of autoantibodies, such as pANCA.

Epidemiology

GENETICS

Genetic factors have been suggested to be important. Multiple studies have shown a strong association between PSC and HLA haplotypes. Associations have been found with HLA-B8 (162a), -DR3, -DR2, and HLA-A1. A striking association with HLA-DRw52a was reported in one study (163) but has not been confirmed by others. Associations with $DRB1^*1301$, $DQA1^*0103$, and $DQB1^*0603$ also have been found. Some haplotypes, such as HLA-DR4, have been associated with aggressive disease. There have been rare reports of familial sclerosing cholangitis and ulcerative colitis (164). This seems surprising because the familial nature of associated ulcerative colitis seems more clear cut.

ENVIRONMENTAL

The prevalence of PSC has not been accurately assessed in the United States. Based on estimates of the prevalence of ulcerative colitis and of PSC associated with ulcerative colitis, the prevalence of PSC has been estimated to be between two and seven cases per 100,000 population. This estimate is similar to the estimated prevalence of PSC in Sweden of 6.3 per 100,000 population. It is widely believed that these figures underestimate the true prevalence. Some cases are not associated with IBD, and clearly some patients are asymptomatic and may have normal serum biochemical laboratory tests early in the course of the disease. It is thought that IBD has important environmental components, possibly represented in the normal bacterial flora of the gastrointestinal tract and reinforced by animal models in which disease is minimal in the germ-free state. However, no specific environmental factor has specifically been associated with PSC. Furthermore, PSC and IBD in individual patients often seem to run independent courses, suggesting that the diseases may have separate but overlapping pathophysiologic mechanisms and environmental triggers.

Clinical Features

The symptoms of sclerosing cholangitis are similar to those of other cholestatic liver diseases (Table 54.4) and include fatigue, pruritus, hyperpigmentation, xanthelasma, and jaundice (164a). Patients also may have symptoms of cholangitis with fever and abdominal pain. Symptoms of underlying IBD may be prominent, but often are mild or absent. Unlike PBC, patients are often young men. Serum AMAs are nearly always absent, and extrahepatic syndromes such as keratoconjunctivitis sicca, arthritis, and thyroid disease are rare. The disease has been found in young children with ulcerative colitis (165,166 and 167).

Etiology	Unknown
Epidemiology	Predominantly young men; most have ulcerative colitis
Genetics	Familial cases rare
Symptoms	Pruritus, fatigue
Physical findings	Often normal early; late: hepatomegaly, jaundice
Laboratory	High alkaline phosphatase and bilirubin; antimitochondrial antibodies absent; p-ANCA in association with IBD
Histologic features	Liver: fibrous obliteration of intrahepatic and extrahepatic bile ducts; rectum, colon: features of ulcerative colitis, minimal to advanced
Cholangiography	Irregular, tortuous, beaded appearance of intrahepatic or extrahepatic bile ducts
Prognosis	Highly variable; may progress to biliary cirrhosis and death
Treatment	No medical treatment proved to arrest disease; supportive: cholestyramine; antibiotics for acute cholangitis; endoscopic procedures or surgery for isolated high-grade obstruction; liver transplantation

p-ANCA, anti-neutrophil cytoplasmic antibody; IBD, inflammatory bowel disease.

TABLE 54.4. Typical Features of Idiopathic Sclerosing Cholangitis

Laboratory Abnormalities

The routine laboratory features of sclerosing cholangitis are similar to those of PBC (329), described in detail earlier. Elevation of serum IgM is not so striking as in PBC, and serum AMAs are nearly always absent. As in other cholestatic liver disease, copper accumulation may be striking in the liver, and results of laboratory tests of copper metabolism are abnormal (168). The most important laboratory test is cholangiography. Cholangiograms demonstrate variable degrees of narrowing of intrahepatic or extrahepatic bile ducts with tortuosity and areas of dilatation, leading to a beaded appearance. This is in contrast to PBC, in which the ducts appear normal until cirrhosis is prominent, in which case, there may be narrowing and irregularity of intrahepatic bile ducts.

Pathologic Findings

The liver biopsy specimen usually appears abnormal, but pathognomonic changes are often not seen. The portal tracts show expansion with edema and fibrosis and relatively modest cellular infiltration. Later, fibrous septa and finally biliary cirrhosis appear. The typical changes in the intrahepatic bile ducts are a fibrous-obliterative process in which segments of bile ducts are replaced by solid cords of connective tissue, leading to an “onion skin” appearance (Fig. 54.3). When the course is associated with bacterial cholangitis, numerous polymorphonuclear cells may be present within and around bile ducts. When extrahepatic ducts are involved, the histologic appearance shows no unique pathognomonic features, revealing only fibrosis with a relatively modest inflammatory component. It has been suggested that the nonspecific hepatobiliary lesions identified in association with IBD, previously alluded to as *pericholangitis*, are in fact part of the spectrum of sclerosing cholangitis. The finding of portal inflammation and fibrosis in association with ulcerative colitis should indicate the need for careful examination of the biliary tree by cholangiography for evidence of sclerosing cholangitis.

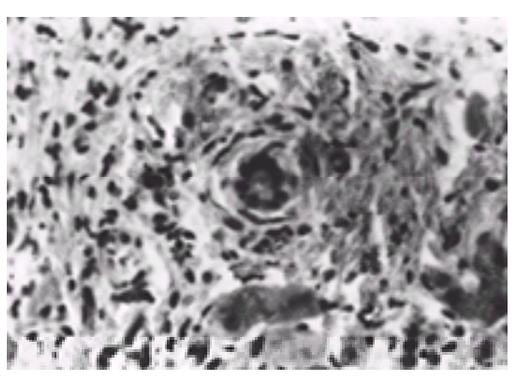

Figure 54.3. Idiopathic sclerosing cholangitis. Bile duct surrounded by lymphocytes and increased collagen.

Treatment and Prognosis

No form of medical treatment has been proved useful at retarding the progressive damage to bile ducts (169). Medical management currently is limited to alleviation of complications of the disease, such as with cholestyramine, fat-soluble vitamins, and antibiotics for episodes of acute cholangitis. Antiinflammatory drugs have not been proved to have any predictable beneficial effect, and as alluded to earlier, colectomy in patients with ulcerative colitis has not been predictably associated with a beneficial effect on the liver disease. For patients with localized areas of high-grade obstruction and symptomatic cholestasis or recurrent cholangitis, a variety of palliative surgical measures directed at the dominant area of obstruction may be useful. The only treatment now for patients with advanced liver damage due to sclerosing cholangitis is liver transplantation.

Immunopathogenesis

The etiology of sclerosing cholangitis is completely unknown. Because of the association with IBD, it has been suggested that absorption of bacteria or toxic materials from the intestine might be important in the etiology. However, sclerosing cholangitis appears to be a rare complication of IBD, and there is no obvious correlation with the extent or severity of bowel disease. Sclerosing cholangitis occurs in patients with minimal IBD, and indeed, from 25% to 50% of patients have no underlying IBD that can be identified. Furthermore, the course of the liver disease appears to be independent of the bowel disease. Patients having undergone colectomy for ulcerative colitis may have relentless progression of the liver disease. This disease may be related to an underlying disorder of fibrogenesis, because other unusual forms of fibrosis have been associated with the disease, including mediastinal fibrosis, Riedel thyroiditis, orbital pseudotumor, and retroperitoneal fibrosis (170,171 and 172).

Because the disease is rare, relatively little is known concerning the immunologic aspects. Antiasialoglycoprotein antibodies are found in some patients, but are not specific for this disease. ANCAs are found in many patients with sclerosing cholangitis and ulcerative colitis, adding further evidence that these diseases share a common pathophysiologic basis (173). Patients with PSC and ulcerative colitis may have anticolon antibodies, and there is evidence of shared cross-reactive epitopes in these two different epithelial cell lineages that could be targets of these antibodies (174). Tests for circulating immune complexes have been reported to be positive in about 70% of patients, but the nature of these circulating immune complex–like materials is unknown, and their relationship to the pathogenesis of the disease is uncertain (175). Peripheral blood lymphocytes show a higher than normal CD4/CD8 ratio in patients with sclerosing cholangitis. Phenotypes of T lymphocytes have been examined in hepatic tissues; whereas $CD8^+$ lymphocytes appear to infiltrate bile duct epithelium, CD4 lymphocytes tend to accumulate around other bile ducts (176). Using a leukocyte migration–inhibition test, it was shown that some patients with sclerosing cholangitis may have leukocyte sensitivity to protein antigen preparations derived from human bile, suggesting that cellular reactivity to biliary epithelial cell–derived antigens might play a role in the disease (177).

The occurrence of biliary cholangiocarcinoma in patients with sclerosing cholangitis suggests that the mechanisms that cause damage to bile ducts might also cause premalignant changes in the biliary epithelium, raising the possibility that the pathogenesis of sclerosing cholangitis may be related to ulcerative colitis, but with a different target epithelial cell, because ulcerative colitis is clearly associated with inflammation and dysplasia of colonic epithelial cells. Further progress in understanding the pathogenesis of ulcerative colitis might provide some future insights into the pathogenesis of sclerosing cholangitis.

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55 IMMUNOLOGIC DISEASES OF THE GASTROINTESTINAL TRACT

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MUCOSAL IMMUNITY

To understand the immunologic changes seen in inflammatory bowel disease (IBD), celiac disease, or other diseases of the gastrointestinal tract that have immune components, it is first necessary to understand normal mucosal immunity (1,2 and 3). Systemic immunity and mucosal immunity are distinct but partially overlapping systems; the two systems are interactive but in a limited way. One distinction is that the induction of peripheral immune responses by peripheral antigen does not result in significant mucosal immunity; however, induction of a mucosal immune response often results in cell-mediated and antibody responses in the systemic immune system.

The mucosal immune system is the major interface of the organism with the outside world. Both the quantity and the diversity of antigens faced by the mucosal immune system are much greater than those faced by the systemic immune system. Moreover, the mucosal immune system must adjust its response to fit the specific antigen. Most of the foreign antigens faced by the systemic immune system are pathogens, which require a vigorous immune response. In contrast, most of the antigens faced by the mucosal immune system are either from the diet or from commensal bacteria. The response of the mucosal immune system to these benign dietary and microbial antigens is to downregulate or suppress the immune response. However, the mucosal immune system is also exposed to bacterial pathogens such as *Salmonella* and *Shigella.* The mechanism by which the mucosal immune system distinguishes between bacterial pathogens, which require a vigorous immune response, and commensal bacteria, which require a suppressed immune response, is not clear. Although the response of the mucosal immune system to normal dietary and commensal bacterial antigens is suppressed, an immune response is generated to these agents nonetheless. In comparison with animals colonized with normal commensal bacteria, histologic evaluation of the small intestine in animals raised in a bacteria-free environment reveals a diminished number of macrophages and lymphocytes, shortening of the epithelial crypts, and elongation of the villi. Thus, one of the characteristics of the normal mucosal immune system is the presence of a chronic low-grade tightly controlled inflammatory response generated against dietary antigens and commensal bacteria. Moreover, this limited inflammatory response affects the turnover of epithelial cells and the architecture of the mucosa.

Parenteral exposure to an antigen results in a much less vigorous systemic immune response if the animal has first been exposed to the same antigen by the oral route (4). This process, termed *oral tolerance,* is related to the suppressed intestinal immune response to elements of the diet and commensal bacteria. The mechanism of oral tolerance is not well understood, but it appears to be T-cell mediated. Clonal deletion of T cells capable of responding to a specific antigen and the generation of regulatory T cells producing downregulatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-b (TGF-b) have been invoked as mechanisms for oral tolerance. Feeding autoantigens has been effective in diminishing the systemic immune response in animal models of autoimmune diseases; whether similar manipulation of oral tolerance will be effective in treating autoimmune diseases in human patients is not known.

The mucosal immune system has a distinct architecture (Fig. 55.1) (1,2). One component of this architecture is the epithelial layer, which stands as an effective barrier to luminal antigens and separates them from macrophages and lymphocytes in the lamina propria just below the epithelium. The lamina propria contains immunoglobulin A (IgA) plasma cells along with large numbers of T cells, B cells, and macrophages. The cell-surface antigens expressed on the lymphocytes of the lamina propria suggest that they are activated. Another component of the mucosal immune system is the Peyer's patch; this structure includes a layer of specialized epithelial cells, M cells, situated immediately above a lymphoid follicle containing T cells, B cells, and macrophages. The immune cells of the Peyer's patch are activated, but less so than those in the lamina propria. The lymphoid follicles have germinal centers with follicular-dendritic cell clusters that are involved in B-cell maturation. M cells take up and transport luminal antigens including proteins, viruses, and bacteria. Some luminal pathogens such as *Salmonella typhimurium* and reoviruses use M cells as a portal of entry. Antigens that pass through the M cell intact are taken up by macrophages and are carried into the underlying lymphoid follicle, where an active immune response can be generated. A third architectural component of the mucosal immune system is the intraepithelial lymphocytes located between the epithelial cells in the small intestine and colon. These are predominately $CD8aa^+$, $TCR\text{-}gd^+$ lymphocytes that express a variety of activation antigens and are $CD45RO^+$ (memory cells). The adherence of intraepithelial lymphocytes to epithelial cells is mediated by an integrin expressed on intraepithelial lymphocytes, $a_E b_7$, whose ligand, E-cadhedrin, is expressed on epithelial cells (5). The function of intraepithelial lymphocytes is not well understood.

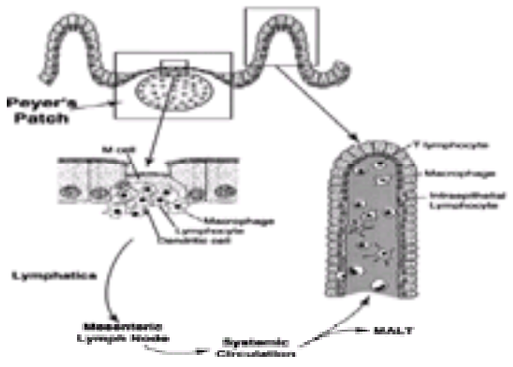

Figure 55.1. The architecture of the mucosal immune system. In Peyer patches, antigens cross the M cells, are taken up by macrophages and dendritic cells, and are presented to lymphocytes. Activated lymphocytes leave Peyer's patches through lymphatics and travel to mesenteric lymph nodes and then through the thoracic duct to enter the systemic circulation. Activated lymphocytes are distributed back to the intestine, where they establish themselves in the lamina propria; they are also distributed to other components of mucosa-associated lymphoid tissue including the respiratory tract, the genitourinary tract, the lactating breast, the lacrimal glands, and the salivary glands. (Adapted from Dean PA, Elson CD. Immunology. In: Nichols RJ, Dozois RR, eds. *Surgery of the colon and rectum.* New York: Churchill Livingstone, 1997:57, with permission.)

An important distinction between the mucosal immune system and the systemic immune system is the prominence of IgA in the mucosal immune system (6). IgG is the most abundant Ig in the serum, whereas the IgA is the most abundant in the intestinal mucosa. IgA is produced by plasma cells in the lamina propria. IgA dimers are

taken up on the basolateral surface of enterocytes and are coated with a glycol protein, secretory component, and are excreted out the apical surface of the enterocyte into the lumen. In the intestinal lumen, IgA binds bacterial and viral surface molecules and mediates their binding to the epithelium. IgA also agglutinates bacteria and viruses, trapping the complexes in the mucus barrier and allowing them to be passed out in the stool.

Lymphocytes in the mucosal immune system have a distinctive trafficking pattern that relates closely to the function of the system (7). Lymphocytes activated in Peyer's patches leave these structures through lymphatics that drain by mesenteric lymph nodes and from there travel through the thoracic duct and enter the systemic circulation (Fig. 55.1). These activated lymphocytes circulate widely through the body, but they “home” back to the intestine and other organs of the mucosa-associated lymphoid tissue (MALT), including the respiratory tract, the genitourinary tract, the salivary glands, the lacrimal glands, and the lactating breast. Homing is the result of interactions of adhesion molecules on the circulating lymphocytes and the endothelial cells of these organs. In the intestine, homing is a product of the interaction between the a_4b_7 integrin expressed on lymphocytes and mucosal addressin-cell adhesion molecule-1 (MAdCAM-1) on endothelial cells. This pattern of lymphocyte trafficking allows activation of the immune response to an antigen in one mucosal organ to result in an immune response to the same antigen in other mucosal organs.

The intestinal immune system was designed to combat infections with enteric pathogens (3). Activation of the intestinal immune system by exposure to enteric pathogens increases intestinal motility and salt and water secretion. In turn, increased motility and secretion cause the intestine to flush the offending pathogens out of the organism. Increased motility and secretion are mediated by inflammatory mediators, including prostaglandin E_2 (PGE_2), produced by activated macrophages and neutrophils. The evolutionary pressure of enteric infections results in the intestinal responses of increased motility and secretion. These responses have become the stereotyped responses of the intestine to any immune-mediated injury. As a consequence, patients with IBD or celiac disease develop increased motility and secretion and, as a result, diarrhea in response to intestinal immune activation, even though they have no pathogens to eliminate.

GASTRITIS

The stomach has three regions: the fundus and body, both of which contain acid-secreting gastric parietal cells and pepsinogen-secreting zymogenic cells; and the antrum, which contains gastrin-producing cells. In contrast to the small and large intestine, B cells, plasma cells, and granulocytes are absent from the normal stomach, and there is only a small population of T cells (CD8>CD4), macrophages, and Langerhans cells. Immune infiltration into the stomach results in two different types of *chronic atrophic gastritis,* based on whether the lesion affects the gastric antrum (8). *Autoimmune atrophic gastritis* (previously known as type A gastritis) is a severe, progressive, atrophic gastritis that shows sparing of the antral mucosa, hyperplasia of gastrin-secreting cells, and autoantibodies to parietal cells and intrinsic factor. Autoimmune atrophic gastritis is the antecedent to pernicious anemia, which is characterized by decreased vitamin B_{12} absorption. *Multifocal atrophic gastritis* (previously known as type B or AB) can present as superficial or atrophic gastritis and is associated with antral involvement, moderate impairment of gastrin secretion, and colonization with *Helicobacter pylori. H. pylori* infection-related gastritis has a strong link with duodenal ulcers and gastric tumors, both carcinomas and lymphomas (9,10,11 and 12). Gastric biopsies from patients with either type of chronic gastritis show a mononuclear cellular infiltrate in the submucosa, which extends into the lamina propria between gastric glands. This infiltrate includes plasma cells, T cells, and B cells and is accompanied by replacement of parietal and zymogenic cells with cells resembling intestinal cells (intestinal metaplasia). Gastritis associated with *H. pylori* infection also has an “active” component with polymorphonuclear leukocytes seen in the infiltrate (13).

Pernicious Anemia

Pernicious anemia is the most common cause of vitamin B_{12} deficiency. About 2% of people who are more than 60 years old have undiagnosed pernicious anemia, which is the end stage of 20 to 30 years of chronic autoimmune atrophic gastritis. The first successful treatment of this anemia was by eating 1 lb of cooked liver daily, and this suggested the lack of an “extrinsic factor” (later identified as vitamin B_{12}) and the discovery of an “intrinsic factor” in gastric secretions. The “discoveries concerning liver therapy in causes of anemia” resulted in the Nobel Prize in Physiology or Medicine in 1934 for George Whipple, George Minot, and William Murphy (14).

This disease is characterized by the presence of autoantibodies to intrinsic factor and parietal cells (15). The major molecular target of these antibodies in human disease is gastric hydrogen, potassium adenosine triphosphate (H^+, K^+-ATPase), the enzyme responsible for acidification of the gastric lumen. Data from small rodent models suggest that these autoantibodies are not pathogenic. Mouse models using either neonatal thymectomy or immunization with mouse H^+, K^+-ATPase demonstrate that gastritis is mediated by CD4 T cells that react with a peptide of the b subunit of H^+, K^+-ATPase (16,17 and 18). The autoreactive T cells are thought to be controlled under normal conditions by a $CD4^+$ $CD25^+$ immunoregulatory cell (19).

The cellular infiltrate in murine autoimmune gastritis results in increased major histocompatibility complex class II (MHC II), Fas, and intercellular adhesion molecule-1 (ICAM-1) expression on gastric epithelial cells and increased expression of IL-2, IL-3, IL-5, IL-6, and IL-10, interferon-g (IFN-g), tumor necrosis factor-a (TNF-a), and granulocyte-macrophage colony-stimulating factor, but not IL-4 (20). Additionally, gastric epithelial cell hyperplasia is seen (21). Neutralizing antibodies to IFN-g reduce disease incidence in murine autoimmune gastritis (22). Based on these findings, the steps leading to the histologic lesion of autoimmune gastritis are thought to be: (a) interaction between parietal cells and $CD4^+$ effector cells; (b) increased expression of MHC II molecules, ICAM-1 adhesion molecules, and Fas antigen on gastric epithelial cells; (c) target cell lysis of parietal cells through Fas-FasL interactions resulting in a loss of parietal cells; and (d) disruption of the normal gastric epithelial cell developmental pathway, resulting in loss of zymogenic cells and amplification of immature epithelial cell types.

Helicobacter pylori Infection

In 1985, *H. pylori* was conclusively shown to be involved in acute gastritis (23). All patients infected with *H. pylori* develop a chronic inflammatory infiltrate, which primarily affects the antral region of the stomach; however, most infected patients are asymptomatic. *H. pylori* is a major factor in the pathogenesis of duodenal and gastric ulcer disease, gastric adenocarcinoma, and MALT lymphoma, with 10% to 20% of infections progressing to serious clinical disease (24). Regardless of disease outcome, all patients mount a vigorous, but ineffective systemic immune response. Therefore, it is important to understand the local immune response and its involvement in disease (24,25). Indeed, studies in mouse models of infection have shown that the cellular immune response is essential for the gastric disease that develops after *Helicobacter* infection (26).

Bacterial virulence factors play a major role in adaptation to the hostile gastric milieu and in the development of gastric disease (9,10,27). Three groups of factors have now been identified: colonization factors, disease-inducing factors, and factors promoting *H. pylori* persistence (13). As the complete genomic sequences of several *H. pylori* strains have become available, rapid progress should ensue on the precise identification of many bacterial virulence factors (28,29 and 30). On infection, *Helicobacter* places itself on or near gastric epithelial cells. Epidemiologic studies have shown an increased incidence of ulcer disease in persons with blood group O. This blood group is associated with $Lewis^b$ blood group antigens (Le^b), which are receptors on gastric mucous cells for *H. pylori.* The ability of bacteria to attach to the gastric epithelium results in a more severe inflammatory response and increased gastric destruction in a mouse model of infection (31).

Innate Immunity

Once the *Helicobacter* has established colonization, it elaborates additional gene products, which interact with the host and induce inflammation. Products of the urease gene and the *cag* pathogenicity island (PAI) are important in direct epithelial destruction and in triggering the host immune response (Fig. 55.2, step 1) (32,33). Strains of *H. pylori* with the *cag* PAI are associated with more severe ulcer disease and preferentially upregulate the expression of IL-8 by gastric epithelial cells 32. The *picB* gene, which is upstream of the *cagA* gene, is essential for induction of IL-8 from gastric epithelial cells *in vitro* (34). This induction of IL-8 is mediated by nuclear factor-kB (NF-kB) in gastric epithelial cells (35). The finding that *H. pylori* is a potent activator of NF-kB has important implications, because other genes important in the immune response (TNF-a, IL-1, IL-6, and ICAM-1) also respond to NF-kB (36). IL-8, in addition to other chemotactic factors released by the bacteria, initiates an influx of neutrophils and activated macrophages (Fig. 55.2, steps 1 and 2) (37). The importance of factors such as lipopolysaccharide (LPS) of *H. pylori* in the modulation of gastritis has been demonstrated in animal models (38).

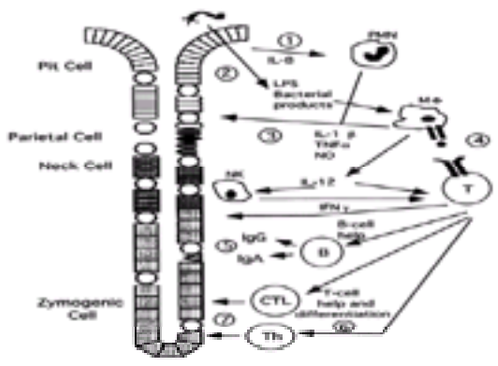

Figure 55.2. Immune-mediated damage in gastric disease. After colonization, *Helicobacter* signals gastric epithelial cells to produce interleukin-8, which, in addition to other chemotactic factors released by the bacteria, initiates an influx of neutrophils and activated macrophages (steps 1 and 2). These recruited mononuclear cells secrete the proinflammatory cytokines and immunomodulatory messenger molecules (step 3). These mediators can directly damage the gastric epithelium, as well as enhance the cytotoxic activity of natural killer cells, support cytotoxic T-lymphocyte generation, and promote the development of T-helper cell (Th1) responses (step 4). The subsequent T-cell activation can lead to production of cytokines, which result in increased T- and B-cell help and differentiation and direct damage to the gastric epithelium (steps 5 to 7).

The recruited mononuclear cells secrete the proinflammatory cytokines TNF-a and IL-1b, and the immunomodulatory messenger molecule nitric oxide (NO) (Fig. 55.2, step 3) (39,40 and 41). These factors not only are important mediators of the subsequent inflammation, but also may play a role in direct epithelial damage because TNF-a and NO are associated with tissue injury. In addition, TNF-a and IL-1b upregulate gastric mucosal Fas antigen expression, which could prime gastric mucosal cells to undergo apoptosis (42). Polymorphisms in both IL-1b and TNF-a predispose to more severe clinical disease after *H. pylori* infection (43,44). One potential explanation is that IL-1b stimulates gastric cell proliferation and directly inhibits gastrin-stimulated histamine secretion, leading to decreased gastric acid secretion (45). IL-12, a cytokine produced by activated macrophages, induces the expression of IFN-g and other cytokines in T and natural killer (NK) cells. In addition, IL-12 enhances the cytotoxic activity of NK cells, supports cytotoxic T lymphocyte (CTL) generation, and promotes the development of naive T cells into T-helper (Th)-1 cells (in conjunction with IFN-g).

Adaptive Immunity

In chronic immune responses, $CD4^+$ Th cells can be divided into two major subsets, termed Th1 and Th2, based on their patterns of cytokine production. Th1 cells predominantly produce IFN-g and IL-2 and elicit delayed-type hypersensitivity. Th2 cells produce IL-4, IL-5, and IL-10 and provide help for some B-cell responses. Differentiation into the Th1 or Th2 phenotype is directed by the cytokines present at the time of specific antigen activation, so IL-12 promotes Th1 development and IL-4 promotes Th2 development. The polarized development of these subsets determines the clinical outcome of many pathologic immune responses, including autoimmune and infectious diseases (46,47). Growing evidence indicates that clinically evident human infection by *H. pylori* induces a predominant $CD4^+$ Th1 response, with the proinflammatory cytokines IFN-g and IL-12 seen, but not the regulatory cytokine IL-4 (Fig. 55.2, step 4) (41,48,49 and 50). These data are consistent with the demonstration of a selective expansion of $CD4^+$ T cells in the gastric lamina propria of patients with *Helicobacter*-associated gastritis (51). This propensity toward a Th1, cell-mediated hypersensitivity type of response, instead of a Th2, humoral-mediated mucosal protection type of response, could combine with other host factors to contribute to epithelial damage and disease.

This theory is supported by results from mouse models of infection, which demonstrate that C57BL/6 mice (which produce a predominant Th1 response) have severe chronic active gastritis, whereas BALB/c mice (predominant Th2 response in other infectious models) exhibit only mild gastritis (52,53). The role of Th1 cells in infected and immunized C57BL/6 mice was addressed by *in vivo* neutralization of IFN-g (54). These studies implicated IFN-g in both protection from *H. pylori* infection and the inflammation induced by this infection (55). In contrast, immunization or infection of IL-4 (Th2 cytokine)–deficient mice (C57BL/6 x 129/Sv) resulted in the inability to protect against *H. felis* infection and a higher load of infectious organisms (56).

The presence of $CD4^+$ Th1 cells that produce or induce the destructive cytokines IFN-g and TNF-a has clearly been shown (41,49). These cytokines could directly contribute to epithelial cell death (Fig. 55.2, steps 3 and 4). In addition, Th1 cells could mediate apoptosis of epithelial cells through Fas-Fas Ligand (FasL) interactions or indirectly through T-cell help to epithelial cell-reactive $CD8^+$ CTLs (Fig. 55.2, step 7). The possibility has been raised that the antigen specificity of $CD4^+$ Th1 clones isolated from patients may play a role in the pathogenesis of the disease. The antigen-recognition repertoire of T-cell clones isolated from a group of six patients with peptic ulcer disease was studied (49,57). The major known *H. pylori* antigens recognized were cagA, vacA, and urease. Because two of these proteins (vacA and urease) have significant homology to subunits of the gastric H^+, K^+-ATPase (58), investigators have postulated that this T-cell response results in autoreactive T-cell clones. In addition, several other *H. pylori*–specific proteins have considerable sequence homology to gastric-specific proteins (59). Consequently, at least some components of the pathogenesis of *Helicobacter* infection must now be considered to result from antigenic or molecular mimicry, which results in an autoimmune attack against specific gastric components. This hypothesis is supported by studies in the *H. felis* mouse model, because the main site of disease (body of the stomach) is away from the area of colonization (antral region) (26,38).

For many years, the classification of human gastritis into two separate groups (autoimmune and multifocal atrophic) has provided a useful framework for investigation into the etiology and pathogenesis of human gastric diseases (8). One of the major differences between these two types of gastritis is the presence or absence of clinically measurable serum autoantibodies. This distinction has been challenged by multiple reports of the presence of autoantibodies in patients with *H. pylori*–induced gastritis (60,61,62 and 63). The first reports of the presence of autoantibodies in *H. pylori*–induced chronic active gastritis were of antibodies that recognized gastrin-producing cells in the gastric antrum (61). This observation was not followed-up for more than 10 years; however, it has become clear that infection with *H. pylori* can lead to the production of anti-*H. pylori* antibodies that cross-react with human gastric mucosa (60,63). That these autoantibodies have the potential to play a crucial role in the pathogenesis of gastric diseases was clearly shown by the experiment of Negrini et al., in which a hybridoma secreting a *H. pylori*–reactive monoclonal antibody caused gastric abnormalities similar to gastritis (Fig. 55.2, step 5) (60). One component of this antigenic mimicry is epitopes contained in the Lewis blood group antigen (64), which are important for adherence of *H. pylori* to surface epithelial cells in the gastric epithelium (31). In addition, Lewis epitopes are present in the b chain of gastric H^+, K^+-ATPase and the LPSs of *H. pylori* (64).

Mucosa-Associated Lymphoid Tissue Lymphomas

H. pylori infection is associated with the development of gastric lymphoid follicles (12). These structures are similar to intestinal Peyer patches, with B cells making up the majority of the lymphoid follicle. Primary non-Hodgkin lymphoma of the stomach accounts for 10% of lymphomas and is the most common extranodal form of non-Hodgkin lymphoma (65). Most of these tumors are diffuse, large-cell lymphomas of the B-cell lineage. Low-grade primary gastric lymphomas recapitulate the structure of Peyer patches (MALT), rather than that of lymph nodes (66). These low-grade MALT lymphomas can evolve into high-grade primary gastric lymphomas, which have classic associations with *p53* inactivation and deletions of *p16* (66). However, some low-grade MALT lymphomas have an additional unique genetic profile, as compared with nodal lymphomas, in that there is a marked increase in the frequency of trisomy 3.

MALT lymphomas have a favorable prognosis and present at an early clinical stage: stage IE or IIE using the Ann Arbor staging system (12). Low-grade MALT lymphomas respond to therapy and have approximately a 78% to 90% survival rate at 10 years (66). This favorable behavior is thought to result from the influence of antigen on tumor growth. Lymphoid tissue accumulates in the gastric mucosa almost exclusively as the consequence of *H. pylori* infection (66); this finding has led to the suggestion that *H. pylori* provides the antigenic stimulus for the growth of gastric MALT lymphoma. Clinical experience demonstrating that 50% to 70% of low-grade gastric MALT lymphomas can be eradicated by antibiotic treatment strengthens this hypothesis (67). However, as tumors evolve into late-stage high-grade lymphomas, the effect of antibiotic treatment is lost. A second line of experimentation has also implicated *Helicobacter* antigens in driving the tumor formation. These experiments have shown that MALT lymphoma B-cell proliferation depends on *H. pylori*–specific T cells and their products, rather than on the bacteria themselves (48,68).

Vaccination

Eradication of chronic *H. pylori* infection with antibiotics markedly alters the natural history of gastroduodenal diseases and reduces clinical symptoms. However, eradication is currently clinically recommended only for patients with peptic ulcer (69,70). Because of the rapid emergence of resistant strains of *H. pylori,* there has been a significant interest in developing a prophylactic or therapeutic vaccine (71). In animal models, a vaccine of crude or purified *H. pylori* antigens can induce a protective immune response against infection with *Helicobacter* species (71). Unfortunately, immunized animals develop severe gastritis when challenged with *Helicobacter* (postimmunization gastritis) (72,73). This gastritis appears to be a prerequisite for protection and underlines the necessity of understanding immune and inflammatory responses in the gastric mucosa. This knowledge will be required for defining immunization regimens or antigens able to elicit the appropriate protective immune response without inducing inflammation.

CELIAC DISEASE

Celiac disease, also known as gluten-sensitive enteropathy, celiac sprue, or idiopathic sprue, is an antigen-driven enteropathy of the small intestine, resulting from an inappropriate immune response to dietary gliadin, a component of wheat proteins. The clinical manifestations of celiac disease are varied in character and severity, with most symptoms attributable to malabsorption resulting from the loss of absorptive capacity of the small intestinal mucosa. As a result of advances in elucidating its pathogenesis, celiac disease is one of the best understood immunologically mediated enteropathies, and it could be considered a model for understanding other T-cell mediated enteropathies driven by luminal antigens.

Clinical Features

The incidence of celiac disease varies dramatically throughout the world, ranging from absence in persons of African and Asian descent to a peak incidence of approximately 0.3% in persons of northern European descent (74). This variance is explained predominantly by the strong genetic linkage of celiac disease with specific MHC II complexes that are not distributed uniformly across all ethnic backgrounds. The clinical manifestations of celiac disease are also variable, with many, if not most, affected persons having mild or no symptoms, thus giving rise to the impression that the true incidence of celiac disease may be much higher than currently appreciated. This impression is supported by studies documenting an increased incidence of antiendomysial antibodies in asymptomatic blood donors from Europe and the United States (75,76 and 77).

The predominant symptoms of celiac disease, when present, are attributable to malabsorption, and they include diarrhea, flatulence, abdominal cramping, abdominal bloating, and weight loss. Patients with extensive involvement of the small intestine may have symptoms attributable to deficiencies in folate, iron, calcium, and vitamins A, D, E, K, and B_{12}, resulting from malabsorption. These deficiencies may lead to myriad extraintestinal manifestations of celiac disease, as outlined in a Table 55.1.

Affected Organ System	Manifestation	Cause
Skeletal	Osteopenia	Malabsorption of calcium and vitamin D
	Pathologic fractures	Osteopenia
	Osteoarthropathy	Unknown
Hematopoietic	Anemia	Iron, folate, vitamin B_{12}, or pyridoxine deficiency
	Hemorrhage	Hypoprothrombinemia, thrombocytopenia, folate deficiency
Nervous	Peripheral neuropathy	Thiamine and vitamin B_{12} deficiency
	Demyelinating central nervous system lesions	Unknown
	Seizures	Unknown
Endocrine	Secondary hyperparathyroidism	Calcium and vitamin D deficiency
	Amenorrhea, infertility, impotence	Malnutrition, ? hypothalamic-pituitary dysfunction
Muscular	Atrophy	Malnutrition
	Tetany	Calcium, vitamin D, and magnesium malabsorption
	Weakness	Atrophy and hypokalemia
Integument	Follicular hyperkeratosis and dermatitis	Vitamin A and vitamin B complex deficiency
	Petechiae and ecchymoses	Hypoprothrombinemia, thrombocytopenia
	Edema	Hypoproteinemia
	Dermatitis herpetiformis	Unknown

TABLE 55.1. Extraintestinal Manifestations of Celiac Disease

Of particular note is the association of celiac disease with dermatitis herpetiformis, IgA deficiency, autoimmune disorders, and malignant disease. Dermatitis herpetiformis is a characteristic pruritic, vesicular skin lesion involving the extensor surfaces of the extremities, trunk, buttocks, scalp, and neck. The skin lesion of dermatitis herpetiformis associated with celiac disease is composed of a subepidermal collection of acute inflammatory cells and fluid with characteristic granular IgA and complement deposits at the dermal-epidermal junction. This skin lesion, like the small intestinal lesion of celiac disease, responds to the withdrawal of gluten from the diet. IgA deficiency and autoimmune disorders including type 1 diabetes and autoimmune thyroid disease are found with increased frequency in patients with celiac disease. The relevance of these associations to the pathogenesis of these diseases is not known. However, the increased occurrence of diabetes and celiac disease could be explained by the increased frequency of persons with human leukocyte antigen (HLA) haplotypes resulting in a susceptibility for diabetes in patients with celiac disease, rather than a common disease pathogenesis. Perhaps most concerning for affected patients is the association of celiac disease with an increased incidence of malignant disease, and in particular malignant diseases involving the gastrointestinal tract. Patients with celiac disease are estimated to be at a tenfold increased risk for the development of any gastrointestinal tract malignant disease and at a many-fold increased risk for developing an enteropathy-associated T-cell lymphoma (78,79,80 and 81). This risk may be decreased by lifelong adherence to a gluten-free diet and thus forms the basis for this recommendation. The complications of celiac disease are mainly limited to the development of malignant disease, as described earlier, and the development of symptoms refractory to withdrawal of gluten from the diet.

Diagnosis

The laboratory abnormalities associated with celiac disease vary with the severity of the small intestinal mucosal lesion and are generally attributable to malabsorption. These findings can be seen in many malabsorptive processes and are not specific to celiac disease. A potential exception to this observation is the presence of antigliadin and antiendomysial antibodies in the serum of patients with celiac disease. Serum IgA antigliadin antibodies have a sensitivity of 80% to 90% for the diagnosis of celiac disease; however, the utility of this test is limited by its low specificity for this diagnosis. Conversely, the presence of IgA antiendomysial antibodies in patients with presentations consistent with celiac disease has a specificity and sensitivity of approximately 90% for this diagnosis (82,83 and 84). The utility of antiendomysial antibody titers is limited by the expense of the test and the difficulty of standardization of testing among laboratories. The discovery that most, if not all, antiendomysial antibodies are directed against tissue transglutaminase (tTG) allowed for the development of a relatively inexpensive, quantitative assay to measure anti-tTG antibody titers (85). This assay is expected to share the relatively high sensitivity and specificity of the more traditional antiendomysial antibody titers. At present, establishing the diagnosis of celiac disease relies on small intestinal biopsy; however, anti-tTG antibody titers may have a role in screening susceptible populations and in following patient responses.

The diagnosis of celiac disease is established by demonstration of the characteristic histopathologic lesion on examination of small intestinal biopsies in patients with the appropriate clinical presentation, in concert with resolution of symptoms on a gluten-free diet. The histopathologic lesion seen in celiac disease, villous atrophy, is characterized by blunting of the small intestinal villi in association with crypt hyperplasia and expansion of the lamina propria mononuclear cells (Fig. 55.3). This lesion is characteristically located in the distal duodenum and proximal jejunum and is believed to decrease absorptive surface area in the small intestine and to result in malabsorption, as described earlier. The lesion does not affect the submucosa, muscularis, or serosa, and it is not associated with ulcers or granulomas. These histologic changes can be patchy, with intervening areas of normal mucosa, thus necessitating multiple small intestinal biopsies to rule out the diagnosis of celiac disease with confidence (86).

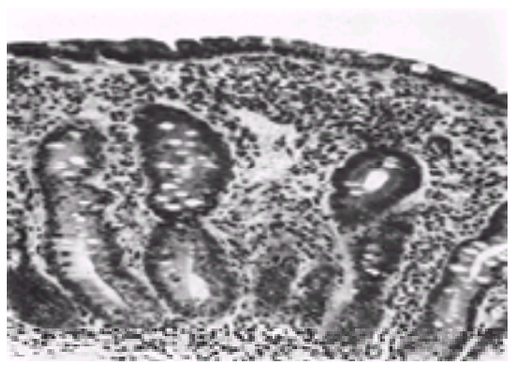

Figure 55.3. Light photomicrograph of a small intestinal biopsy from a patient with celiac disease. The biopsy shows the characteristic lesion of celiac disease: blunted villi, epithelial cell crypt hyperplasia, and expansion of the lamina propria with mononuclear cells. These changes result in a loss of absorptive surface area in the small intestine and give rise to malabsorption.

Therapy

Therapy for celiac disease, although conceptually simple, represents a difficult task with significant social and financial burdens. Within a few weeks of adherence to a gluten-free diet, the patient affected with celiac disease should note a diminution of symptoms. An exception to this rule is the rash of dermatitis herpetiformis, which resolves slowly on a gluten-free diet and is therefore treated with dapsone in appropriate patients. Patients who fail to respond as anticipated should have their dietary history reviewed meticulously, because incomplete removal of gluten is the most common cause of treatment failure. In patients who fail to respond despite strict adherence to a gluten-free diet, alternative diagnoses should be considered, as well as the possibility of associated diseases that are not responsive to a gluten-free diet such as refractory sprue, collagenous colitis, or enteropathy-associated T-cell lymphoma. Some patients with refractory sprue respond to corticosteroids or other

immunosuppressive agents; however, these should be employed only after other potential diagnoses have been investigated.

Mechanism of Disease

Celiac disease was first described in children in 1888 (87), and it was reported in adults in 1932 (88). A major advance in understanding the pathogenesis of this disease occurred in the 1950s, when it was demonstrated that dietary wheat was harmful to children and adults with celiac disease (89). Subsequently, the harmful portion of dietary wheat was shown to be the water-insoluble portion of wheat protein or gluten (90). The component of gluten playing a role in the pathogenesis of celiac disease was identified as the alcohol-soluble portion of wheat gluten, or gliadin, a complex mixture of glutamine- and proline-rich polypeptides (91). Initially, the relevant pathophysiologic mechanism of celiac disease was believed to involve the “susceptible” mucosa that was postulated to arise as a result of an intestinal enzyme deficiency or as a result of a defect in mucosal permeability. As our understanding of celiac disease has progressed, the concept of the “susceptible” mucosa has lost favor. Current evidence suggests that celiac disease is predominantly an immunologically mediated disorder involving an inappropriate T-cell mediated immune response to dietary gliadin.

The initial clues to understanding the immunologic mechanism of celiac disease arose from genetic studies documenting a concordance of celiac disease and specific HLA antigens (92,93). Follow-up to these observations revealed that approximately 95% of patients with celiac disease express the MHC II complex HLA-DQ2. In most persons, the HLA-DQ2 complex is either encoded in *cis* by alleles DQA1*0501 and DQB1*0201, in patients with the DR3-DQ2 haplotype (94), or in *trans* by alleles DQA1*0501 and DQB1*0202, in patients with the DR5-DQ7/DR7-DQ2 haplotype. Alleles DQB1*0201 and DQB1*0202 differ at one amino acid and are believed to be functionally equivalent. Most patients with celiac disease lacking the HLA-DQ2 complex express the HLA-DQ8 complex encoded by alleles DQA1*0301 and DQB1*0302 (95,96). The association of celiac disease and these MHC II complexes is so strong that most patients with celiac disease express either HLA-DQ2 or HLA-DQ8. Among the primary functions of MHC II are thymic selection of $CD4^+$ T lymphocytes and presentation of antigen to $CD4^+$ T lymphocytes in the peripheral immune system. Therefore, the strong genetic linkage of specific MHC II complexes with the requirement for the exposure to gliadin implied a role for T-lymphocyte responses specific for gliadin as a mechanism of disease.

Further advances in the understanding of celiac disease were facilitated by the development of techniques allowing *in vitro* assays of intestinal biopsies from patients with celiac disease. Using these assays, investigators showed that small intestinal biopsy tissue from patients with celiac disease that was exposed to gluten in culture produced a proinflammatory cytokine response dominated by the production of IFN-g (97). Further studies documented that inhibition of T-cell activation in this *in vitro* system could prevent the production of IFN-g and antiendomysial antibodies by small intestinal biopsies from patients with celiac disease on exposure to gliadin (98). In further support of these findings, $CD4^+$ T lymphocytes bearing T-cell receptors (TCRs) specific for a naturally occurring peptide of gliadin in association with HLA-DQ2 were cloned from the intestine of patients with celiac disease, but not from control populations (99).

The understanding of celiac disease was further advanced by the demonstration that most antiendomysial antibodies from patients with celiac disease are directed against the self-protein tTG (85). The role of antibodies directed against tTG in causing celiac disease is unclear; however, tTG may play a role in augmenting the immune response to gliadin. tTG has the capacity to deamidate neutral charged glutamines, converting them to negatively charged glutamate residues. Deamidation of gliadin peptides allows them to bind HLA DQ-2 with increased affinity and more effectively to drive an immune response mediated by small intestinal T cells in patients with celiac disease (100). In addition, tTG may generate novel antigenic epitopes by cross-linking gliadin peptides with self-proteins and may potentially initiate an autoimmune response (101). This work has been extended to identify the immunodominant tTG-modified gliadin epitopes predominantly responsible for driving the inappropriate T-cell responses in some patients with celiac disease (102,103).

Taken together, this body of work suggests the following model for the pathogenesis of celiac disease (Fig. 55.4). In persons expressing the specific MHC II complexes DQ2 and DQ8 along with other as yet undefined susceptibility factors, small amounts of dietary gliadin cross the epithelial cell layer and initiate an immune response. The gliadin peptides may undergo deamidation by tTG present in the intestinal mucosa, and cross-linking of these peptides to other nonself-proteins and self-proteins including tTG. These peptides are taken up by antigen-presenting cells and are processed and presented in association with MHC II complexes DQ2 and DQ8, notably the deamidated gliadin peptides bind DQ2 with increased affinity; the result is more efficient intestinal T-cell stimulation. $CD4^+$ T lymphocytes specific for these gliadin peptides in association with MHC II are then stimulated by TCR ligation and undergo proliferation, clonal expansion, and cytokine production. T-cell activation and cytokine production result in a cascade of yet undescribed downstream events, damaging the mucosa and resulting in the characteristic pathologic lesion of celiac disease: villous blunting, crypt hyperplasia, and lamina propria expansion. Studies have shown that most antiendomysial antibodies produced in patients with celiac disease are specific for tTG, and investigators have proposed that this occurs because of cross-linking of gliadin to tTG that eventually results in the production of antibodies directed toward tTG. The requirement for anti-tTG antibodies or T-cell responses specific for self-protein in the development of celiac disease remains to be proven.

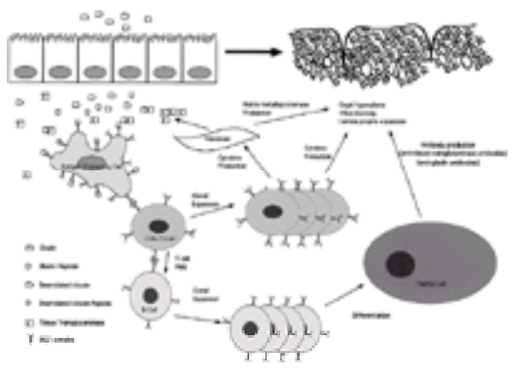

Figure 55.4. Immunologic mechanism for the pathogenesis of celiac disease. In susceptible persons expressing the HLA-DQ2 complex, small amounts of gliadin in an immunogenic form traverse the intestinal epithelium after the ingestion of dietary wheat products. Tissue transglutaminase present in the mucosa may deamidate gliadin, and/or crosslink gliadin with other proteins, including tissue transglutaminase. Gliadin, deamidated gliadin, and gliadin linked to other proteins may be taken up by professional antigen-presenting cells, processed, and presented to $CD4^+$ T cells in association with major histocompatibility complex class II (HLA-DQ2). Notably gliadin peptides bind HLA-DQ2 with increased affinity and have been shown to stimulate intestinal T cells from patients with celiac disease more efficiently. Gliadin-specific T cells proliferate, expand, and produce cytokines that can facilitate B-cell proliferation and differentiation to plasma cells. Activated T cells can further induce tissue transglutaminase production by fibroblasts; the result is further deamidation of gliadin on dietary wheat ingestion. B cells with B-cell receptors specific for gliadin cross-linked to other proteins, including self-proteins, may be preferentially expanded by presenting these antigens to T cells, and subsequently differentiate into plasma cells producing antibodies directed against self-proteins such as tissue transglutaminase. This continual immune activation results in yet undefined downstream events leading to mucosal damage and the characteristic lesion consisting of villous blunting, crypt hyperplasia, and lamina propria expansion.

Despite the major advances in our understanding of the pathogenesis of celiac disease, many questions remain, including the role of autoimmune responses in the development of celiac disease, the relevant responses downstream of T-cell activation resulting in the pathogenesis of celiac disease, and the reason most persons expressing MHC complexes DQ2 and DQ8 do not develop celiac disease. At present, celiac disease represents one of the best understood immunologically mediated enteropathies, and it may suggest a framework to understand other immunologically mediated diseases in the intestine.

INFLAMMATORY BOWEL DISEASE

IBD includes *ulcerative colitis* and *Crohn's disease,* which are chronic inflammatory diseases of the gastrointestinal tract (104). They are diagnosed by the appearance of a set of clinical, endoscopic, and histologic characteristics. The inflammatory response in ulcerative colitis is largely confined to the mucosa and submucosa, but in Crohn's disease, inflammation extends through the intestinal wall from mucosa to serosa. Ulcerative colitis is confined to the colon, whereas Crohn's disease has the potential to involve the patient's entire gastrointestinal tract, even though only a small segment is involved initially. Despite these differences in distribution, no single finding is absolutely diagnostic of one disease over the other. Moreover, there is a group of patients whose clinical picture falls between the two diseases; these patients are said to have indeterminate colitis.

Genetics and Environmental Factors

The pathogenesis of IBD involves a complex set of interactions among susceptibility genes, the environment, and the immune system (105). The most important risk factor for IBD is a family history; approximately 15% of patients with IBD have affected first-degree relatives, and the incidence among first-degree relatives is 30 to 100 times that of the general population. The best estimate of the lifetime risk of developing IBD among first-degree relatives of affected persons is 3% to 9%. Concordance among monozygotic twins is high, especially in Crohn's disease. Susceptibility to IBD does not follow any simple mendelian mode of inheritance. Although no specific

genetic abnormalities have been demonstrated to predispose to the development of IBD, the available data suggest oligogenic inheritance; that is, more than one gene may predispose to the development of IBD. Moreover, both ulcerative colitis and Crohn's disease are genetically heterogenous. Work is under way to identify predisposing genes using both the candidate gene approach and systematic genomewide scans. HLA class II genes are associated with IBD. The DR1-DQW5 complex and the DRB3*0301 allele are associated with Crohn's disease, and HLA-DR2 is associated with ulcerative colitis. Linkage analysis in multiply affected families suggests that genes outside the HLA complex are also important determinants of susceptibility in ulcerative colitis and Crohn's disease. A susceptibility locus for Crohn's disease is found on chromosome 16; localization is centered around loci D165409 and D165419 (106). Other major susceptibility loci map to chromosomes 1p, 3q, and 4q. The next step is to identify candidate genes in these regions that may be relevant to IBD.

Clear evidence indicates activation of the immune response in IBD. The lamina propria is infiltrated with lymphocytes, macrophages, and other cells of the immune system, and high levels of proinflammatory cytokines and inflammatory mediators are found in the mucosa. The earliest histologic changes in Crohn's disease are the changes of immune activation. Lymphocytes, macrophages, and plasma cells accumulate near mucosal crypts. Immune activation next to a crypt can lead to the development of a crypt abscess with destruction of the epithelium. An inflammatory process involving adjacent crypts leads to the development of an aphthous ulcer, the earliest macroscopic evidence of disease. This sequence is important in that it demonstrates that in IBD immune activation leads to epithelial injury, rather than epithelial injury leading to immune activation.

Although clear evidence indicates activation of the immune response in IBD, the specific antigen that triggers this response has not been identified, despite an intensive search over the past 40 years. In IBD, immune activation is largely confined to the gastrointestinal tract; therefore, the search for an antigenic trigger has focused on the intestinal lumen, where most of the foreign antigens are either microbial or dietary in origin. Three major hypotheses of the antigenic trigger in IBD have been postulated (104). One is that the antigenic trigger is a microbial pathogen that has not yet been identified because of fastidious culture requirements. According to this hypothesis, the immune response in IBD is an appropriate but ineffective response to a pathogen. Various viruses, bacteria, and mycobacteria have been proposed as candidate organisms, but little evidence has been found to support any of these as a causal agent in IBD.

The second hypothesis of the antigenic trigger in IBD is that it is one expressed on the patient's own cells, particularly intestinal epithelial cells. In this autoimmune hypothesis, the patient mounts an appropriate immune response against some luminal antigen, either dietary or microbial; however, because of similarities between proteins on the epithelial cell and the luminal antigen (molecular mimicry), the patient's immune response also attacks the epithelial cells. Anticolon antibodies have been identified in patients with IBD. The best characterized of these is an IgG antibody to tropomyosin; this antibody was eluted from resected, washed ulcerative colitis tissue (107). Although this antibody has been well characterized, no evidence indicates that it plays a role in the pathogenesis of ulcerative colitis.

The third, and best supported, hypothesis of the antigenic trigger in IBD is that it is some common dietary or usually nonpathogenic microbial agent against which the patient mounts an inappropriate immune response. In healthy people, a finely tuned, low-grade chronic inflammation is present in the intestinal lamina propria. This chronic inflammation is a product of exposure of immune cells in the lamina propria to luminal antigens. Failure to suppress this inflammatory response would result in an inappropriately vigorous and prolonged immune response to normal luminal antigens. The uncontrolled immune activation seen in IBD may reflect the failure of these normal suppressor mechanisms. The genetic basis of IBD may relate to an inability to mount a suppressor response to a specific luminal antigen. Perhaps patients with IBD are genetically programed to mount an intense immune response to some common luminal antigen, either dietary or microbial, to which immunologically normal persons mount a suppressor response. Whether this failure of suppression is specific to a single antigen on a single strain of bacteria or is a global failure of suppression to large groups of bacterial strains is not clear. In genetically based mouse models of colitis, defects in T-cell function or cytokine production result in uncontrolled immune responses to normal colonic bacteria. It may be that in IBD a defect in immune regulation results in an inappropriately active immune response to normal colonic bacteria.

An insight into the breakdown of suppressor mechanisms in IBD comes from an *in vitro* study of the immune response to intestinal bacteria. Peripheral blood mononuclear cells and lamina propria mononuclear cells from immunologically normal persons do not proliferate when they are incubated with bacterial sonicates from autologous intestine. However, lamina propria mononuclear cells from inflamed areas of IBD intestine proliferate when cocultured with bacteria (108). These data do not suggest that patients with IBD have a specific defect in their immune response to bacteria, but rather they suggest that the tolerance to intestinal bacteria is broken in IBD. The regulatory events that downregulate the mucosal immune response to normal luminal antigens have failed. In this sense, there is no antigenic “trigger” in IBD, but rather the inappropriate immune response is initiated by a failure of the suppressor response to luminal antigens.

Immune System

Analysis of circulating leukocytes shows only modest differences between immunologically normal persons and patients with IBD (109). The number of circulating lymphocytes and the proportions of $CD4^+$ and $CD8^+$ T cells subsets are normal. The lymphocyte proliferative response to mitogens is also normal. The most obvious difference is an increase in the number of neutrophils. Immunohistochemical analysis of the lamina propria reveals that the total number of T cells is increased in IBD, but the $CD4^+$:$CD8^+$ ratio is the same, at 2:1 (110). The number of intraepithelial lymphocytes in IBD mucosa is normal. Lamina propria T cells in IBD are more activated than in normal intestinal tissue, with increased cell-surface expression of activation markers, increased Ig secretion, and increased proliferation (111,112).

Most patients with IBD have normal serum levels of all the Ig classes (IgG, IgA, IgM, and IgE). Turnover studies demonstrate increased turnover of IgG and IgM with increases in both synthesis and catabolism. Although serum antibody levels are largely normal, Ig production in the lamina propria is markedly enhanced in IBD. Isolated lamina propria mononuclear cells from IBD surgical resections synthesize and secrete IgG, IgA, and IgM at elevated levels. Some of these antibodies are directed to luminal bacteria (113). There are marked differences in IgG subclass production in IBD, with increased IgG1 production in ulcerative colitis and increased IgG2 in Crohn's disease.

Immunoregulation

Although the antigenic trigger that activates the immune response in IBD has not been identified, the sequence of events involved in the immune activation and tissue destruction in IBD has been characterized (109,114). This sequence, presented in Fig. 55.5, is used to organize a review of normal events in mucosal immune activation and the abnormalities seen in IBD. Several genetic manipulations of the immune system have been shown to induce chronic colitis in mice. These manipulations include targeted disruption of the genes encoding IL-2, IL-10, and the TCR, alterations in T-cell subsets and reconstitution of severe combined immunodeficiency (SCID) mice with T-cell subsets (115,116,117,118,119 and 120). The sequence of events in Fig. 55.5 is used to integrate these mouse models into what is known about normal and dysregulated immune activation in the intestine.

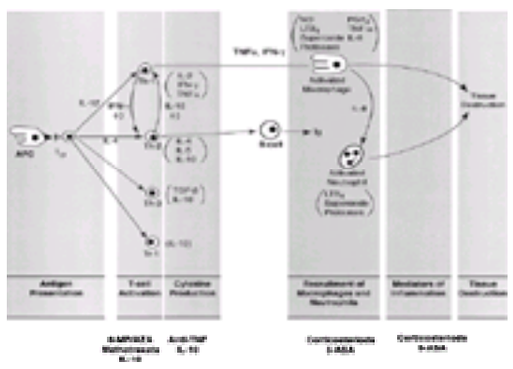

Figure 55.5. The pathogenesis of inflammatory bowel disease. The mucosal immune response develops through a series of steps (e.g., antigen presentation, lymphocyte activation, cytokine production). The drugs used to treat this disease act by affecting some of these steps. The names of the drugs are listed below the name of the step they affect. Among these drugs are azathioprine (AZA), 6-mercaptopurine (6-MP), and 5-aminosalicylic acid (5-ASA). Antigen-presenting cells (APCs) present antigen to naive $CD4^+$ T cells (T), which differentiate to four different $CD4^+$ T-cell subsets (Th1, Th2, Th3, Tr1) under the control of various cytokines (interleukin-4 (IL-4), IL-10, IL-12). These $CD4^+$ T-cells subsets, in turn, produce a variety of cytokines. Two of these cytokines, tumor necrosis factor-a (TNF-a) and interferon-g (IFN-g), activate macrophages and result in the production of nitric oxide (NO), leukotriene B_4 (LTB_4), superoxide, proteases, prostaglandin E_2 (PGE_2), TNF-a, and IL-8. Neutrophils, in turn, are activated by IL-8. Both activated macrophages and activated neutrophils contribute to tissue destruction through the production of superoxide and proteases.

The first step in the immune response to antigen in the intestine consists of the uptake and processing of antigen by professional antigen-presenting cells (primarily macrophages and dendritic cells). $CD4^+$ T cells undergo stimulation by TCR ligation by antigenic peptides presented in conjunction with HLA class II molecules on macrophages and dendritic cells. Optimal T-cell stimulation requires “costimulation” or ligation of CD28 on T cells by CD80 or CD86 on antigen-presenting cells.

Evidence also indicates that intestinal epithelial cells can express class II and may play a role in antigen presentation within the intestinal immune compartment; however, it is unclear if they can efficiently provide costimulatory signals (121). HLA class II is expressed on small intestinal epithelial cells in Crohn's disease; whether antigen presentation by epithelial cells plays any role in the pathogenesis of Crohn's disease remains to be seen. The antigen specificity of $CD4^+$ T cells is determined by their TCRs. One could imagine that a germline abnormality in the TCR could result in oral reactivity and could contribute to disease susceptibility. However, carefully performed studies have failed to demonstrate differences between patients with IBD and control subjects in their TCRs. Azathioprine, 6-mercaptopurine, and methotrexate are all used to treat IBD, particularly Crohn's disease, and all appear to act by inhibiting T-cell activation.

Activation of T cells results in cytokine production. In mice, differentiated $CD4^+$ T cells may be grouped into subsets based on the cytokines they produce after antigenic stimulation. This paradigm may have significant importance in IBD, because cytokines produced by one subset of $CD4^+$ T cells (Th1) are believed to have pathogenic roles, whereas cytokines produced by other subsets (Th2, Th3, Tr1) are believed to be immunoregulatory or beneficial. Th1 cells produce IL-2, IFN-g, and TNF-a, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. Activation of Th2 cells promotes B-cell activation. Naive Th0 cells differentiate down either the Th1 or the Th2 pathway when they ligate antigen. The pattern of differentiation is determined by the cytokines that are present in the environment at the time of differentiation. IL-12 promotes differentiation down the Th1 pathway, whereas IL-4 promotes differentiation down the Th2 pathway. The development of a dysregulated Th1 response has been demonstrated in genetically engineered mouse models of colitis (114,115,116,117,118,119 and 120). T cells secreting IFN-g and TNF-a are found in intestinal lesions in these models, and therapy with anti–IFN-g or anti-TNF is effective. The importance of Th1 activation in mediating intestinal inflammation is supported by the demonstration that antibodies to IL-12 improve both the clinical and histopathologic aspects of trinitrobenzene sulfonic acid colitis in mice (122). In this model of colitis, lamina propria T cells demonstrate a Th1 pattern of cytokine production. Administration of monoclonal antibodies to IL-12 results in marked improvement in this model even if given therapy is after the inflammatory response is well established (122). The ability of IL-12 antibodies to downregulate the immune response in this model suggests that preventing lymphocytes from differentiating down the Th1 pathway blocks the development of colitis.

In all these models, colitis does not develop if the genetically manipulated animals are raised in a germ-free environment (123). Moreover, transfer of Th1 cells that react with bacterial antigens from mice with colitis to immune-deficient mice induces colitis (124). These findings demonstrate that a genetically determined defect in systemic immunity can have as its only manifestation colitis that results from an inappropriately robust immune response to normal colonic bacteria. Whether this is a global response to all colonic bacteria or a selective response to particular bacteria has not been established. These data could support the suggestion that these models result from a failure to mount a suppressor response to colonic bacteria. These findings also support the suggestion that colonic bacteria are the targets of the immune activation in IBD.

Whether the Th1/Th2 model can be applied to IBD in humans is not clear. In one study, lamina propria mononuclear cells isolated from IBD surgical resections were stimulated with anti-CD2/CD28, a stimulus that would determine their capacity to produce T-cell cytokines (125). T cells from Crohn's disease mucosa produced IFN-g, whereas those from ulcerative colitis mucosa produced IL-5, a finding supporting the suggestion of an overactive Th1 response in Crohn's disease. Cytokine production in IBD mucosa has been measured in several studies. The most striking finding is that IFN-g and IL-12 are produced at high levels in Crohn's disease mucosa but not in ulcerative colitis mucosa. Further support for the role of cytokines in the pathogenesis of Crohn's disease comes from the demonstrated therapeutic efficacy of anti-TNF antibody and from the suggestion that exogenous recombinant IL-10 is also therapeutic in Crohn's disease (126,127). IL-10 promotes the differentiation of Th0 cells to non-Th1 subsets, which, in turn, may inhibit the differentiation of Th1 subsets; therefore, one may expect that exogenous IL-10 would diminish Th1 activation in Crohn's disease. Taken together, these data suggest that the gastrointestinal immune activation in Crohn's disease is mediated by activation of Th1 cells.

In health, the intestinal inflammatory response is maintained under tight control despite exposure of lamina propria immune cells to an enormous number of luminal antigens. The tight check on the mucosal immune response is mediated by populations of regulatory T cells. Most studies of regulatory T cells in the intestine have been performed in mice, and the applicability of these studies to human IBD is not clear. One population of regulatory T cells, designated Th3, produces TGF-b, which downregulates Th1 cells (128). A second population of regulatory T cells, designated Tr1, produces large amounts of IL-10 (129). The immunomodulatory function of Tr1 cells was confirmed using the $CD45RB^{high}$ transfer model in mice. Splenic $CD4^+$ T cells can be divided on the basis of their expression of CD45RB; naive $CD4^+$ cells are $CD45RB^{high}$, whereas primed or memory $CD4^+$ cells are $CD45RB^{low}$. Transfer of $CD4^+$, $CD45RB^{high}$ splenocytes into SCID mice induces colitis, whereas transfer of $CD4^+$, $CD45RB^{low}$ splenocytes causes no disease (118,119). Transfer of the two populations together also causes no disease. $CD45RB^{high}$ $CD4^+$ splenocytes produce IFN-g, whereas $CD45RB^{low}$ $CD4^+$ splenocytes produce TGF-b and IL-10, which inhibit the production of IFN-g. The $CD45RB^{low}$ subset contains Tr1 cells, which produce IL-10. These experiments demonstrate that colitis can be induced by transfer of a population of lymphocytes that occur in normal mice; moreover, this colitis can be prevented by transfer of Tr1 T cells.

In a particularly interesting set of studies, investigators demonstrated that colitis induced in SCID mice by transfer of $CD45RB^{high}$ $CD4^+$ T cells can be prevented by cotransfer of murine Tr1 clones derived from $CD4^+$ T cells expressing a transgenic TCR for ovalbumin (129). Immune suppression of this system depends on antigen-induced activation of Tr1 cells *in vivo* because these cells inhibit colitis only when recipients are receiving ovalbumin in their drinking water. By contrast, the effect of Tr1 cells is not antigen specific because these cells can inhibit the function of T cells responding to unknown intestinal antigens. Thus, IL-10 and TGF-b produced by Tr1 cells in response to one antigen can suppress T-cell activation in response to a wide variety of other antigens. It is likely that this phenomenon, termed *antigen-driven bystander suppression,* accounts for some of the global downregulation of the immune response in the normal intestine. Failure of antigen-driven bystander suppression could account for the inappropriate immune activation seen in IBD.

Macrophages

The next step in immune activation in the intestine is the activation of lamina propria macrophages by cytokines produced by activated T cells (130). IFN-g produced by Th1 cells activates mucosal macrophages and causes them to produce TNF-a. In contrast, the activation of mucosal macrophages is downregulated by TGF-b and IL-10, which are produced by Th3 and Tr1 regulatory T cells.

Intestinal macrophages, like macrophages in other organs, are derived from monocytes in the peripheral blood. The phenotype and functional capacity of macrophages are influenced by the tissue microenvironment in which they reside. Normal intestinal macrophages have a downregulatory phenotype and cannot be easily induced to mediate acute inflammatory responses. Perhaps the cytokine environment of the intestinal mucosa induces migrating monocytes to differentiate to a down regulatory phenotype, or it may be that monocytes predisposed to differentiate toward a downregulatory phenotype selectively migrate into the intestine. One example of the downregulatory properties of normal intestinal macrophages is their failure to express CD14, which mediates the response to LPS (131). Compared with macrophages in other organs, intestinal macrophages make large amounts of IL-10 and relatively small amounts of IL-12. This pattern of cytokine production should upregulate the Th2 response and downregulate the Th1 response. Intestinal macrophages are also relatively resistant to activation by IFN-g; they fail to generate a respiratory burst when they are stimulated with IFN-g. In contrast, macrophages isolated from the intestines of patients with IBD act to promote rather than to suppress the inflammatory response. Many lamina propria macrophages in IBD express CD14 and are therefore more responsive to activation by LPS (131). In contrast to macrophages from normal intestine, many macrophages isolated from IBD mucosa can be triggered by incubation with IFN-g to undergo a respiratory burst generating reactive metabolites of oxygen including superoxide, hydrogen peroxide, and hydroxyl radical. There are also important differences in the abilities of normal and IBD lamina propria macrophages to express cytokines. Normal lamina propria macrophages do not express IL-1 or IL-18 when they are activated with LPS, whereas lamina propria macrophages from patients with IBD do express these cytokines in response to LPS.

In health, lamina propria macrophages secrete factors, including IL-10 and PGE_2, that downregulate the immune response. Lamina propria macrophages express cyclooxygenase-2 and produce large amounts of PGE_2 through cyclooxygenase-2 (132). The PGE_2 produced by lamina propria macrophages acts as an immunomodulator. In T-cell responses to antigen, PGE_2 modulates the immune response. Clinical exacerbation of IBD by nonsteroidal antiinflammatory drugs may be the result of inhibition of the synthesis of immunomodulatory prostaglandins in the intestinal mucosa.

Inflammation

The next step in immune activation in the intestinal tract is the activation of macrophages and neutrophils to produce proteases, reactive oxygen species, and inflammatory mediators (3). Macrophage activation results in the production of IL-8 and other chemotactic factors that enhance the migration of additional peripheral blood neutrophils into the inflamed mucosal. In IBD, enormous numbers of neutrophils leave the circulation and enter the inflamed mucosa and submucosa of the bowel. Some of these neutrophils migrate across the epithelium into the lumen and are passed into the stool, and others are destroyed in the inflamed tissue before they have a chance to migrate into the lumen. This constant flux of neutrophils is mediated by the expression of adhesion molecules on circulating neutrophils and vascular endothelial cells; these adhesion molecules allow the neutrophils to bind to the endothelium before migrating into the tissue. Both the expression of adhesion molecules and the migration of neutrophils are regulated by inflammatory cytokines (IL-8 and TNF-a) and by lipid mediators of inflammation including platelet-activating factor and leukotriene B_4. IL-8, TNFa, platelet-activating factor, and leukotriene B_4 are all found at elevated levels in IBD mucosa. Neutrophil activation in the inflamed gut results in the release of granule-bound proteases and the production of superoxide and other reactive oxygen species. These products of activated neutrophils play an important role in the destruction of the epithelium in IBD. Functional and macroscopic changes seen in IBD mucosa, including hyperemia and edema, are changes typical of any inflammatory state and are the products of soluble mediators released in the process of inflammation. Inflammatory mediators cause tissue edema by increasing vascular permeability to albumin and other macromolecules; hyperemia results from mediators that induce vasodilation. PGE_2 is a mediator of both enhanced vascular permeability and vasodilation in IBD. Both corticosteroids and 5-aminosalicylate have certain pharmacologic effects that could contribute to their

efficacy in IBD. All these pharmacologic effects are focused on blocking either the recruitment or activation of macrophages and neutrophils.

Immune activation in IBD also induces epithelial cells to express proteins they do not ordinarily express. Epithelial cells in areas of active inflammation in IBD express inducible NO synthase (iNOS), cyclooxygenase-2 (an enzyme involved in prostaglandin synthesis), and the neutrophil chemotactic agent epithelial neutrophil-activating peptide 78 (133,134 and 135). The expression of these proteins is regulated by IL-1 and TNF-a, and the high levels of these cytokines in IBD mucosa may account for the expression of these proteins in epithelial cells.

The last step in the pathogenesis of IBD is the destruction of the epithelium. The major mediators of epithelial destruction appear to be reactive oxygen species and proteases produced by activated neutrophils and macrophages. Proinflammatory cytokines including TNF-a and IFN-g may also contribute to the destruction. Activation of neutrophils and macrophages is mediated in part by the transcription factor NF-kB. The expression of IL-8, iNOS, and cyclooxygenase-2 is regulated by NF-kB activation. Each of these proteins plays a role in the pathogenesis of IBD. Administration of an antisense phosphorothioate oligonucleotide to a subunit of NF-kB abrogates the inflammatory response in the trinitrobenzene sulfonic acid model of colitis in the mouse (136). This raises the question whether pharmacologic manipulation of NF-kB would be useful in human IBD.

This sequence of events that occurs in the development of IBD (Fig. 55.5) is much the same as the sequence of events that occurs in response to infection with *Salmonella* or *Shigella* (3). However, in *Salmonella* or *Shigella* infection, this sequence of immune activation is followed by a downregulation of the inflammatory response and epithelial repair. In these infections, the inflammatory response is downregulated when the organisms driving the upregulation of the inflammatory response have been eliminated. In contrast, in IBD, there are no pathogenic organisms to eliminate and thus no easy mechanism for shutting down the immune response.

WHIPPLE DISEASE

G.H. Whipple first described *Whipple disease* in 1907 as lipodystrophia intestinalis (137,138). Although he described the presence of a rodlike bacillus in the lamina propria of the intestine, it was not until the early 1990s that molecular biologic techniques were used to identify the organism as a gram-positive actinomycete, which was given the name *Tropheryma whippleii* (137,138).

Although primarily thought of as a gastrointestinal disorder, Whipple disease is a chronic, systemic illness. The presentation is variable and can include many different signs and symptoms; however, the most common clinical presentation is a combination of diarrhea, weight loss, and malabsorption, which has been preceded for months to years by other symptoms such as arthralgias (139). Whipple disease has a low incidence (frequency less than 0.1%) and occurs primarily in white, 40- to 50-year old men, although all age groups (including children) can contact the disease. A genetic predisposition can be seen in the HLA-B27 positive population because 28% to 44% of patients with Whipple disease are HLA-B27 positive (versus 8% in the general population) (138,140).

Patients with Whipple disease often have laboratory evidence of malabsorption (deficiency of fat-soluble vitamins, hypoalbuminemia, hypocholesterolemia, mineral deficiency) and normocytic hypochromic anemia of chronic disease. However, small intestinal biopsy and histologic demonstration of characteristic lesions make the diagnosis of Whipple disease. Endoscopy shows areas of thickened folds with the typical granular, yellow-white shaggy covering (from thickened or blunted edematous intestinal villi). The first and second portions of the duodenum are characteristically involved, and disease extends variably throughout the distal small intestine. The histologic hallmark of Whipple disease consists of foamy macrophages, which are characteristically periodic acid-Schiff (PAS)–positive (141). The substance in macrophages that stains positive with PAS is thought to be remnants of the cell walls of phagocytosed bacilli. These PAS-positive monocytes also contain gram-positive, acid-fast bacillus–negative *T. whippleii.* These PAS-positive macrophages can be detected in almost all tissues in patients; however, the presence of PAS-positive macrophages is not pathognomonic of Whipple disease, because they have also been identified in other infections *(Mycobacterium tuberculosis, M. avium-intracellulare).* The intestinal mucosa demonstrates widened villi with numerous PAS-positive macrophages in the lamina propria and decreased numbers of lamina propria plasma cells, lymphocytes, and eosinophils. Small intestinal mucosal or submucosal lymphatic vessels are dilated and have easily visualized lipid droplets, a finding suggesting that the malabsorption is secondary to lymphatic stasis.

The suspicion of Whipple disease based on characteristic histologic lesions can be confirmed by polymerase chain reaction (PCR) based on the 16S ribosomal RNA of *T. whippleii* (142). The recommended treatment is daily intravenous streptomycin and penicillin for 14 days, followed by oral trimethoprim-sulfamethoxazole for 1 year (138). Under this treatment, the joint symptoms disappear in days, whereas intestinal symptoms take weeks to improve. Even with this extended antibiotic regimen, the rate of relapse is significant (35%), and investigators have recommended that PCR should be used to monitor response to therapy, with a negative result on PCR after 1 year predicting a low likelihood of clinical relapse (142,143).

The pathogenesis of Whipple disease is still unclear; however, it has been hypothesized that patients with the disease have an altered host response. Most patients show an impairment of delayed-type hypersensitivity and appear to have an inability to eliminate the *T. whippleii* bacilli effectively (i.e., persistence of PAS-positive macrophages) (137). One immunologic defect in this disease is the reduced ability of monocytes to produce IL-12 on activation (144). This primary defect in IL-12 production results in a secondary effect of reduced IFN-g production by Th1 cells. Because IFN-g is important for activating macrophages to kill organisms more effectively, this may explain the inability of patients with Whipple disease to control *T. whippleii* infection. This monocyte-macrophage cytokine defect is supported by an absence of the cell-surface marker CD11b on PAS-positive macrophages in the lamina propria of patients with Whipple disease (137,144).

Until recently, *T. whippleii* could not be isolated or propagated; however, investigators have reported that IL-4 deactivated macrophages and human fibroblasts are permissive to *T. whippleii* growth (145,146). This use of IL-4 to induce permissive growth conditions for *T. whippleii,* when considered in the light of the reported monocyte IL-12 production deficiency, allows an intriguing hypothesis to be proposed. These two cytokines have well-described effects on the development of differentiated Th subsets; IL-12 enhances T-cell development to IFN-g–producing Th1 cells, whereas IL-4, in the absence of IL-12, enhances the development of IL-4–producing Th2 cells. Evidence based on PCR analysis of healthy people has shown that 11% to 35% of people are colonized with *T. whippleii* (147,148). In light of this finding, the genetic predisposition to produce Th2 more than Th1 cells may allow a commensal bacillus to become pathogenic.

IMMUNOPROLIFERATIVE SMALL INTESTINAL DISEASE

Immunoproliferative small intestinal disease (IPSID) is a clinical syndrome of malabsorption, chronic diarrhea, and weight loss that is endemic in Middle Eastern and Mediterranean countries (149). This syndrome is thought to represent a spectrum of diseases, which in the past were known as a-heavy-chain disease and Mediterranean lymphoma. It is similar in clinical presentation to celiac sprue, which predominates in other parts of the world. Both syndromes involve a chronic stimulation of the intestinal MALT and are associated with an increased risk of malignant disease.

IPSID was first described in the mid-1960s in Israel in young adults with dietary malabsorption (149). The histologic appearance is characterized by plasmacytic or lymphocytic infiltrate in the lamina propria of the small intestine. The intestinal villi are broadened, and the crypts are shortened, whereas the intestinal epithelial cells appear relatively normal (150). Over time, this early lesion evolves into late-state IPSID, which is characterized by broadening of the villi to the point of complete effacement and infiltration by malignant lymphocytes. This lesion was originally known as Mediterranean lymphoma.

In some cases, plasma cells in the intestinal lamina propria secrete an IgA heavy-chain protein (a-CP) devoid of light chains (151,152). This unique a-CP is an approximately 30,000 MW protein that consists of a free a_1-heavy chain that is missing the variable (V_H) and constant (C_H1) regions because of a structural mutation. This deletion allows a-CP to be secreted in the absence of light chains. a-CP can be detected in serum by immunofixation electrophoresis or in tissue sections by immunohistologic staining of plasma cells. The less sensitive diagnostic test of serum electrophoresis often cannot detect a-CP. The presence of this monoclonal Ig, in conjunction with clinical and histologic signs of Mediterranean lymphoma, led physicians to term the disease a-heavy-chain disease.

Most patients with IPSID originate from developing countries in the Mediterranean basin, the Middle East, Africa, and the Far East. They are usually young, can be either male or female, and live in areas of poor sanitation and hygiene (149). In addition to malabsorption, patients often present with clubbing, abdominal pain, and growth retardation. The diagnosis of IPSID is made by intestinal biopsy, which reveals the characteristic histologic picture described earlier. Endoscopy variably shows a nondistensible intestine resulting from submucosal infiltrates and thickened mucosal folds or ulcers. Bacterial overgrowth and intestinal parasitosis (primarily with *Giardia*) are common, and serum a-CP may be present (20% to 87%) (153,154).

The high prevalence of intestinal microbial overgrowth in IPSID and its endemic nature in areas of poor sanitation in the Middle Eastern and Mediterranean countries have led to the proposal that IPSID represents a chronic immunoproliferative response of MALT B cells to bacteria or parasites that eventually becomes monoclonal (155,156). This is similar to the association between *H. pylori* infection and primary gastric lymphoma (MALT lymphomas) (66). This association has led to the recommended treatment for early stages of IPSID with tetracycline or other broad-spectrum antibiotics (157), which has been reported to yield a 20% to 71% response rate. Patients with late-stage disease have been treated with total abdominal radiation or multiple combination chemotherapy regimens (157). Overall, complete remission rates have averaged 64%. One report has linked the regression of refractory IPSID to the eradication of *H. pylori* (158).

GRAFT-VERSUS-HOST DISEASE

The term *graft-versus-host* describes an immunologic entity characterized by skin abnormalities and diarrhea and caused by immunologically competent cells introduced into an allogenic immunoincompetent host (159). *Graft-versus-host disease* (GVHD) is a significant cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation. Acute GVHD usually presents within the first 100 days after transplantation with a distinctive syndrome characterized by a triad of dermatitis, hepatitis, and gastroenteritis (159). The incidence of acute GVHD is increased in older patients and in patients who received marrow transplants from related, but HLA-nonidentical donors or transplants from HLA-matched unrelated donors (159). The risk of GVHD has been correlated with minor HLA antigen disparities, which act as alloantigens that can be recognized by donor lymphocytes. The diagnosis of acute GVHD involving the intestinal tract is made most accurately by upper gastrointestinal endoscopy with biopsy (160). The endoscopic appearance of the stomach and duodenum ranges from subtle mucosal erythema and edema to frank ulceration and mucosal sloughing. The histologic findings from intestinal biopsy include crypt epithelial cell apoptosis and dropout, crypt destruction, and lymphocytic infiltration (160).

GVHD is caused by an attack of donor T cells against alloantigens of the recipient (161). Depletion of T cells in the donor marrow prevents GVHD, but it results in a higher rate of graft failure and an increased incidence of leukemic relapse (i.e., loss of the graft-versus-leukemic effect) (161). Animal models of GVHD indicate that dysregulated cytokine production is responsible for many of the manifestations of acute GVHD. This dysregulation is postulated to occur in three sequential phases. The agents used to prepare the recipient for marrow transplantation initiate phase 1. This includes total body irradiation or chemotherapy, which activates host cells to secrete increased levels of inflammatory cytokines (e.g., TNF-a, IL-1, and IL-6) and injures the mucosal surface of the gastrointestinal tract such that bacterial breakdown products (e.g., endotoxin or LPS) can enter the systemic circulation (162). In phase 2, the activation of alloreactive donor T cells in the presence of inflammatory cytokines such as IL-12 results in activated donor T cells that predominantly secrete IL-2 and IFN-g (“type 1” cytokines). These cytokines control and amplify the alloantigen response in T cells and NK cells. These inflammatory mediators, along with specific antihost cytotoxicity mediated by Fas pathways, NK cell cytolysis, and NO, result in the pathophysiology of GVHD. Murine allogeneic bone marrow transplant models have demonstrated a link between excessive TNF-a production and the manifestation of acute GVHD. This knowledge has led to several novel approaches to GVHD prophylaxis. The most direct of these treatments is the use of TNF inhibitors, which directly block the effects of TNF-a (163). However, agents such as keratinocyte growth factor and IL-11, which both work upstream of the release of TNF-a, also effectively decrease acute GVHD in mouse models (164,165). The advantage of these therapies is that they reduce the incidence of GVHD while preserving the graft-versus-leukemic effect.

SMALL BOWEL TRANSPLANTATION

Small bowel transplants are performed less commonly than other solid organ transplants (heart, lung, liver, kidney), but the number performed has increased markedly over the past few years. The usual indication for small bowel transplantation is irreversible failure of the intestine's ability to support the patient's nutritional needs (166). This failure is most often the result of short bowel syndrome after extensive surgical resection of the intestine or, less commonly, absorption and motility disorders. In children, the common causes of intestinal failure resulting in transplantation are volvulus, gastroschisis, intestinal atresia, and necrotizing enterocolitis. In adults, the common causes are thrombotic disorders, Crohn's disease, desmoid tumors, and trauma. Most patients with intestinal failure are managed first with long-term total parenteral nutrition. Small bowel transplantation is usually considered only when long-term total parenteral nutrition has failed because of complications including line sepsis, thrombosis of access sites, and hepatic failure.

Grafts are obtained from ABO-matched cadaveric donors. Recipients and donors are not usually closely HLA-matched. Isolated intestinal grafts and combined liver-intestinal grafts are both common. Immunosuppression after transplantation is achieved with a combination of tacrolimus and low-dose steroids to which PGE_1 is added briefly during the early postoperative period. Rejection is managed with prednisone, OKT3, azathioprine, and adjustment of the tacrolimus dose.

The immunologic aspects of small bowel transplant are complicated. There are large numbers of lymphocytes in graft and complex interactions between activated and regulatory T cells. After transplantation, the host lymphocytes rapidly infiltrate Peyer patches and eventually replace the donor lymphocytes in the lamina propria (167). Donor intraepithelial lymphocytes are not replaced unless there is an episode of rejection (this finding supports the suggestion that intraepithelial lymphocytes are highly differentiated and turn over slowly). Successful transplantation requires the development of a chimeric state with both donor and recipient lymphocytes in the graft. Inadequate immunosuppression results in rejection, but overly aggressive immunosuppression results in failure of the host immune cells to populate the graft mucosa and, as a result, failure to develop the tightly controlled downregulated immune response that is characteristic of the normal mucosal immune system.

Events associated with organ procurement, preservation, and transplantation promote a less tightly controlled and more vigorous mucosal immune response (168). Epithelial injury secondary to ischemia and reperfusion leads to loss of epithelial integrity and stimulation of neutrophils and macrophages by products of dying epithelial cells. Exposure to bacterial products leaking through the damaged epithelium causes activation of lamina propria macrophages with production of cytokines that promote graft rejection through recruitment of additional leukocytes and increased MHC expression.

In acute rejection, the graft is infiltrated with lymphoblasts. The inflammatory reaction extends into the lamina propria and results in epithelial injury and crypt cell apoptosis. Acute histologic rejection is associated with pericryptic infiltration with $CD3^+$ $TCRab^+$ T cells containing clusters of $CD8^+$ cells, numerous $CD25^+$ cells, and $CD68^+$ macrophages (169). The presence of clusters of $CD8^+$ cells around and in necrotic crypts suggests that these are CTLs and play a role in the epithelial destruction characteristic of intestinal rejection. Rejection may be widespread, but it is typically worst in the ileum. As rejection becomes more chronic, obliterative arteriopathy develops. Episodes of rejection that start later, more than 100 days after transplantation, tend to have more crypt cell apoptosis and less mucosal injury.

Success rates with small bowel transplantation are improving but are still disappointing. The largest series reported the results of 71 transplants in 63 recipients (170). Of the 63 recipients, 32 were alive and 28 had functioning grafts; 4-year survival was a little less than 50%. Causes of graft loss included technical and management errors, cytomegalovirus infection, lymphoma, rejection, and sepsis. Predictors of graft loss included positive donor cytomegalovirus serology, inclusion of the colon in the graft, OKT3 use, steroid recycle, and high tacrolimus blood levels. Lymphomas developed in 12 of the 63 recipients and caused 8 deaths. These B-cell lymphomas, also termed lymphoproliferative disorders, are associated with Epstein-Barr virus infection.

EOSINOPHILIC GASTROENTERITIS

Eosinophilic gastroenteritis is a pathologic entity marked by idiopathic infiltration of eosinophils in the gastrointestinal mucosa (171). Eosinophilic infiltration can affect any part of the gastrointestinal tract, although the stomach and small bowel are most commonly involved. Infiltration with eosinophils can be predominately mucosal or predominately muscular. With mucosal infiltration, the primary complaints are diarrhea and crampy abdominal pain; gastric involvement results in nausea and vomiting. In a few cases, mucosal infiltration is extensive enough to result in malabsorption and weight loss. Mucosal infiltration can be diagnosed by endoscopic biopsy. With mucosal involvement, barium studies show thickened folds, reflecting eosinophilic infiltration and edema secondary to eosinophil activation. Muscular involvement presents as obstruction with nausea, vomiting, and abdominal pain. Endoscopic biopsies may not be helpful if the eosinophilic infiltrate is confined to the muscularis propria. With muscular infiltration, barium studies show irregular narrowing of the antrum or small bowel, depending on the distribution. The eosinophil count in the peripheral blood is usually modestly elevated in eosinophilic gastroenteritis. Extremely high levels of peripheral eosinophilia should suggest a different diagnosis.

The differential diagnosis of abdominal pain and diarrhea associated with eosinophilic infiltration of the gastrointestinal mucosa is limited. Certain parasitic infections, including ascariasis, strongyloidiasis, trichinosis, and trichuriasis are associated with peripheral eosinophilia, gastrointestinal eosinophil infiltration, and gastrointestinal symptoms. In contrast to worm infections, which are associated with eosinophilia, protozoan infections, such as amebiasis and giardiasis, are not associated with eosinophilia.

About half of patients with eosinophilic gastroenteritis have significant allergic histories with childhood food allergies, eczema, allergic rhinitis, asthma, or urticaria. Although an association with allergic disease exists in some patients, sequential withdrawal of food substances is not likely to yield relief of symptoms in eosinophilic gastroenteritis.

The mechanism of the eosinophilic infiltration in the intestine is not well understood. IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor all promote eosinophilic infiltration and may be involved (172,173). The mechanisms of eosinophil-mediated intestinal injury are also poorly understood. In eosinophilic gastroenteritis, many of the intestinal tissue eosinophils are degranulated. Eosinophil granules contain major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase. Activated eosinophils produce cytokines including IL-1, IL-5, and TNF-a.

Treatment of eosinophilic gastroenteritis is not particularly satisfactory. Patients who have a strong indication of allergy to a specific food, such as positive radioallergosorbent tests for specific IgE antibodies for specific foods, should be given a trial of an elimination diet. Oral corticosteroids are frequently effective; however, they should be used in the lowest effective dose. The duration of the requirement for steroids is variable, and, in many patients, these drugs can be successfully withdrawn after a course of therapy.

IMMUNODEFICIENCIES

The gastrointestinal mucosa is a primary interface of an individual with the outside world. The importance of the mucosal immune system in host defense is demonstrated by the wide spectrum of mucosal opportunistic infections that affect immunocompromised individuals. However, individuals with milder or more selective immunodeficiencies are frequently asymptomatic. This observation may be explained by the presence of nonimmunologic components of host defense as well as the multiple overlapping functions of various components of the immune system, such that loss of one component results in only mild effects. Primary immunodeficiencies resulting in absence or malfunction of bone marrow–derived components can be divided into those affecting the innate immune system and those affecting the acquired immune system. The latter group can be subdivided further into disorders affecting T-cell function, B-cell function, and antibody production and those affecting both T-cell and B-cell function. Selective immunodeficiencies affecting antibody production can have varied gastrointestinal manifestations ranging from primarily asymptomatic (selective IgA deficiency) to frequent opportunistic infections resulting in diarrhea and malabsorption (common variable immunodeficiency). Patients with immunodeficiencies resulting in the loss of T-cell function or combined B-cell and T-cell function frequently have more severe manifestations of diarrhea and opportunistic infections of the gastrointestinal tract; however, in these syndromes, gastrointestinal manifestations do not form a dominant part of the presentation because of the overall severity of the clinical picture in affected patients. In addition, gastrointestinal diseases resulting in protein-losing enteropathy may give rise to immunodeficiencies. Immunodeficiencies secondary to gastrointestinal enteropathies are generally not believed to be of clinical significance, except in the case of intestinal lymphangiectasia, in which hypogammaglobulinemia is accompanied by lymphopenia. For an overview of gastrointestinal manifestations in immunodeficiencies, the reader is referred to Table 55.2. For a more in-depth discussion of primary immunodeficiencies and secondary immunodeficiencies.

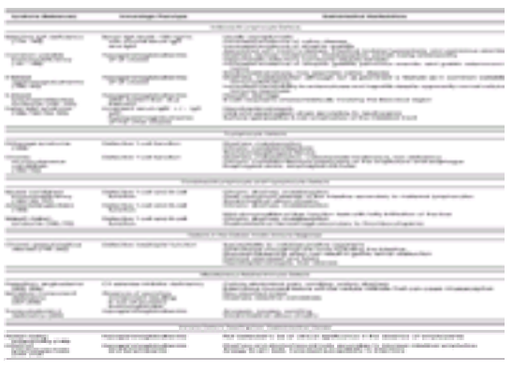

TABLE 55.2. Immunodeficiencies and Gastrointestinal Manifestations

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56 MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is a chronic inflammatory illness affecting central nervous system (CNS) pathways and leading to progressive neurologic dysfunction. It represents the principal adult demyelinating disease and remains the leading cause of neurologic disability in young adults in the Western Hemisphere. More than 1 million people in North America and Europe have the illness, with prevalence rates ranging from less than 30 to 150 cases per 100,000. Accumulating evidence indicates that MS is caused by the activation of myelin-reactive T cells by cross-reactive microbes in genetically susceptible hosts. Common pathologic features include CNS perivascular infiltration of inflammatory cells, plaques of demyelination, astrogliosis, and axonal injury.

Historically, support for the view that MS is an immune-mediated disease has been largely based on the pathologic appearance of the lesions, the common presence of abnormally elevated immunoglobulin (Ig) levels in the cerebrospinal fluid (CSF), the magnetic resonance imaging (MRI) demonstration of focal fluctuations in the integrity of the blood–brain barrier (BBB), the genetic linkage to immune-related molecules, and similarities to the animal model experimental autoimmune encephalomyelitis (EAE). Several contemporary observations warrant a rethinking of the mechanisms underlying the development of MS. The suggestion of treatment responders and nonresponders in therapeutic trials and descriptions of the pathologic heterogeneity that exists among individual patients raise the possibility that the spectrum of clinical MS spans several distinct pathophysiologic processes (1,2). Alternatively (or more likely in combination), the clinical and pathologic heterogeneity may reflect the diversity of unique host attributes.

Axonal damage is increasingly recognized as a prominent pathologic feature in both acute and chronic lesions as well as in normal-appearing white matter in the brains of patients with MS. Although these observations do not preclude the role of inflammatory demyelination in MS pathogenesis, modern MRI modalities demonstrate that axonal compromise may predate the inflammatory lesions, a finding that raises the possibility that an independent axonal disorder may contribute to the primary pathobiology of the disease.

Nonetheless, based on accumulating data from immunologic studies of patients with MS and a wealth of animal model data, autoimmune dysregulation has been viewed as the major contributor to tissue damage. The clinical features of relapses and spontaneous remissions seen in the classic form of MS are shared with other inflammatory diseases and implicate the immune system in the underlying pathophysiology of acute attacks. Long-standing experience with immunosuppressive treatments and the more recent development of immunomodulating therapies that affect the course of MS further support the important pathophysiologic contribution of the immune system.

This chapter focuses on MS as an autoimmune disease of the CNS. Topics to consider relate to the following: mechanisms by which tolerance to CNS components may be broken; sites and processes involved in recognition of putative antigens; cellular and humoral responses; dynamics of immune-cell infiltration across the BBB; immune-neural interactions; mechanisms of immune-mediated tissue pathology; and mechanisms of repair.

HISTORICAL PERSPECTIVE

Although the disease was first depicted in the late 1830s (3), the earliest cohesive accounts of MS are attributed to Jean Martin Charcot, a French neurologist at the Salpetrière in Paris, who coined the term *sclérose en plaques disseminés* and was first to highlight the prominent accumulation of inflammatory cells in a perivascular distribution within the brain and spinal cord white matter of patients (4,5 and 6). Early in the twentieth century, in his studies of acute and fulminant forms of MS, Otto Marburg emphasized the vascular orientation of demyelinated lesions (7), work that further contributed to the concept that MS was a primary inflammatory demyelinating disease. Marburg also described the prominent axonal disorder associated with acute lesions. Controversies soon developed regarding the pathobiology of MS, and the field was variably divided into “immunologic” and “developmental” schools of causality.

In 1916, James Dawson presented a detailed clinicopathologic correlation of the disease and provided a comprehensive summary of preexisting reports (8). His work was quoted extensively in Russell Brain's important textbook *Diseases of the Nervous System,* which proposed in its earliest edition that the disease was mediated by perivascular infiltration of lymphocytes and plasma cells with subsequent phagocytosis of myelin, glial overgrowth, and axonal damage (9). The identification of increased levels of Igs in the CSF of patients with MS (10), and the use of isoelectrofocusing (IEF) to demonstrate the typical oligoclonal pattern of Ig migration (11), further implicated responses of the adaptive immune system in the disease.

Parallel developments in the field of glial cell biology eventually identified oligodendroglia as the myelin-forming cells of the CNS (12,13,14,15,16,17 and 18), and further studies implicated oligodendrocytes, myelin components, and axons as the probable targets of immune-mediated pathologic features in MS (19,20,21,22 and 23).

The long-standing observations that MS is nonrandomly distributed across populations and geographic zones spurred a wealth of epidemiologic studies into the relative contributions of genetic predisposition and environmental factors to the pathobiology of the illness. More recently, molecular epidemiologic studies have consistently implicated the major histocompatibility complex (MHC) gene, and in particular the HLA-DR2 allele, as risk conferring. Population-based studies, as well as clinical trials in MS, have faced major challenges given the fluctuating and unpredictable course of the illness within any given individual patient as well as the great variability that exists across individual patients. Refinements in disease classification and diagnostic criteria, and the development of ancillary testing, have improved both the sensitivity and specificity of diagnosis. In particular, MRI has unveiled substantial subclinical fluctuations in disease activity attributed to local inflammation and has provided several powerful parameters to follow both the natural history of CNS involvement and the response to therapy. Since 1991, three immunomodulating treatments have been approved for the treatment of relapsing-remitting MS (RRMS) in the United States, based on a combination of clinical and MRI efficacy data. These major advances have established MS as a treatable neurologic illness. Nonetheless, the development of more effective and safer treatments that can be used at the time of diagnosis for this potentially disabling illness is paramount, and it is predicated on a more thorough understanding of the underlying immunopathology.

IMMUNOLOGIC OVERVIEW

Our current understanding of MS immunopathology is that autoreactive proinflammatory T cells are critical to the propagation of CNS tissue injury. Myelin-reactive T cells appear to be in an enhanced state of activation in the periphery of patients with MS, a finding suggesting a peripheral breach in tolerance to CNS antigen (Fig. 56.1). Selective expression of adhesion molecules, chemokines and chemokine receptors, and matrix metalloproteinases (MMPs) has also been demonstrated on inflammatory cells, endothelial cells, and glial cells in samples from patients with MS. These molecules are likely to be important in mediating the transmigration of effector cells across the BBB and into the CNS perivascular tissue. Invading autoreactive T cells can then become reactivated on encounter with their antigen in the CNS. The effector profile of such cells is likely to depend on several factors in the microenvironment, including the cytokine milieu and the costimulatory profile of local and infiltrating antigen-presenting cells (APCs). Further release of local cytokines, chemokines, and MMPs may support the recruitment of subsequent waves of infiltrating effector cells, including T cells, monocytes, and B cells. Mechanisms of myelin destruction and axonal damage are likely to be multiple and include direct effects of proinflammatory cytokines, oxygen radicals and complement fixing antibodies, antigen specific and nonspecific cytotoxicity, and apoptosis. Activation of resident CNS glial cells, such as microglia, may provide the basis for the generation or maintenance of pathologic responses, even in the absence of further infiltration of exogenous inflammatory cells. In contrast, evidence that glial elements may play a role in the repair and recovery from myelin injury may lead to the development of novel therapeutic approaches in MS and underscores the importance of elucidating the complexities of glial-immune interactions.

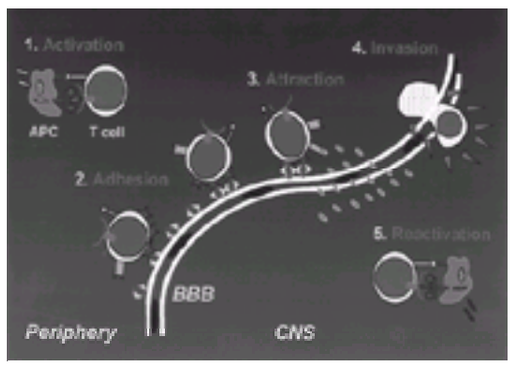

Figure 56.1. A model of multiple sclerosis (MS) immunopathogenesis. Myelin autoreactive T cells are found in an enhanced state of activation in the circulation of patients with MS. Upregulation of adhesion molecules *(1)*, chemoattraction *(2),* and elaboration of matrix proteinases *(3)* results in invasion of activated autoreactive T cells across the blood–brain barrier *(4)* (BBB). Inside the central nervous system (CNS), reactivation of T cells by local or infiltrating antigen-presenting cells (APCs) results in release of proinflammatory and cytotoxic mediators and leads to tissue injury. (See Color Figure 56.1)

Studies in EAE, the most commonly used animal model of MS, have provided important insights into mechanisms of T-cell–mediated CNS autoimmune disease. It appears likely that when a genetically susceptible host immune system encounters a common environmental antigen (e.g., an infectious organism), a process of *molecular mimicry* results in the peripheral activation of cross-reactive T cells that can migrate to the CNS and mount proinflammatory responses to myelin epitopes. Local damage results in exposure of additional myelin components that may become primary targets of subsequent waves of infiltrating cells, leading to the phenomenon of *epitope spreading.*

Studies of the mode of action of immunosuppressive and immunomodulating therapies in MS have further contributed to our understanding of the immunopathology of the disease. Putative mechanisms of action include the following: downregulation of costimulatory molecules and decreased peripheral activation of autoreactive T cells; interference with migration of inflammatory cells into the CNS by downregulation of adhesion molecules and MMPs, shifts in cellular responses from inflammatory to suppressive, or antiinflammatory effector profiles; or the generation of regulatory cells that may abrogate proinflammatory responses in the CNS by “bystander suppression.”

The successful search for safer, more effective therapies in MS will be predicated on our ability to determine the relative contributions of genetic and environmental factors, their respective impact on immune responses in health and disease, and the delicate balance between injury and repair that exists at the neural-immune interface.

EPIDEMIOLOGY

The broad clinical spectrum of MS and its fluctuating, unpredictable course make it an especially challenging illness to study from an epidemiologic perspective. In the absence of a definitive test, accurate diagnosis and reliable case ascertainment are not always possible. The long latency of expression of clinically evident disease poses a particular challenge to both retrospective and prospective studies of etiologic factors. Despite these complexities, much has been learned about MS through well-designed epidemiologic studies. Overall, the accumulating data support a model in which one or more environmental exposures in a genetically susceptible individual may result in the triggering of a CNS autoimmune disease.

The prevalence rates of MS in North America range between 30 and 150 per 100,000. Based on a weighted mean of several studies, the average annual incidence of MS in the United States is 3.2 per 100,000 per year. The median age of onset of symptoms is 23 to 24 years of age, with a peak age of onset for women in the early 20s and for men in the late 20s (24,25 and 26). As in most diseases classified as autoimmune, there is a clear female predominance in MS cases. In a summary of 30 incidence-prevalence studies, the cumulative female-to-male ratio was approximately 1.8 to 1.0 (27). The sex ratio of MS cases may have a nonrandom geographic distribution, much like the disease itself. For example, most studies conducted in Scandinavia and Canada (28) report female-to-male sex ratios that are near 1.5:1, whereas reports from Taiwan (29), Malaysia (30), and Northern Australia (31) find a ratio of 2.3:1 or greater. An exception to these is the Pharsee population in India, in which one sees a strong male predominance (32).

Environment

Global maps of MS prevalence rates, constructed based on multiple descriptive epidemiologic studies, reveal a nonrandom geographic distribution of the disease. A diminishing north-to-south gradient of MS prevalence was described in the Northern Hemisphere (33,34,35 and 36), with an opposite trend identified in the Southern Hemisphere (34,37). In general, investigators have observed that the highest prevalence rates are found in the temperate zones of both the Northern and Southern hemispheres, and the prevalence rises as one moves away from the equator. This nonrandom geographic distribution of MS likely reflects a combination of genetic and environmental influences. In both Europe and the United States, the prevalence of MS may reflect the degree of Scandinavian and northern European heritage in resident populations (38). Nonetheless, the severalfold difference in the south-north prevalence in Australia, across a genetically homogeneous population, argues for a nongenetic effect (39). The identity of such a latitude-based risk factor remains elusive. Cluster studies, in which an apparent excess of MS cases is reported within a small geographic area, have not contributed greatly to the etiologic understanding of MS (40,41). Likewise, ecologic studies, although useful in generating and providing early support for a wide range of etiologic hypotheses, are not directly amenable to inferences of causality (41,42). Hundreds of studies have attempted to explore the possible correlation between geoclimatic exposures (climate, natural radiation, geologic features, water, soil content, forestry, and rainfall) or sociocultural exposures (industry, agriculture, housing, diet, toxins, occupation, and religion) and the occurrence of MS (43). None of these putative risk factors have been substantiated.

Migration studies in MS have suggested that migrants from high-risk to low-risk areas show a decreased rate of MS (41,44,45 and 46). Furthermore, the age of emigration appears to influence whether the migrants acquire the risk of the new environment or retain the risk of their home country. Although such comparison studies are fraught with difficulties because of differences in case definition, case ascertainment, treatments, and survival, and the potential for nonrepresentative sampling (41,47,48), they do suggest that some environmental exposure during a particular window of time (likely in early adolescence) may contribute to the risk of developing MS.

These observations, together with studies of proposed “MS epidemics” (41), have contributed to the hypothesis that MS has an infectious origin. Despite multiple candidate organisms, to date, no infectious agent has been established as the cause of MS. A distinction must be made between the proposed role of an infectious agent as the specific cause of the disease and a cross-reactive interaction or even a relatively nonspecific interaction with a susceptible host immune system. Each of these may result in the onset, or exacerbation, of an immune-mediated disease. An infection may also unmask a preexisting deficit, rather than be involved in its pathophysiology. Indeed, several studies have identified an increased risk of MS exacerbation after a viral illness (41,49,50 and 51). Further discussion of the possible mechanisms underlying these observations is provided later in this chapter.

Genetics

Race has been identified as an independent factor affecting susceptibility to MS and, as noted, is likely to contribute to the nonrandom geographic distribution of the disease. European whites are at highest risk of the disease. Hispanic origin appears to confer a lower risk; in one study, persons of Hispanic origin comprised only 1% of MS cases, although they represent 8% of the total population. Likewise, African Americans comprised approximately 5% of MS cases in the United States, although they represent 12% of the general population (52). MS is also uncommon in Asia. Japanese persons and other Asians retain their relatively low susceptibility after emigration to the United States (53). Australian aborigines (39), natives of New Zealand (54), and black South Africans (55) are rarely affected, whereas MS is more common in the respective comingled white populations.

The combined results of several of the larger population-based MS twin studies (56,57,58,59 and 60) (Table 56.1, part A) demonstrate a concordance rate of approximately 27% in monozygotic twins and a 2.4% concordance in dizygotic, same-sex pairs (61). The dizygotic concordance rate is essentially the same as that found in nontwin sibships, and it exceeds the general population risk (approximately 0.2% lifetime risk). The further tenfold increase in risk observed in monozygotic twins provides clear evidence for a genetic contribution to MS risk, but it also underscores the importance of environmental factors.

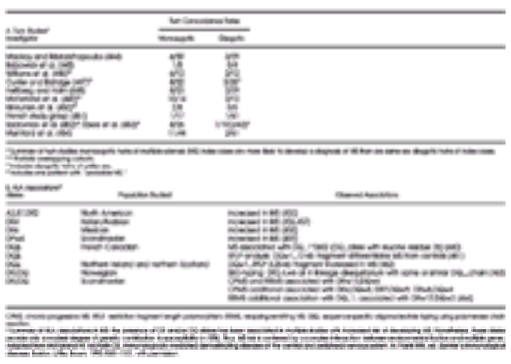

TABLE 56.1. Genetics of Multiple Sclerosis

Linkage analysis studies in MS multiplex families (with more than one affected member) have revealed an association of the MHC alleles with increased risk of developing the illness (Table 56.1, part B) (41,62). Extended MHC haplotype analysis concluded that class II DR2 conferred true susceptibility to the disease, rather than just being marker of an “at risk” population (63). More specifically, the class II MHC alleles DRB1*1501 or DRB5*0101, DQA1*0102 and DQB1*0602 (64,65) on chromosome 6p have been consistently implicated. Increased frequencies of these haplotypes are seen in northern Europeans, a finding suggesting that these genes may contribute to the geographic trends in MS prevalence. Nonetheless, these genes provide only a modest degree of genetic contribution (less than 10%) to susceptibility (66). Weaker associations have been reported, although not always replicated, for other MHC alleles, the T-cell receptor (TCR) and Ig chain encoding regions, myelin basic protein (MBP), and tumor necrosis factor (TNF) genes (67,68 and 69). There is no established evidence for a genetic marker that confers protection from the disease.

Three full genome searches, applying microsatellite polymorphism markers in large numbers of affected sibling pairs (70,71,72 and 73), identified chromosomes 2p23, 5q13, 6p21, and 19q13 as susceptibility regions, a finding supporting the existing hypothesis that genetic susceptibility to MS is conferred by multiple interacting genes, each with relatively modest individual contribution. From investigations of the EAE model, it is also likely that minor differences in protein structure identified by single nucleotide polymorphisms may have major consequence in immune function that are translated into disease susceptibility.

A general genetic predisposition to autoimmune diseases (an “autoimmune disease haplotype”), is suggested by some studies (74,75), but it would account for only a few patients with MS. Scattered reports note the concurrence of MS and systemic lupus erythematosus, scleroderma, myasthenia gravis, ankylosing spondylitis, uveitis, inflammatory bowel disease, autoimmune thyroid diseases, and diabetes (76,77,78,79,80,81,82,83,84 and 85), although none of these associations have been confirmed in rigorous population-based surveys except for thyroiditis.

CLINICAL PRESENTATION

Symptoms and Physical Findings

The hallmark of MS is the great variability in the clinical presentation and course of the illness. Inflammatory lesions may develop at any site along myelinated CNS white matter tracts, and symptoms therefore depend on the functions subserved by the pathways involved. The most common symptoms and signs involve alteration or loss of sensation resulting from involvement of spinothalamic or posterior column fibers, visual loss from optic neuritis, limb weakness and spasticity related to disruption of corticospinal tracts, tremors and incoordination of gait or limbs, largely related to cerebellar or spinocerebellar fiber involvement, and abnormalities of cranial nerve function (such as double vision from disturbance in conjugate eye movement) secondary to brainstem lesions (86). Trigeminal neuralgia and other paroxysmal sensory disturbances are not uncommon. The Lhermitte symptom is described as a sensation of electric shock radiating down the spine or into the limbs on neck flexion. It is a common symptom among patients with MS and is thought to reflect a lesion of the cervical spinal cord. Bowel, bladder, and sexual dysfunction occurs in more than two thirds of patients at some time during the course of their illness (87,88), largely because of disruption in spinal cord pathways. Fatigue is a common symptom of elusive origin that may significantly interfere with daily functioning (89). Depression in MS may occur in as many as half of patients (90,91), and it has been variably attributed to the direct effect of CNS lesions, to medications, or to affliction with a chronic, unpredictable illness. Cognitive impairment is increasingly recognized as an important problem in MS. Patients often complain of memory loss, distractibility, and difficulty in sustaining mental effort. Neuropsychologic testing reveals deficits in more than half of the patients tested, with most frequent abnormalities involving attention, recent memory, processing speed, and abstract conceptualization (92). A correlation with progressive brain atrophy on MRI has implicated axonal loss as the pathologic substrate of the cognitive deterioration in MS (93,94 and 95). Disability in MS is most often the result of fatigue, ataxia, spastic paresis, mood and cognitive changes, and gait disorders.

Most patients experience some degree of spontaneous improvement in symptoms after an acute attack. This has been attributed to the resolution of inflammation and edema at the site of the responsible lesion, rather than to the reversal of demyelination, which may persist even in the absence of symptoms (96). Patients often report transient worsening of existing symptoms, or a transient recurrence of old symptoms, when their body temperature increases. This form of heat sensitivity most likely results from a reversible deterioration in conduction in previously injured fibers and was classically described as the Uhthoff phenomenon, in which a hot bath led to transient visual deterioration in a patient with a prior history of optic neuritis. Exercise, fever, and a hot climate can similarly unmask preexisting lesions.

Clinical Course and Prognosis

MS is generally categorized as being either *RRMS* or *primary-progressive* in onset. A relapsing-remitting onset is observed in 85% to 90% of patients, and this form of the disease is characterized by a series of exacerbations that result in varying degrees of disability from which patients recover partially or completely. An exacerbation is followed by a remission period of variable duration (weeks to many years) before another attack. In keeping with the inflammatory substrate, attacks typically present subacutely. Symptoms develop over hours to several days, persist for days to several weeks, and gradually dissipate. Later in the disease, the extent of recovery from attacks is often decreased, and baseline neurologic disability accrues. The course of disease in about 40% of patients with RRMS ultimately changes to a progressive form known as *secondary-progressive* MS (SPMS). The progressive forms of disease (SPMS and primary-progressive MS) lack the acute attacks and instead typically involve a gradual clinical decline. Assessment of disability in MS has most often employed the Expanded Disability Status Scale (EDSS), ranging from 0 (no disability) through 10 (death from MS) (97). Several limitations of the EDSS scale (including its nonlinearity and underemphasis of upper extremity and cognitive functions) have led to the development of newer tools, such as the MS Composite, which is being validated in clinical trials (98).

The outcome of MS is highly variable. Untreated, approximately 50% of all patients with MS require the use of a walking aid (EDSS of 6) by 10 years after clinical onset (99), although the consequences on prognosis of newer treatment regimens are not yet clear. Men tend to have a more severe course of MS than women (100). Increased attack frequency and poor recovery from attacks in the first years of clinical disease predict a more rapid deterioration. Multiple T2-weighted and gadolinium-enhancing lesions on the presenting MRI scan also predict a more severe subsequent course. Conversely, minimal MRI T2-weighted or gadolinium-enhancing lesions on the initial scan, as well as first presentation with optic neuritis, the presence of sensory rather than motor symptoms, and good recovery from relapses, have all been associated with a more benign course (41,101). The life expectancy for patients with MS is mildly reduced compared with the general population, with important causes of MS-related mortality including depression, suicide, and severe neurologic disability (102).

Diagnosis

In the absence of a specific defining assay, the diagnosis of MS continues to be predicated on the clinical history and neurologic examination, although use of the MRI has had a major impact on early diagnosis. The old criteria for the diagnosis of *clinically definite* MS require evidence for the involvement of two or more separate CNS white matter sites as well as historical evidence of at least two episodes of neurologic dysfunction, separated in time. With the common use of MRI scanning, newer criteria for the diagnosis of MS will be required for patients presenting with monophasic symptoms. In one prospective study, patients experiencing their first episode suggestive of CNS demyelination and having corroborative MRI evidence of at least three typical lesions were followed for an average of 42 months (range, 13 to 84 months). Within the follow-up period, 83.4% of patients developed an additional relapse and thus qualified for the diagnosis of clinically definite MS. More than half of those patients developing clinically definite MS experienced the additional relapse within 1 year of their first episode (103). In another study, treatment of a similar group of patients (with a single demyelinating episode and abnormal MRI) with interferon-b1a (IFN-b1a) significantly delayed the occurrence of the disease-defining second episode (data reported at the annual American Academy of Neurology meeting, San Diego, 2000). The availability of treatments that are able to affect the course of MS favorably early on underscores the importance of timely and accurate diagnosis.

Acute disseminated encephalomyelitis is a monophasic demyelinating illness that can present with clinical, imaging, and laboratory manifestations indistinguishable from an acute MS attack. However, typical acute disseminated encephalomyelitis is seen in pediatric populations, it has a more explosive course associated with alterations in mental status, and a postviral or postvaccination history is often elicited. Although one should reasonably exclude alternate diagnoses that may also cause fluctuating neurologic dysfunction, with particular attention to treatable disorders, this is usually not a major issue. More common differential diagnostic considerations include collagen vascular diseases, sarcoidosis, vasculitides, and Lyme disease; however, patients with biopsy-proven sacoidosis, or with clear laboratory evidence of Lyme disease or systemic lupus erythematosus, may still exhibit what also clearly fits the clinical and MRI criteria of MS. Even after antibiotic treatment for Lyme disease, these patients often follow the course of typical MS, a finding that raises the possibility that two common diseases occasionally occur in the same patient. Other diseases that should be excluded include vitamin B_{12} deficiency, syphilis and human immunodeficiency virus (HIV) infection, and, rarely, mitochondrial disorders or late-onset leukodystrophies. Paraclinical tools such as visual, auditory, or somatosensory evoked responses show abnormal responses in 85%, 67%, and 77% of patients with MS, respectively (104), and these findings may support the diagnosis by providing evidence of involvement of additional CNS sites, although these additional test are often not needed with the use of the MRI. When the neurologic examination (alone or with supportive electrophysiologic evidence) demonstrates the involvement of two or more sites, but no clear history is elicited for multiplicity of events in time, the identification of certain CSF abnormalities may be used to make the diagnosis of *laboratory-supported* MS. The CSF of patients with MS typically shows normal glucose, a few lymphocytes, normal to mildly elevated total protein, and oligoclonal bands (OCBs) (Fig. 56.2 and discussion later in this chapter). Often absent early in the disease, OCBs can eventually be detected in more than 90% of patients with clinically definite MS (105), but they also occur in several other infectious, inflammatory, and lymphoproliferative disorders. Patients with human T-cell lymphotropic virus type I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) exhibit high levels of intrathecally synthesized IgG (106). CSF OCBs have also been described in conditions such as subacute sclerosing panencephalomyelitis (SSPE) (107), neurosyphilis (108), varicella-zoster virus infection (109), and HIV infection (110), as well as in collagen vascular diseases, cerebrovascular accidents, and up to 5% of healthy persons. The frequencies of immunologic abnormalities in the CSF of patients with MS are shown in Table 56.2.

Cerebrospinal Fluid Abnormality (References)	Frequency (%)
Increased IgG (465,466)	50
Increased IgA, IgM (467)	25
Increased IgG index (465,468)	80–90
Increased IgG synthesis rate (466,469)	80–90
Oligoclonal bands (466,470,471)	90
Increased free κ light chains (472)	80–90

Adapted from McFarland HF, McFarlin DE. Immunologically mediated demyelinating diseases of the central and peripheral nervous system. In: Frank MM, ed. Samter's Immunological diseases. Boston: Little, Brown, 1995:1081–1101, with permission.

TABLE 56.2. Cerebrospinal Fluid Abnormalities in Multiple Sclerosis

Role of Magnetic Resonance Imaging

MRI has become established as the optimal imaging modality for MS. From a diagnostic standpoint, the typical appearance of multiple hyperintense lesions on T2-weighted imaging is not specific for MS. In the appropriate clinical setting, however, this appearance provides an important ancillary diagnostic tool that may establish the multifocality of CNS involvement. MRI has also been used to assess MS disease activity, disease burden, and the dynamic evolution in these parameters over time (111) (Fig. 56.3). MRI is four to ten times more sensitive than the clinical evaluation in capturing CNS lesions (112), and serial studies have unequivocally demonstrated that clinically apparent changes reflect only a minor component of disease activity. Lesions in the cerebrum are much more likely to be clinically silent, as compared with lesions in the brainstem or spinal cord.

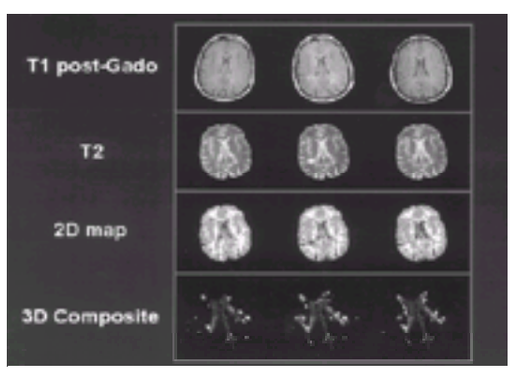

Figure 56.3. Serial magnetic resonance imaging (MRI) studies in multiple sclerosis (MS) highlight the dynamic disease pathophysiology. Serial brain MRI studies of a single patient with MS were taken at three different times **(left column:** study performed day 0; **middle column:** day 266; **right column:** day 362). **Top row:** Axial T1-weighted sequences obtained after gadolinium-contrast administration. Corresponding anatomic levels are compared at the three time points. A ring-enhancing lesion, absent in the first scan, is present on day 266 and disappears again by day 362. **Second row:** T2-weighted images at the same anatomic level demonstrate the appearance and subsequent shrinkage of this lesion, which remains visible on T2, but no longer enhances, at day 362. **Third row:** Computed algorithms enable the construction of accurate two-dimensional (2D) maps that distinguish and quantify white matter *(white),* gray matter *(gray),* ventricles *(blue),* and lesions *(yellow).* The ring-enhancing lesion is red. **Bottom row:** Three-dimensional (3D) composite model of the whole brain. The gray matter and white matter are stripped away to reveal the dynamic changes in lesional activity and burden over time (see text for further discussion). (From Weiner HL, Guttmann CR, Khoury SJ, et al. Serial magnetic resonance imaging in multiple sclerosis: correlation with attacks, disability, and disease stage. *J Neuroimmunol* 2000;104:164,165,166,167,168,169,170,171,172 and 173, with permission from Elsevier Science.) (See Color Figure 56.3)

Serial imaging studies, together with studies that correlate pathologic findings with recent MRI lesions, have been particularly informative and suggest that acute inflammation represents an early event in the development of an MS lesion (113). Newly appearing (acute) MS lesions on MRI show contrast enhancement on T1-weighted imaging after intravenous administration of gadolinium-chelate (114). This appearance reflects focal breakdown of the BBB, and correlative pathologic studies demonstrate acute inflammatory infiltrates at the same sites (115,116,117 and 118). Each contrast-enhancing lesion can also be seen as an area of hyperintense signal on T2-weighted MRI. This is thought to reflect a relatively nonspecific increase in water content at the site of the lesion. The contrast enhancement of a typical new MS lesion gradually dissipates over 4 to 12 weeks, a finding reflecting local restoration of an intact BBB that has been correlated with a decrease in the focal inflammatory profile. Concurrently, the T2-weighted hyperintense signal of the same lesion typically shrinks, leaving a smaller, often permanent T2-weighted abnormality that marks a site of previous inflammation (Fig. 56.3). Pathologically, regions corresponding to areas of increased T2 signal on MRI have a heterogeneous appearance ranging from interstitial edema without evidence of tissue damage through variable degrees and combinations of demyelination, axonal injury, axonal loss,

and astrogliosis (115,119,120 and 121). This lack of specificity may explain in part why T2-weighted lesion burden correlates poorly with disability (122,123). Newer imaging approaches, including magnetic transfer imaging (124) and MR spectroscopy (125,126), as well as measurements of T1-weighted hypointense lesions (127) and atrophy on MRI (119), have demonstrated improved correlation with disability and are likely to provide more specific quantitative measures of tissue injury. Based on these imaging studies and on pathologic observations (128,129), cumulative axonal injury is emerging as a major contributor to neurologic disability in MS.

Pathology

Gross examination of classic MS specimens reveals multiple irregular but sharply demarcated foci of discoloration and shrinkage scattered throughout the brain and spinal cord (Fig. 56.4). There is a predilection to the optic nerves and white matter tracts of the periventricular regions, brainstem, and spinal cord. Small plaques in cortical regions reflect involvement of intracortical myelinated fibers. In a single brain, lesions of variable age and size may be identified with the naked eye: fresh plaques may be pink (related to the hyperemia of active inflammation) or white to yellow (reflecting lipid breakdown products); more chronic plaques are retracted and grayish tan (from focal gliosis). Typical lesion size is 0.5 to 2.0 cm, although some lesions may be confluent whereas others remain microscopic. Global atrophy and *ex vacuo* hydrocephalus (predominantly from white matter tissue loss) correlate with disease duration.

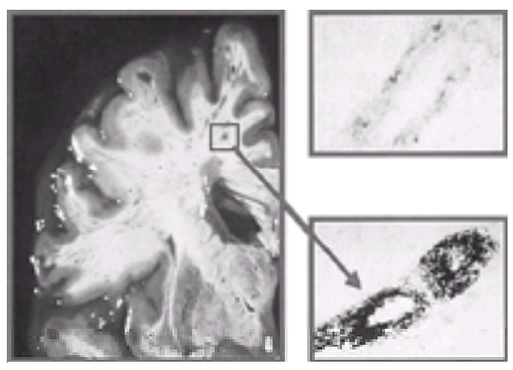

Figure 56.4. The pathology of multiple sclerosis (MS). **Left**: Coronal section of a cerebral hemisphere of a patient with MS. Multiple areas of discoloration, such as the one outlined in the **box,** are seen in the periventricular and subcortical regions and represent the classic MS plaques. **Lower right:** Microscopic view of a cryostat section from outlined subcortical plaque, stained with anti-CD4 antibody (magnification × 98). The hallmark perivascular inflammatory infiltrate is demonstrated. **Upper right:** Antikeratin control stain. See the text for a discussion of the pathologic heterogeneity of MS lesions. (From Windhagen A, Newcombe J, Dangond F, et al. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J Exp Med* 1995;182:1985–1996, with permission of the Rockefeller University Press.) (See Color Figure 56.4)

Microscopically, MS lesions may be classified as active, chronic-active, or chronic-inactive or as “shadow plaques.” Since the earliest pathologic studies of MS in the 1830s, there has been general agreement that the hallmark of the disease is the inflammatory perivascular demyelinated plaque with reactive glial scar formation (19,130,131 and 132) (Fig. 56.4). More recent studies have elegantly demonstrated that substantial axonal injury occurs, and axonal transection is abundant throughout active MS lesions, along the hypercellular edge of chronic-active lesions and to a lesser, but significant, extent also in the hypocellular core of chronic-inactive lesions (128,129).

The inflammatory cell profile of active lesions is characterized by perivascular infiltration of predominantly T cells ($CD4^+$ a/b and g/d as well as $CD8^+$) and monocytes with occasional B cells and infrequent plasma cells (Table 56.3). Endothelial activation and perivascular edema are common. Lymphocytes may be found in normal-appearing white matter beyond the margin of active demyelination (20). Macrophages are most prominent in the center of the plaques and are seen to contain myelin debris, whereas oligodendrocyte counts are reduced. In ultrastructural studies, macrophages appear to strip myelin lamellae from axons, a feature that implicates them as important mediators of tissue injury. In the peripheral nervous system, clearance of myelin debris after nerve injury is a prerequisite to subsequent regeneration, and this raises the possibility that some macrophage functions may, in fact, be beneficial in the CNS as well (133).

Cellular
T cells, (CD4, CD8, TcRα/β and TcRγ/δ)
Macrophages, B cells and plasma cells
Reactive microglia, reactive astrocytes, reactive endothelial cells
Molecular
Immunoglobulins (variable isotypes)
Complement components (including membrane attack complex)
Proinflammatory cytokines (TNF-α, IFN-γ, IL-2, IL-12, LT, etc.)
Chemokines and matrix metalloproteinases
Adhesion molecules (on endothelia, leukocytes and glia)
Evidence of injury
Axonal damage
Myelin stripping and myelin debris
Oligodendrocyte loss (apoptosis, necrosis)

IFN, interferon; IL, interleukin; LT, leukotriene; TNF, tumor necrosis factor.
[a] The diversity of cellular and molecular elements found in MS plaques and the identification of unique patterns of plaque pathology suggest that several distinct pathogenic processes may be contributing to tissue injury in MS.
From Hickey WF. The pathology of multiple sclerosis: a historical perspective. *J Neuroimmunol* 1999;98:37–44, with permission from Elsevier Science.

TABLE 56.3. Variable Elements Identified in Multiple Sclerosis Plaques[a]

In chronic-active lesions, the inflammatory cell infiltrate is less prominent and may be largely restricted to the rim of the plaque, a finding suggesting some ongoing inflammatory activity along the lesion edge. Plasma cells may be more frequent than lymphocytes along the vessels of chronic-active lesions (134,135). In the center of a chronic plaque, astrocytic proliferation and denuded axons are found, with scattered foamy macrophages and microglia (containing lipid debris) and a paucity of oligodendroglia. Chronic-inactive plaques are generally characterized as sharply demarcated glial scars.

“Shadow plaques” are areas of thinly myelinated axons in which the myelin appears to be healthy, albeit thinner and less dense than usual. These are thought to represent regions of new myelin formation at sites of recent injury (136,137). The reason that this process is not uniformly seen in all cases of MS remains unclear.

Serial imaging studies with MRI and MR spectroscopy have provided important insights into the dynamic evolution of MS lesions and the extent of inflammatory activity, BBB compromise, and axonal injury, thus calling for a reevaluation of MS pathology (113,126,138,139). Indeed, the application of criteria defining MS lesional activity and the detailed analysis of plaques from both early and late stages of the disease with respect to the patterns of demyelination, cellular activation, and oligodendrocyte pathologic features have demonstrated that a spectrum of distinct lesion types may exist. These, in turn, may reflect divergent mechanisms of tissue injury (1,2,137,140,141). In certain plaques, T cells predominate, whereas in others, monocytes and Igs are principally involved at the site of damage. Some severely demyelinated plaques contain largely intact oligodendroglia, whereas in other plaques, demyelination is associated with significant oligodendrocyte destruction. In some lesions, the predominant mechanism of oligodendrocyte injury is necrosis, whereas in other lesions, apoptosis is much more prominent.

No correlation exists between either the extent of oligodendrocyte destruction or the predominant pattern of oligodendrocyte injury (apoptosis versus necrosis) and the stage of demyelinating activity. Furthermore, neither the extent nor the pattern of oligodendrocyte destruction correlates with the overall severity of the lesions. Thus, the distinct patterns of oligodendrocyte injury do not appear merely to reflect the temporal progression or the severity of a single pathogenic process, a finding suggesting that multiple immunopathogenic mechanisms are acting in parallel. In the comprehensive study by Luchinnetti et al. (1), however, the pattern of disease tended to be the same in multiple lesions from any single patient with MS. These observations raise the possibility that distinct immunopathogenic mechanisms may be operating in different patients with MS to produce variable patterns of tissue injury (1). MS classifications based solely on clinical criteria may fail to capture important nuances of the underlying pathophysiologic processes.

This hypothesis is supported by accumulating data from *in situ* and immunohistochemical analyses of MS plaques. Such studies reveal a broad range of cellular and molecular elements (Table 56.3) that may define a variety of immunologically distinct processes, rather than support a convergent, uniform model of MS immunopathogenesis. Both environmental exposures and polygenetic susceptibilities contribute to MS pathogenesis, and the combination and complex interactions of these factors may dictate the predominant immunopathogenetic response in a given individual. If this hypothesis is true, it could in part explain the broad heterogeneity that is seen in clinical course, disease severity, and response to therapy. Ultimately, the most effective treatment in a given MS patient may require insights into the

injurious mechanisms most prominent in that individual person at that time.

IMMUNOPATHOGENESIS

Lessons from the Animal Model

EAE is an immune-mediated disease of the CNS that is characterized by multifocal demyelinating plaques and perivascular inflammatory infiltrates, primarily comprised of T cells and monocytes, and associated with a paralytic illness. These pathologic and clinical features provide the impetus for studying EAE as a model of MS. EAE is mediated by $CD4^+$ T cells that recognize a variety of CNS autoantigens and secrete proinflammatory T-helper (Th1)-associated cytokines, such as IFN-g and TNF-a. It can be induced in several species of animals by injection of adjuvant with homogenized whole myelin or myelin components such as MBP, proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG). The ability to induce EAE by passive transfer of $CD4^+$ MHC class II–restricted T cells that are reactive with these antigens (142,143 and 144), but not by serum from affected littermates, established EAE as a T-cell–mediated disease. The role of B cells is less well established. EAE has been successfully induced in B-cell–deficient animals, a finding suggesting that B cells are not critical for disease expression (145,146 and 147). In one of these studies, recovery from EAE was slower in the B-cell–deficient mice compared with wild-type littermates, a finding suggesting that B cells, in fact, may have a role in recovery (146). In other studies, however, B cells were required for the complete expression of the disease (148), with variable roles attributed to the presence of complement fixing antibodies or to the role of B cells as APCs (149). The observation that EAE can be induced in the marmoset model by injection of Igs directed against the myelin antigen MOG has rekindled interest in the humoral responses in EAE (150).

Susceptibility to EAE depends on the strain of the animal (151,152 and 153) (Table 56.4). For example, mice such as B10.Pl, SJL/J, and PL/J are susceptible , whereas BALB/c and C57BL/6 mice are relatively resistant. Disease severity and course are also influenced by the genetic background and may range broadly from a mild, uniphasic illness to aggressive relapsing-remitting and progressive disease patterns. Investigators now recognize that the development of EAE and the pattern of EAE pathology are dictated by the coordinated expression of certain genes that are involved in the activation and effector functions of inflammatory cells. The expression of these genes, in turn, is modulated by the animal's environment such that genetically identical animals with different infectious exposures may manifest distinct profiles of EAE induction and phenotype. An important component of susceptibility to EAE relates to the MHC background of the animal, which influences the T-cell repertoire, as well as which portions of the myelin proteins are encephalitogenic in any given strain (154). Genes that encode for costimulatory molecules, cytokines, chemokines, and adhesion molecules have been the focus of intense study. Molecular genetic techniques have enabled the dissection of the effects on disease phenotype of underexpression (knock-out) and overexpression (transgenic) of an ever-growing array of immune-related molecules (153,155,156,157 and 158). Table 56.5 provides a partial summary of this rapidly expanding body of work.

Mutation	Effect on Disease Susceptibility Relative to Wild Type	Reference
Knockouts		
TNF-α	Increase	473
TNF-α	Decrease	474
LT-α	Decrease	475
TNF-α + LT-α	Increase	476
LT-β	No change	475
β2-microglobulin	No change	477
CD8	Increase in relapses, decrease in mortality	478
IL-4	No change	479
IFN-γ	No change	473
IFN-γ	Increase	477
Interferon regulatory factor-1	Decrease	480
Transgenics		
TCR (MBP-specific)	Increase	481
TCR (MBP-specific)	Spontaneous by 12 wk	482
Rag-1 disrupted		
TNF-α	Increase	483
Bcl-XL	Earlier onset and more chronic form	484
Diagnostics		
IFN-γ	Increase	485
Mutations		
Fas	Decrease	486
FasL	Decrease	486

IFN, interferon; IL, interleukin; LT, lymphotoxin; MBP, myelin basic protein; TCR, T-cell receptor; TNF, tumor necrosis factor.
Adapted from Encinas JA, Kuchroo VK. Genetics of experimental autoimmune encephalomyelitis. In: Theofilopoulos AN, ed. Current directory of autoimmunity. Basel: Karger, 1999:247–272. with permission.

TABLE 56.4. Comparison of Reported Experimental Autoimmune Encephalomyelitis Susceptibility Loci

Chromosome	(487) RIII/J × B10.RIII F2	(488) NOD × Biozzi ABH backcross	(489) SJL/J × B10.S backcross	(490) SJL/J × B10.S F2
2			Eae7	
3	Eae3		Eae8	Eae3
7		Eae4		Eae4
8			Eae8	
11		Eae5		Eae5
16	Eae2 (Eae1 (H-2))		Eae2	
17				Eae9 (non H-2)
18		Eae6 (telomeric)	Eae6 (telomeric)	Eae10 (centromeric)

[a] Reported loci with linkage at a significance of $p < .0004$ (except Eae1, which was not analyzed in these studies). The study of BALB/c Biozzi ABH backcross mice had no loci that fit the criterium for suggestive linkage. Loci are named according to the order in which they first appeared in the literature.
Adapted from Encinas JA, Kuchroo VK. Genetics of experimental autoimmune encephalomyelitis. In: Theofilopoulos AN, ed. Current directory of autoimmunity. Basel: Karger, 1999:247–272. with permission.

TABLE 56.5. Specific Molecular Mutations and Their Effects on Susceptibility to Experimental Autoimmune Encephalomyelitis[a]

The observation that IFN-g knock-out animals developed worse EAE than wild-type littermates led to a trial of IFN-g in MS that resulted in disease exacerbation (159). Likewise, anti–TNF-a receptor antibody, shown to ameliorate EAE (and rheumatoid arthritis), caused worsening disease in patients with MS (160). Another example relates to the therapeutic application of altered peptide ligands (APLs). In the animal model, a single amino acid substitution within the peptide sequence of an encephalitogenic myelin epitope resulted in an APL that induced TCR antagonism, partial agonism, and anergy when used to stimulate T-cell clones generated against the original encephalitogenic antigen. Treatment of EAE with the APL induced a myelin-reactive T-cell repertoire that was shifted with respect to its cytokine responses from an inflammatory (Th1) toward an antiinflammatory (Th2) profile and was associated with improvement (161). Based on these results, an APL of the immunodominant MBP (83,84,85,86,87,88,89,90,91,92,93,94,95,96,97,98 and 99) epitope was used in a trial of MS. Unfortunately, clinical deterioration and a dramatic increase in new gadolinium-enhancing lesion formation was seen in several patients receiving the APL (see further discussion later).

Thus, although EAE in a single inbred strain of animals may reflect single patients, the wide range of genetic diversity and the broad environmental exposure of highly outbred humans are clearly not well modeled by EAE. In spite of these limitations, studies of EAE have been used to define fundamental mechanisms underlying immune-mediated CNS demyelination and have helped to elucidate novel themes in autoimmunity, such as molecular mimicry, epitope spreading, and bystander suppression (described in detail later). The emphasis in the following section of this chapter is on data generated from human studies of MS. When appropriate, reference is made to the EAE literature to highlight general principles of immune system–nervous system interactions, elucidated in the animal model, that may have relevance to MS.

Autoreactive T Cells

The earliest demonstration that self-antigen may induce an autoimmune CNS inflammatory disease came about in the 1880s, when persons receiving the neural tissue containing Pasteur rabies virus vaccine were unfortunate enough to develop encephalomyelitis (162). The EAE model was developed in an effort to study this phenomenon, and based on the observation that $CD4^+$ T cells directed against myelin epitopes mediate EAE, there has been a long-standing effort to characterize myelin autoreactive cells in MS. In several studies (typically employing limiting dilution analysis), the calculated frequencies of T cells reactive to MBP (163,164,165,166,167,168 and 169), myelin-associated glycoprotein (MAG), and MOG (170,171 and 172) were similar or slightly higher in the circulation of patients with MS compared with normal subjects. Increased frequencies of PLP-reactive T cells in the blood and CSF of patients with MS compared with controls were also reported (172,173). Although the development of tetramer technology should permit a more comprehensive characterization of myelin antigen-specific cells, it is clear that myelin-reactive T cells can be found in the peripheral blood of both healthy persons and patients with MS (167). Thus, the mere presence of autoreactive cells in the periphery is an insufficient explanation for the development of autoimmune disease. If myelin-reactive T cells are nonetheless involved in MS immunopathogenesis, the prediction would be that these cells must differ in some way from those found in healthy persons. Indeed, Zhang et al. found that the interleukin-2 (IL-2) receptor, a hallmark of activated T cells, was expressed on MBP-reactive T cells from patients with MS but not on those obtained from healthy persons (174). Similar frequencies of MBP- and PLP-reactive $CD4^+$ T cells were obtained from peripheral blood mononuclear cells (PBMCs) of patients with RRMS and controls after primary stimulation with antigen. However, when the PBMCs were first expanded with IL-2 and then were further expanded with antigen, much higher frequencies of MBP- and PLP-reactive $CD4^+$+ T-cell activation requires two signals, one delivered through the TCR interacting with the MHC-peptide (signal 1) and the other provided by engagement of costimulatory molecules on the APC with their ligands on the T-cell surface (signal 2) (175). One important T-cell costimulatory pathway is represented

by the engagement of CD28 on T cells by the B7-1 or B7-2 molecules, typically expressed on the surface of activated APCs. Two reports have demonstrated that MBP-reactive T cells from the peripheral blood of patients with MS are less dependent on B7 costimulation for their activation, compared with MBP-reactive T cells from healthy persons. Stable cell transfectants expressing the MHC class II DR2 molecule, either alone or in combination with the human B7-1 or B7-2 costimulatory molecules, were used to present the immunodominant myelin peptide MBP (p85-99) to highly purified $CD4^+$ T cells obtained from DR2-matched patients with RRMS or controls (176). As expected, MBP-reactive T cells from healthy persons did not expand to transfectants expressing the DR2 molecule alone, but these cells readily proliferated when B7-1 or B7-2 molecules were also present. In marked contrast, transfectants expressing the DR2 molecule alone could expand MBP-reactive T cells obtained from patients with MS. In other experiments, antibodies that blocked the engagement of B7 molecules inhibited the expansion of MBP-reactive T cells from healthy persons but did not inhibit the expansion of these T cells from patients with MS (177). Together, these studies demonstrate that MBP-reactive T cells in patients with MS are in an enhanced state of activation and have less stringent stimulation requirements, a finding suggesting a breach in peripheral tolerance to this autoantigen. An important extension to these observations has been the demonstration that MBP-reactive T cells in patients with MS are more likely to belong to the memory T-cell pool than the autoreactive T cells in healthy persons (178).

Although myelin-reactive T cells in MS are less dependent on costimulation for activation, evidence suggests that upregulation of costimulatory molecules may contribute to disease pathogenesis. Immunohistochemical staining of MS plaques and inflammatory stroke lesions from the same brain reveals that whereas B7-2 is expressed in both types of lesions, the expression of B7-1 is restricted to the MS plaques (179,180). In other studies, enhanced expression of B7-1 was detected on B cells in peripheral blood and CSF of patients with MS compared with controls, whereas levels of B7-2 expression were the same. The increased expression of B7-1 appeared to correlate with disease activity, and, in one study, treatment response to IFN-b1b was associated with normalization of the levels of B7-2 expression on B cells of patients (181). In EAE, the B7:CD28/CTLA-4 costimulatory pathway is more directly implicated in disease pathogenesis and regulation (182). In an adoptive transfer model of EAE, *in vivo* treatment with CTLA-4–Ig (that prevents engagement of both B7-1 and B7-2 molecules with CD28 on T cells) during EAE induction or *in vitro* treatment of myelin autoreactive T cells before transfer of cells ameliorated the disease induced in the recipient animals. However, CTLA-4–Ig treatment of recipient mice after the transfer of autoreactive T cells did not influence either disease course or disease severity. Thus, CTLA-4–Ig was assumed to block the induction phase but not the effector phase of T cells in EAE. Local CNS delivery of CTLA-4–Ig using a nonreplicating adenoviral vector was able to ameliorate ongoing EAE (183). Members of the B7:CD28 costimulatory pathway are differentially regulated during the course of EAE (184), and the patterns of B7-1 and B7-2 expression appear to be distinct when comparing peripheral immune organs with the CNS. *In vivo* blockade of B7-1 during induction of relapsing-remitting EAE protected from the disease and skewed myelin autoreactive cells toward a Th2 (antiinflammatory) profile. In contrast, B7-2 blockade exacerbated disease and skewed autoreactive T-cell responses toward a Th1 (inflammatory) cytokine profile (185,186). Blockade of B7-1, but not of B7-2, after the first remission of relapsing-remitting EAE significantly decreased the incidence of subsequent relapses, a finding suggesting that B7-1 blockade prevented epitope spreading (187). However, it has become clear that the strength of signal delivered through the TCR influences the role of B7 molecules in the type of costimulatory signal delivered to the T cell.

Cytokines

T cells have been classified according to the cytokine profiles they produce on activation (188,189). MHC class II–restricted $CD4^+$ IL-2, lymphotoxin, and TNF-a, have been defined as Th1 (inflammatory) cells and have been clearly shown to be pathogenic in EAE. IFN-g induces APCs to secrete high levels of IL-12, which is a powerful proinflammatory cytokine by virtue of its ability to differentiate naive (Th0) cells into Th1 cells (190,191). Mice deficient for the IL-14p40 gene are resistant to EAE, and neutralizing antibodies to IL-12 inhibit the development of EAE *in vivo* (192,193). In contrast, $CD4^+$ 197). TGF-b producing cells have been termed Th3 cells and are also protective in EAE. Th2 cytokines promote humoral immune responses, and although defined as antiinflammatory cells in the context of EAE (a well-established cell-mediated disease), Th2 responses may be pathogenic in settings such as antibody mediated, immune complex, or allergic disorders (198). Multiple approaches used to manipulate *in vivo* cytokine functions, including neutralizing antibody administration and the development of cytokine transgenics and knock-out animals, have demonstrated that cytokines may have divergent, unpredictable effects on EAE (193,199,200). Such results may reflect differences in systemic versus CNS immune responses or, in the case of genetic engineering approaches, developmental redundancy or compensation (197).

The Th1/Th2 paradigm of T-cell responses appears to be less stringent in human $CD4^+$ cells as compared with mice, and defining MS as a Th1 disease has been less straightforward than in EAE. This may relate to differences in regulation of the IL-12 receptor b chain between humans and mice. Several reports have suggested that myelin-reactive T-cell lines derived from patients with RRMS have a Th1 bias (201,202 and 203), although it appears that most myelin-reactive T cells secrete both Th1 and Th2 cytokines, and cytokine secretion depends on the culture conditions (204). In other words, depending on *in vitro* conditions, MBP-specific cytokine responses can be shifted to a Th1 or Th2 phenotype in both patients and controls, an observation that may have important therapeutic implications (204). In a study of T-cell clones generated to PLP from patients with MS at different clinical stages of disease, cytokine profiles of clones were related to the stage of disease (205). During acute attacks, T-cell clones had Th1-like phenotypes with no TGF-b secretion. During remission in the same patients, however, most clones showed Th0, Th1, and Th2 cytokine profiles. However, these studies have not been replicated in other laboratories, and without MRI examinations of patients, it is not clear whether so-called “stable” patients are truly stable. In other studies, intracellular cytokine staining confirmed that PBMCs from patients with chronic-progressive MS express more IL-12 on activation than do those from healthy persons (206). Moreover, treatment of these patients with cyclophosphamide and methylprednisolone reduced the frequency of IL-12–staining monocytes to normal levels. In the same study, a greater frequency of T cells from untreated patients secreted IFN-g and TNF-a compared with T cells from normal control subjects. Frequencies of T cells expressing Th2 cytokines were comparable among patients with MS and healthy persons.

Several further lines of evidence support the hypothesis that Th1 cells may be pathogenic in MS. Th1 and Th2 cells express distinct profiles of chemokine receptors. An increased proportion of T cells from patients with MS was shown to express the characteristic Th1 chemokine receptor pattern, and MS plaques were found to express increased levels of the corresponding chemokines (207,208 and 209). Analysis of cytokine mRNA in CSF from patients with MS showed a bias toward Th1 cytokines (210). Immunohistochemical studies of MS plaques *in situ* have demonstrated the presence of the proinflammatory cytokines TNF-a and IL-12 (180,211,212). TNF-a was detected in chronic lesions from patients with MS, but it was not detected in the CNS of neurologic controls or in the spleen or PBMCs of patients with MS, a finding suggesting a specific association of TNF-a with the CNS lesions (212). Within the MS plaques, astrocytes and macrophages were found to be the source of the TNF-a (211). Thus, although direct evidence that MS is a Th1 disease is still lacking, there has been an ongoing effort to evaluate existing therapies as well as emerging therapies with respect to the profile of cytokine responses that they induce. Interventions that shift or deviate the cytokine responses away from a Th1 and toward a Th2 profile have been thought to be favorable. As discussed later, the ability to shift myelin autoreactive T-cell responses toward a Th2 cytokine profile may limit putative Th1-mediated injury. Furthermore, autoreactive T cells migrating into the CNS and elaborating Th2 cytokines at the site of inflammation may provide further benefit by suppressing local Th1 responses of other autoreactive T cells, in a process known as *bystander suppression.*

Humoral Immune Response

While accumulating studies of MS and EAE have led to the characterization of MS as a cell-mediated immune disorder and have directed the focus to the role of T cells in disease pathogenesis, an abnormal humoral immune response has also been well described in patients with MS. Indeed, a renewed interest in the possible contribution of B cells to MS immunopathology has been sparked by nonhuman primate and MS pathologic studies. Whether the abnormal humoral responses described in MS are directly involved in disease pathogenesis or whether these reflect epiphenomena of a dysregulated immune system remains to be elucidated.

Intrathecal Immunoglobulins

Intensive investigation into the role of the humoral response in the pathogenesis of MS followed the report by Kabat et al. in the 1940s that the CSF of patients with the disease contained elevated levels of Igs (10). This seminal observation has been consistently reproduced, such that detection of elevated levels of CSF Ig is still used to support the diagnosis of MS in some cases. The elevated Ig levels mostly result from increased synthesis of IgG (213), with lesser elevations also observed for the IgM (214), IgD (215), and IgA (216) isotypes. In pathologic studies, B cells identified in MS plaques are mostly surface IgG positive (217), and a population of plasma cells appears to reside in long-established plaques from patients with chronic progressive disease (134). Contrasting their common presence in the CSF of patients with MS, OCBs are only rarely found in the circulation of the same patients (218). These findings, and the observations that Ig-to-albumin ratios are higher in the CSF compared with the serum of patients (219,220), together provide evidence for the intrathecal production of the Ig. IEF separates proteins according to their isoelectric point. Thus, Igs derived from a clonal population of B cells migrate to the same position on an IEF gel. CSF Ig from most patients with MS separated by IEF reveal a distinct pattern of bands not observed in healthy persons (11). These OCBs correspond to a limited set of charged Ig proteins (Fig. 56.2). These bands are believed to represent Igs derived from an oligoclonal population of B-cell clones within the CNS. OCBs can be detected in more than 90% of patients with clinically definite MS (221,222 and 223). Additional techniques, such as immunoelectrophoresis (218), two-dimensional electrophoresis (216), and idiotype analysis (224), have supported the oligoclonal origins of the CSF Igs in MS. As noted earlier in this chapter, CSF OCBs and evidence of intrathecal Ig synthesis are found in conditions other than MS and are likely to reflect nonspecific CNS inflammation.

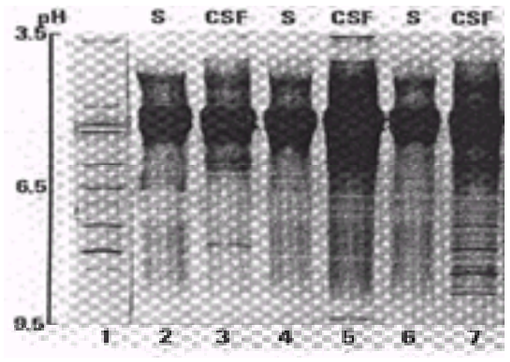

Figure 56.2. Oligoclonal bands (OCB) in multiple sclerosis (MS). Demonstration of OCB pattern of MS cerebrospinal fluid (CSF). Agarose isoelectric focusing of CSF and serum (S) from a patient with paraesthesia *(lanes 2 and 3),* myopathy of unknown cause *(lanes 4 and 5),* and multiple sclerosis *(lanes 6 and 7).* The *dark bands* in the lower part of *lane 7* are also present in immunofixation of the same isoelectrofocusing gel using antiserum against immunoglobulin chains, a finding indicating that these bands are largely composed of immunoglobulin G. *Lane 1* shows the pI reference standards. (From Link H. B-cells and autoimmunity. In: Russell WC, ed. *Molecular biology of multiple sclerosis.* New York: John Wiley, 1997:161,162,163,164,165,166,167,168,169,170,171,172,173,174,175,176,177,178,179,180,181,182,183,184,185,186,187,188,189 and 190, with permission.)

Substantial effort has been invested to elucidate the antigenic specificity of MS CSF Ig. Much of the earlier research in this field focused on exogenous antigens and on the identification of antibodies, within the elevated CSF Ig, directed against viruses and bacteria. This was fueled by early reports that measles antibodies could be detected in MS sera (225). Since then, oligoclonal IgG antibodies that react with a range of viruses, including measles, mumps, herpes simplex virus type-1 (HSV-1), varicella-zoster virus, cytomegalovirus, and rotavirus, have been reported in some patients with MS (226). Antibodies reactive toward Epstein-Barr virus antigens are present in more than 80% of CSF samples from patients with MS (227). Antibodies directed against b-hemolytic streptococcus, *Haemophilus influenzae* type B, *Escherichia coli,* and *Enterococcus* have also been reported (228). However, in all these studies, the viral and bacterial antibodies constitute only a minor fraction of the elevated MS CSF Ig (229). In contrast, in SSPE, much of the intrathecal Ig is directed against the measles virus (230), and in HSV-1 encephalitis, the elevated CSF Ig is largely accounted for by antibodies directed against the herpes virus (231). Furthermore, the antimicrobial antibodies variably identified in MS CSF have a lower antigen affinity than their counterparts in SSPE and HSV encephalomyelitis (232). These observations imply that the production of CSF Ig in SSPE and HSV-1 is antigen driven and is directed against the causative infectious agent. In MS, however, the driving mechanism behind the Ig elevation has not been successfully assigned to a single exogenous antigen.

The possibility that CSF Ig in patients with MS is generated as a response to myelin self-antigens has also been considered. Antibodies specific for MBP have been identified in the CSF (233,234), as well as in CNS tissue (235), of patients with MS. In another study, autoantibodies to MBP were detected in the CSF of more than 90% of patients with MS with active disease, whereas they were undetectable in 98% of non-MS CSF (236). Antibodies directed to PLP are also found in MS CSF; however, curiously, it seems that anti-MBP and anti-PLP antibodies are not present simultaneously in a given patient (237,238). Whether this observation reflects the gradual spread of the antigenic focus in MS (epitope spreading) remains to be established. Autoantibodies reactive with MAG (239), with the enzyme transaldolase (TAL) (240), and with oligodendrocyte-specific protein (OSP) (241) have also been detected in the CSF of some patients with MS. In one study, high-affinity autoantibodies directed against TAL were detectable in CSF of 15 of 20 patients with MS, whereas they were absent in 145 healthy persons and in patients with other autoimmune and neurologic diseases (240). TAL is expressed in oligodendrocytes and is of interest because it shares amino acid sequence homologies with core proteins of human retroviruses. The putative B-cell epitope of OSP shares sequence homology with several viral peptides. These reports suggest a role for molecular mimicry in the development of MS CSF Igs. In all these studies, however, only a minor component of the total Ig measured in MS patient CSF samples could be explained by antibodies directed against the myelin self-antigens under question. Standard approaches to the detection of antibodies in body fluids and tissues may be limited, in part, by the formation of immune complexes. This potential limitation has been addressed in studies in which CNS tissue has been subjected to acidification to effect elution of bound immune complexes. After such treatment of MS plaques, otherwise undetectable OCBs have been revealed (107,242,243).

Autoantibodies reactive with MOG were also detected is MS CSF in previous work (244). The potential role of anti-MOG antibodies came under sharper focus with the development of a marmoset EAE model. Marmosets immunized with antibodies against MOG developed EAE (150), an outcome that in other EAE models generally requires immunization with the actual antigen and adjuvant or the passive transfer of activated autoreactive T cells. No demyelination occurred when animals were similarly immunized with antibodies against MBP. Disease-inducing T cells enhanced the lesions in this model. In a follow-up to these studies, autoantibodies to MOG were found bound to disintegrating myelin segments in actual MS lesions (245). This body of work has contributed to the renewed interest in humoral responses in MS.

An intriguing, although poorly understood, humoral phenomenon in many MS CSF samples is the appearance of free light chains (218). These free k and l chains do not appear to result from degradation of whole Ig, but rather are thought to be produced by intrathecal plasma cells (246). Indeed, free light chains have been reported in MS CSF that does not contain oligoclonal IgG (247). More recently, investigators reported that the same, or similar, k light chain variable regions can be shared between CSF B cells from different patients with MS (248). Light chains were originally described as a product of multiple myeloma, in which a B-cell neoplasm produces the dimeric light chains known as Bence Jones proteins. Whether B cells within the MS CNS behave as though they have undergone a form of malignant transformation has not been established. Finally, although frequently found in MS, CSF light chains are not specific to MS and have also been reported in cases of neurosyphilis (249) and in HIV-1–infected patients (250).

Despite much effort, the bulk of the specific activity of intrathecal Ig in MS has not been definable. Nonetheless, evidence suggests that an antigen-driven response may be at work. Examination of CNS Ig in MS reveals that much of the IgG present is of the IgG1 subclass (251,252). The CNS Igs that react with various bacterial or viral antigens also have IgG subclass restrictions (252). This finding implicates, in both cases, an antigen-driven response rather than nonspecific activation. Analysis of the Ig V_H domains from MS samples reveals that these gene segments have accumulated replacement mutations in the complementary determining regions (CDR) (253). These findings have since been reproduced and strongly suggest an antigen-driven response, with associated somatic hypermutation, rather than polyclonal B-cell activation (254,255 and 256).

B Cells

Relatively little research has been performed regarding the B-lymphocyte population in MS. As noted earlier, hypersomatically mutated (presumably memory) B cells have been detected in the CSF of patients with MS (253). Several studies have examined the relative numbers of autoreactive B cells in the CNS. This was done by using ELISPOT to enumerate the autoantigen specific Ig-secreting B cells in MS plaques. Elevated numbers of CNS cells from patients with MS secreting antibodies to MBP (257), PLP (172), MAG (258), and MOG (171) have been reported. These autoreactive B cells were typically found in much lower numbers in the peripheral blood of the same patients with MS and in control subjects, a finding suggesting a preferential accumulation in the MS CNS. Several studies using fluorescence-activated cell sorter analysis have demonstrated that the relative number of peripheral B cells ($CD19^+$) in MS is not abnormally elevated (259). The subset of $CD19^+CD5^+$ lymphocytes, implicated in autoantibody production, has also been studied, and although cells with this phenotype have been detected in MS CSF, a conclusive role in autoantibody production in this compartment has not been established (260,261).

Serum Autoantibodies

The detection of autoantibodies directed against myelin antigens in the serum of patients with MS has been elusive. This may in part relate to a tendency to form immune complexes that hampers their detection (262). Serum anti-MBP antibodies have been reported (263,264), although not in all studies (257,265,266). In one study, 54% of MS sera tested was positive for MOG autoantibodies, whereas 22% of sera from healthy individuals were also positive (267). As with MBP, reports are conflicting regarding the detection of anti-MOG antibodies in MS serum (244). Investigators have reasoned that negative results reported from searches for autoantibodies may be caused by the deposition of the relevant antibodies in the CNS that may act as a sink. Certainly, low affinity and low concentration may be hampering detection. Sporadic reports have noted detecting serum autoantibodies against recombinant TAL (240), and B cells secreting autoantibodies directed against PLP have been identified in the peripheral blood of patients with MS (172). Autoantibodies to other putative MS antigens, such as myelin oligodendrocyte glycoprotein (MOBP), 2',3' cyclic nucleotide 3' phosphodiesterase (CNPase), myelin oligodendrocyte glycoprotein, ab-crystalline, and S-100b have not been analyzed in sera (268). Autoantibodies directed against nonmyelin antigens have also been detected in the sera of some patients with MS. Antinuclear antibodies (269), anticardiolipin antibodies (270), and antibodies against b_2-glycoprotein I (271) are detected in MS sera more frequently than in the sera of healthy persons. Elevated autoantibody levels toward a large panel of organ-specific and non–organ-specific antigens were measured in patients with MS relative to control subjects (272). The pathogenic relevance of these observations is not established, and a form of nonspecific systemic immune dysregulation is suspected.

Complement

Elevated levels of the membrane attack complex (C5b-9) have been reported in the CSF of patients with MS (273,274). C5b-9–enriched vesicles have also been demonstrated in the MS CSF (275). Consistent with these findings is the observation that CSF C9 concentrations are decreased in patients with MS relative to control subjects (276), a finding implying increased consumption of C9 in MS, as occurs during formation of the C5b-9 attack complex. These results suggest that complement components may participate in the tissue-damaging processes in MS. Overall, however, reports on the involvement of complement in MS have been conflicting, and a pathogenic role has not been established.

Migration of Inflammatory Cells into the Central Nervous System

Perivascular infiltration of inflammatory cells in the CNS represents one of the pathologic hallmarks of MS lesions and requires adhesion and transmigration of these cells across the BBB (277,278) (Fig. 56.1). The concept that the CNS is an immunologically privileged site has been challenged and modified over the years, and it is now accepted that small numbers of lymphocytes continuously migrate across the BBB in the normal state (196,279,280 and 281). This process appears to be unrelated to the antigenic specificity of the infiltrating cells and is thought to represent a random surveillance function of the immune system. Activated T cells, however, express upregulated levels of adhesion molecules and are able to migrate across the BBB much more efficiently than naive, resting T cells. Extravasation of cells has been characterized as a multistep and highly regulated process (282). This involves a sequence of overlapping molecular interactions between inducible ligand-receptor pairs on the surface of the migrating cell and the endothelial barrier (277,283). According to this *rolling-adhesion model,* initial tethering is achieved by selectin-carbohydrate binding that results in a reversible rolling of the leukocyte along the endothelial surface. Firm adhesion may be achieved if activated leukocyte integrins interact with Ig superfamily members, expressed on the endothelium. Locally generated chemokines promote integrin activation and contribute to cellular arrest (284). Chemokine-chemokine receptor interactions further drive the migration of cells toward the source of the chemical gradient, and elaboration of MMP effects a breakdown of the basement membrane and facilitates tissue infiltration (285). Evidence from EAE and MS studies has implicated each of these molecular interactions in disease pathogenesis and identifies potentially exciting novel therapeutic targets.

Selective Expression of Adhesion Molecules

Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), both members of the Ig superfamily, play important roles in endothelial-leukocyte interactions and in leukocyte extravasation (286,287). ICAM-1 binds to the integrin receptors leukocyte factor antigen-1 (LFA-1) (CD11a/CD18) and type 3 complement receptor-1 (CD11b/CD18) on the surface of leukocytes (288). VCAM-1 binds with a_4 integrins, including very late antigen-4 (VLA-4) a_4b_1 integrin, which is constitutively expressed on most mononuclear cells (289,290). Elevated levels of ICAM-1 have been identified on endothelial cells of both acute and chronic-active MS lesions, and these levels were shown to correlate with the extent of leukocyte infiltration (283,291,292 and 293). VCAM-1 was detected in chronic-active MS lesions, on both endothelial cells and on microglia, in contrast to normal brain, in which no VCAM-1 was identified (294). The ligands for ICAM-1 and VCAM-1 (LFA-1 and VLA-4, respectively) have been identified on the perivascular inflammatory cells of MS lesions (295). More direct implication in pathophysiology is derived from observations in the EAE model. Myelin autoreactive T cells fail to cross the BBB in VLA-4–deficient animals (296,297). Moreover, treatment of wild-type animals with monoclonal antibody directed against VLA-4 results in diminished infiltration of the CNS by inflammatory cells with a concomitant diminution in disease severity (298,299). The specificity of these molecular interactions is underscored by the demonstration that whereas the interaction between the a_4b_1 integrin (VLA-4) and VCAM-1 is required for the development of EAE, blockade of the interaction between the a_4b_7 integrin and VCAM-1 does not influence disease development (300).

Several studies have demonstrated adhesion molecules on the surface of CNS glial cells. ICAM-1–positive astrocytes are found both within and around active MS lesions, but not in normal brain (291,294). VCAM-1 and LFA-1 are detectable on microglial cells in chronic-active MS lesions (294). In addition to the possible role in inflammatory cell migration, investigators have proposed that glial cell expression of adhesion molecules may play roles in antigen presentation and T-cell costimulation (295,301,302), as well as in glial–extracellular matrix interactions (303). The local immune microenvironment may have important ramifications on the expression of adhesion molecules, influencing, in turn, the recruitment of further inflammatory cells. ICAM-1 expression on cultured human astrocytes is enhanced by proinflammatory cytokines (304). A similar observation is made in murine systems in which this upregulatory effect can be countered by antiinflammatory cytokines (305,306). This cytokine-mediated regulation of adhesion molecule expression on glial cells appears to be cell-type specific (306), and the molecular mechanisms underlying these effects are currently under active study (286). Elevated levels of the soluble forms of adhesion molecules including sICAM-1 and sVCAM-1 have been detected in the serum (307,308 and 309) and CSF (310,311 and 312) of patients with MS compared with control subjects. These levels appear to correlate with clinical and MRI indicators of disease activity and with the pattern of MS (308,309,313). Proteolytic cleavage of membrane-associated adhesion molecules appears to be the most likely source of sICAM-1 and sVCAM-1, and MMPs (see later) may be involved in this process (314,315). Although no consistent association has been found between levels of soluble E-selectin (sE-selectin) and disease activity (309,312,316), there appears to be a selective elevation of sE-selectin in patients with primary progressive MS that is not seen in patients with the RRMS (317,318).

During treatment with IFN-b1b (which has been shown to decrease MS activity), levels of sVCAM and sICAM were increased, and these elevations correlated with a decrease in the MRI lesion burden (319,320,321 and 322). In view of these seemingly contradicting observations, it remains difficult to assign a clear pathophysiologic role to soluble adhesion molecules. A possible explanation may stem from the additional observation that VLA-4 expression on peripheral blood lymphocytes is decreased in patients with MS during treatment with IFN-b (323). Conceivably, the elevated levels of soluble adhesion molecules (e.g., sVCAM-1) associated with IFN-b treatment may result in quenching or downregulation of their ligands (e.g., VLA-4) on the inflammatory cells and thereby may inhibit pathologic cellular infiltration of the CNS (286,323).

Selective Expression of Chemokines

Chemokines can enhance immune cell migration through direct chemoattraction and by activating leukocyte integrins to bind their adhesion receptors on endothelial cells. Certain a and b chemokines have been identified that appear to recruit T cells and monocytes selectively into the CNS and are associated with EAE disease activity (324). Karpus and colleagues directly compared the roles of macrophage inflammatory protein-1a (MIP-1a), membrane cofactor protein-1 (MCP-1), and MIP-2 in the induction of EAE (325,326). *In vivo* administration of monoclonal antibodies against MIP-1a inhibited adoptively transferred EAE, whereas antibodies directed against MCP-1 inhibited relapses. Consistent with these observations, immunohistochemical analysis of postmortem brain tissue from patients with MS has demonstrated that astrocytes, but not perivascular or parenchymal microglia, express MCP-1 in both active demyelinating and chronic active lesions (327). In another study, mRNA levels of RANTES (regulated on activation normal T cell expressed and secreted), chemotactic for lymphocytes and monocytes, were examined in brain samples of patients with MS (328). RANTES was expressed by activated perivascular T cells that were localized predominantly at the edge of active plaques. A comprehensive study by Sorensen and colleagues examined the expression levels of chemokines and chemokine receptors on cells in the CSF of patients with MS, neurologic control patients, and healthy persons, as well as the expression of chemokines and their receptors by brain tissue obtained from patients with MS (209). The authors noted an increase in RANTES levels and a threefold increase in interferon inducible protein 10 (IP-10), chemotactic for activated T cells, in the CSF of patients with MS compared with control subjects. There were no differences among the study populations in the levels of IL-8 or GRO-a, which are chemotactic for neutrophils. An increased frequency of $CD4^+$ and $CD8^+$ T cells from the CSF expressed the IP-10 receptor CXCR3. Immunohistochemical staining confirmed these results. There was an increased perivascular expression of CXCR3 in MS lesions, whereas RANTES staining was also upregulated in MS plaques but was expressed in a more diffuse manner. CCR5, the receptor for RANTES, was also detected on lymphocytes, macrophages, and microglia in actively demyelinating lesions. Thus, in addition to MIP-1a and MCP-1, the data suggest that the IP-10/CXCR3 and RANTES/CCR5 pathways have selective roles in MS pathogenesis.

Data indicate that some of the chemokines and chemokine receptors that have been implicated in both EAE and MS are preferentially chemotactic for the proinflammatory Th1 cells (208,209,328). Th1 cells preferentially migrated in response to the CC chemokines MIP-1a and RANTES, whereas neither Th1 cells nor the antiinflammatory Th2 cells responded to CXC chemokines (207). The extent to which chemokines and their receptors contribute to MS pathogenesis is unclear. For example, absence of a functional RANTES/CCR5 system alone is not sufficient to protect against the development of MS, because several patients with MS have been identified in whom a homozygous mutation prevents the expression of CCR5 (329).

Selective Expression of Matrix Metalloproteinases

MMPs comprise a family of tightly regulated proteolytic enzymes that are secreted into the extracellular matrix. Degradation of the extracellular matrix plays an important role in many normal physiologic processes, but under pathologic conditions, extracellular matrix degradation may promote tissue invasion by neoplastic or inflammatory cells (330). MMPs are expressed by activated T cells, monocytes, astrocytes, and microglial cells (331,332,333 and 334), and they are typically secreted as proenzymes, requiring proteolytic cleavage for their own activation and undergoing downregulation by tissue inhibitors of MMPs (TIMPS). Potential mechanisms of MMP contribution to MS pathophysiology include the following (335,336): (a) disruption of basement membrane of the BBB (337,338), thereby facilitating transmigration of inflammatory cells; (b) breakdown of extracellular matrix enabling infiltration into the neurophil (331,339); (c) proteolytic cleavage of membrane-bound proinflammatory cytokines such as TNF-a (340,341); and (d) direct damage to the myelin sheath (342).

In pathologic studies of MS tissue, gelatinase B (MMP-9) was expressed in white matter perivascular mononuclear cells and, together with other MMPs, was associated with both monocytes and astrocytes in demyelinating lesions (333,334). Microglia in active MS lesions express a range of inflammatory cytokines that have been shown to induce gelatinase B expression by inflammatory cells *in vitro* (293). Participation of MMPs in MS lesion development may result from aberrant

overproduction of the proteases or from failure to downregulate their actions sufficiently.

Increased activity of CSF proteolytic enzymes has been reported in patients with MS compared with healthy persons (343,344). Gelatinase B levels are increased in both serum and CSF of patients with MS during acute relapse, and the elevated levels correlate with the degree of BBB disruption as evidenced by the numbers of gadolinium-enhancing lesions on MRI (345,346). Furthermore, treatment with corticosteroids that are known to suppress MMP transcription is associated with a reduction in both CSF gelatinase levels and in the number of enhancing MRI lesions (347).

In vitro studies demonstrate that IFN-b1b inhibits T-cell expression of MMPs and subsequent T-cell migration. These observations may explain in part the ability of IFN-b1b to diminish the gadolinium-enhancing burden and to modify the progression of disease in patients with MS (348,349 and 350). In EAE, the interaction between T cells and endothelial cells, mediated by the adhesion molecule VCAM-1 (see earlier), induces T-cell secretion of the MMP gelatinase (351). MMPs, in turn, are able to participate in the release of adhesion molecules from the cell surface (315). Furthermore, MMPs have been shown to cleave surface-bound proinflammatory molecules including TNF-a, which has been shown to promote BBB breakdown and tissue injury (340). Ongoing studies in EAE allow more direct examination of the complex interactions among chemokines, adhesion molecules, MMPs, and proinflammatory cytokines and provide the opportunity to study novel therapeutics aimed at these molecular targets.

Central Nervous System Reactivation and Immune-Neural Interactions

A more complete understanding of the immune responses that take place within the unique CNS environment requires an appreciation of the complex interplay between immune and neural elements. In this context, the CNS should not be regarded merely as a passive target of immune-mediated injury. Rather, investigators now recognize that CNS elements may initiate, regulate, and sustain local immune responses (352). Immune cells percolate through the CNS at a low baseline rate as part of the normal process of immunosurveillance. Thus, a network of immune-neural interactions exists even under physiologic conditions and may serve to maintain the normal CNS microenvironment.

Autoaggressive T-cell responses directed against myelin elements require reactivation of these T cells on entry into the CNS that, in turn, is predicated on appropriate antigen presentation. This may occur at the BBB interface (by endothelial cells or by “perivascular microglia”) or within the CNS parenchyma (by resident microglia, astrocytes, or infiltrating monocytes or macrophages). BBB endothelial cells have been shown to process and effectively to present myelin antigen to T cells (353). Although the endothelial cells do not express MHC class II molecules in the resting state, they can be induced to do so on exposure to proinflammatory cytokines (e.g., IFN-g), which concurrently upregulate B7 costimulatory molecules (352,354). In studies using human brain microvascular endothelial cells (HBVECS) as APCs to autologous T cells, proliferative responses of the T cells could be either enhanced or inhibited, depending on the *in vitro* microenvironment, a finding suggesting that BBB endothelial cells may have a regulatory role in CNS inflammatory responses (354). In the context of CNS T-cell reactivation, “perivascular microglia/macrophage” may be particularly important APCs, based on their unique location at the interface between the circulation and the CNS, their apparent high basal level of expression of MHC class II molecules, and their ability to phagocytose and process antigens efficiently (355). In addition, perivascular macrophage may regulate the ability of T cells to migrate beyond the perivascular space and into the CNS parenchyma (356).

Within the CNS, parenchymal microglia, derived from hematogenous precursors during embryogenesis (357,358), also constitutively express MHC class II molecules (359) and can phagocytose and present antigen to T cells (360,361). These resident CNS microglia share several phenotypic and functional characteristics with professional antigen-presenting dendritic cells, including the constitutive expression of B7-2 costimulatory molecules and the propensity to upregulate B7-1 molecules on activation (359). Microglia studied within MS plaques *in situ* were shown to express B7-1 in addition to B7-2, whereas microglia from inflammatory stroke lesions in the same brain expressed B7-2 but not B7-1 (179,180). This selective upregulation points to a role for microglial activation and B7-1 signaling in the immunopathogenesis of MS. Activated CNS microglia are therefore poised to present myelin antigens to autoreactive T cells efficiently.

Investigators have further shown that, on contact with activated T cells, human microglia are capable of producing large amounts of IL-12, which, as noted previously, is perhaps the most potent inducer of Th1 responses (352). The IL-12 production depends on the engagement of CD40 (on microglia) by CD40-ligand (upregulated on activated T cells) and can be enhanced by IFN-g from the T cells (362) as well as by autocrine secretion of TNF-a from activated microglia or infiltrating macrophages (363,364). Microglia express several “pattern recognition” receptors (such as the lipopolysaccharide receptor, CD14) and can become rapidly activated in response to a range of CNS insults including infection, trauma, and tissue degeneration (352). The subsequent elaboration of proinflammatory cytokines (including IL-1b, IL-6, IL-12, and TNF-a) may trigger an inflammatory cascade that self-perpetuates long after the original insult is gone. These interactions place microglia at the interface between innate and adaptive immune responses and highlight the possible roles of these resident CNS cells in initiating, propagating, and maintaining MS inflammatory responses.

Astrocytes, although capable of upregulating MHC class II molecules on activation (365,366), have generally not been viewed as efficient APCs. Activated human fetal astrocytes expressing MHC tend to induce T-cell anergy, perhaps because of the absence of B7 expression (367,368 and 369). Astrocytes have also been shown to mitigate the inflammatory responses induced by microglia, and they may have a downregulatory role on T-cell inflammatory responses in the CNS (370,371). The elaborate “cross-talk” that exists among T cells, microglia, and astrocytes underscores the importance of immune-neural interactions in the disease immunopathogenesis.

Mechanisms of Tissue Injury and Recovery

The following is a summary of the major themes of immune-mediated injury in MS; a more comprehensive discussion of the multiple mechanisms of tissue damage in MS is provided elsewhere (129,372,373). Although MS can be regarded as a systemic autoimmune disease, the distribution of tissue injury is remarkably specific. Disease is largely limited to CNS myelin or its cell of origin, the oligodendrocyte, and to the underlying axons. Axonal injury may be secondary to immune responses or the loss of trophic support from myelin, and evidence suggests that a primary process of axonal injury may play an important role (129,372). The selective injury pattern is likely conferred by features of both the effector elements of the immune system and the target tissue itself. Responses of the adaptive immune system (mediated by a/b T cells, B cells, and specific Igs) provide a high degree of selectivity based on recognition of particular tissue antigens. In contrast, effector elements of the innate immune system, such as a/b T cells, natural killer (NK) cells, macrophages, and complement, are less restricted by specific antigens, and selective responses depend on properties of the target microenvironment, including interactions with elements of the adaptive immune system, such as complement fixation of bound antibodies. Table 56.3 lists the various cellular and molecular elements found in MS plaques and suggests that distinct pathologic mechanisms may be playing a role.

Initial lesion development appears to depend on infiltration of T cells and may be largely driven by autoantigen-specific responses. These may involve both MHC class II–restricted a/b $CD4^+$ T-cell responses and MHC class I–restricted cytotoxicity mediated by $CD8^+$ cells. Antibodies directed against myelin or oligodendrocytes may induce injury by both antibody-driven cytotoxic responses and by complement fixation of bound antibody. NK cells, NK T cells, and a/b T cells may damage cells by perforin-granzyme release. Oligodendrocytes are also susceptible to apoptotic cell death (mediated by both Fas and TNF pathways); the degree of susceptibility is, in part, determined by additional factors in the microenvironment as well as by the internal signaling environment of the target cells (129,372,374,375 and 376). A range of toxic proinflammatory cytokines and other soluble elements such as nitric oxide and reactive oxygen species may contribute to nonspecific injury and oxidative stress (340,372,377,378,379,380,381 and 382). Mononuclear phagocytes are the major effector cells responsible for removing myelin in MS lesions. The role of resident microglia, discussed earlier, may represent a mechanism by which damaging inflammatory processes may be maintained, long after the initiating events have dissipated. Finally, reports also note that both cellular (e.g., astrocytes) (383) and humoral (e.g., TNF-a neurotrophin receptor/NGF) (372,384,385,386,387,388,389 and 390) elements in MS lesions can exert a dual role, promoting either injurious proinflammatory or protective antiinflammatory responses in a context-dependent fashion.

Recovery in MS may involve resolution of active inflammation, remyelination of surviving oligodendrocytes, or maturation of progenitor cells. The rapid improvement typically observed in the first week or so after a relapse is likely to reflect resolution of local edema and removal of potentially toxic immune mediators that may adversely affect axonal transmission. In contrast, the more gradual recovery that some patients experience over months after an exacerbation probably reflects a limited degree of remyelination and reorganization of sodium channels on demyelinated axons (129,391). Shadow plaques, originally mentioned by Marburg (7) and Dawson (8), are described earlier and are thought to represent attempts at remyelination.

Several studies have suggested that the humoral response in MS may promote remyelination after damage. Proposed mechanisms include binding of antibodies promoting oligodendrocyte proliferation, binding of antibodies promoting oligodendrocyte clearance and replacement with viable cells, and antibody-mediated blockade of otherwise pathogenic molecule (392). The Theiler murine encephalomyelitis virus (TMEV) model of MS produces chronic, progressive, inflammatory CNS demyelination. Chronically infected SJL/J mice show limited spontaneous remyelination, which is in part due to T-cell–mediated immune responses that are shown to inhibit myelin repair. Several antibodies with specificity toward unidentified oligodendrocyte antigens promote CNS remyelination when they are passively transferred into syngenic mice (392,393 and 394). Analysis of these antibodies revealed that they shared characteristics with natural autoantibodies (393) in that they are of the IgM isotype and had few, if any, somatic mutations. Collectively, these results suggest that natural autoantibodies may have the potential to promote remyelination in animal models of MS. Natural autoantibodies have been shown to provide a beneficial effect on other autoimmune animal disease models such as myasthenia gravis (395), diabetes (396), and lupus (397). Whether remyelinating oligodendrocytes are derived from proliferation and subsequent differentiation of progenitor cells or whether they arise from dedifferentiation of mature oligodendrocytes remains unclear. A more thorough discussion of this important question is presented elsewhere (398).

Disease Initiation and Progression

One of the fundamental difficulties in studying the initiating events in MS relates to the prolonged time interval that separates the onset of the pathogenic processes from the development of clinical disease. There has been an ongoing effort to link the known genetic susceptibility conferred by the HLA-DR2 allele with the observation that myelin-reactive T cells are abnormally activated in MS. Triple-transgenic mice were developed that express the relevant human HLA-DR2 molecule, the TCR from an MBP-specific, DR2-restricted human T-cell clone, and the human CD4 coreceptor. The mice could be induced to develop EAE, but, more important, some of the animals developed spontaneous disease without introduction of exogenous antigen. This work demonstrates that a risk-conferring MHC allele can mediate both induced and spontaneous disease by presenting an MBP self-peptide to T cells (399). Elegant studies have elucidated the three-dimensional crystal structure of the complex formed by the HLA-DR2 molecule and the immunogenic MBP peptide (Fig. 56.5) (400). The primary sequence of the MBP peptide was a good predictor of its orientation within the binding groove of the HLA-DR molecule, and it was hoped that similar structural analyses would provide a reliable approach to understand and predict both normal and aberrant immune responses. Subsequent work, however, revealed that structure-function correlates are not always reliable. A possible explanation for this apparent failure may relate to the observations that T-cell responses appear to be far more degenerate than previously appreciated, and cross-reactivity may be the rule rather than the exception (401).

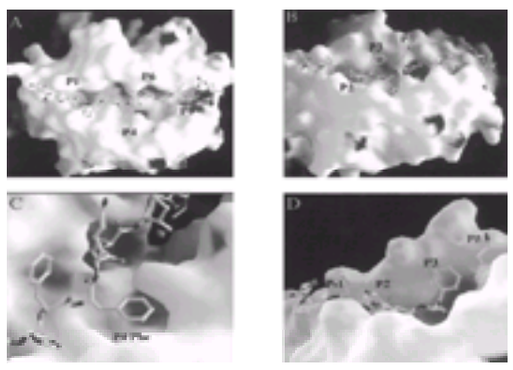

Figure 56.5. Structure of the HLA-DR2–myelin basic protein (MBP) peptide complex. **A** and **B**: Top and side views of the complex; 14 residues are included for the MBP peptide. P1 and P4 are hydrophobic pockets in the DR molecule and are occupied by the P1 Val and the P4 Phe of the MBP peptide, respectively. These interactions anchor the peptide in the groove. Peptide atoms are shown as ball-and-stick structures. **C**: Close-up view of the P4 pocket. The Gln b70 of the DR2 molecule (not labeled) is positioned over the P4 Phe of the MBP peptide. **D**: The T-cell receptor contact residues of the MBP peptide. P2 His, P3 Phe, and P5 Lys (previously shown to be important for T-cell recognition of the MBP peptide) are prominent, solvent exposed residues. (From Smith KJ, Pyrdol J, Gauthier L, et al. Crystal structure of HLA-DR2 (DRA*0101, DRB1*1501) complexed with a peptide from human myelin basic protein. *J Exp Med* 1998;188:1511–1520, with permission of the Rockefeller University Press.) (See Color Figure 56.5.)

In this context, molecular mimicry has been proposed as one mechanism that may underlie the induction of MS or the triggering of a flare. According to this hypothesis, an infectious agent may express antigenic epitopes that have sufficient functional homology with a host antigen that peripheral activation of T-cell responses to the pathogen may inadvertently induce autoreactive T cells. The latter may migrate across the BBB in their activated state where they would recognize the CNS autoantigen and mediate tissue injury. Molecular mimicry has been convincingly demonstrated in several animal models of autoimmune disease, including EAE (402,403,404 and 405), thus making it a highly attractive hypothesis of the cause of MS in a genetically susceptible individual. However, the profound limitations of human clinical immunology may preclude the possibility of providing strong evidence for this hypothesis in patients.

Another possible mechanism of MS initiation is represented by the induction of a CNS autoimmune demyelinating disease after infection with TMEV. In this model, direct CNS infection by the virus leads to an initial T-cell response directed to the TMEV-infected resident APCs, whereas the subsequent chronic T-cell–mediated disease involves the activation of myelin-reactive $CD4^+$ T cells (406,407 and 408). Superficially, the expansion of myelin-reactive T cells after viral infection may be viewed as another example of molecular mimicry. However, in an important extension to this model, investigators showed that microglia, isolated directly from the CNS of TMEV-infected animals, were able to present PLP epitopes to T-cell lines efficiently. Thus, direct viral infection of the CNS resulted in endogenous processing and presentation of myelin antigen and the subsequent expansion of previously naive myelin autoreactive T cells (408,409).

These two models of disease initiation need not be mutually exclusive. Peripheral activation of autoreactive T cells by molecular mimicry, which alone may not precipitate a CNS autoimmune disease, may result in disease if a prior insult to the CNS results in exposure of cross-reactive self-antigens that would otherwise not be accessible to immune surveillance. Indeed, these two models were shown to work in concert in EAE, in which prior expansion of T cells to myelin self-antigen was necessary for disease induction by T cells primed with a cross-reactive environmental antigen (410). In both models, CNS tissue injury would lead to exposure of additional epitopes and the subsequent development of autoreactive T cells recognizing those new targets. The clinical diagnosis of MS is often made years after the immunologic onset of the disease, by which time activated T cells recognizing multiple myelin epitopes are likely to have developed. This process of epitope spreading may explain why, to date, it has not been possible to identify a single antigenic target that drives the pathogenic immune response in MS (174,411). Not all inflammatory insults of the CNS (e.g., stroke) are followed by a progressive CNS autoimmune disease. The explanation may lie in the demonstration that blocking the interaction between B7-1 on CNS APCs and CD28 on T cells prevents epitope spreading and abrogates the development of relapses in murine EAE (412). The notion that B7-1 expression is critical for the process of epitope spreading is in keeping with the *in situ* immunohistochemical demonstrations, described previously in this chapter, that B7-1 is selectively expressed in MS plaques but not in inflammatory stroke lesions from the same brain.

TREATMENTS AND IMMUNOLOGIC MECHANISMS ACTION

Therapeutic approaches in MS may be broadly divided into treatments that are symptomatic or supportive and treatments that are directed at the underlying pathophysiology of the disorder. Effective medical management of spasticity, bladder symptoms, dysesthesias, fatigue, and depression may significantly improve daily functioning and may enhance the patient's quality of life. Ambulatory aids, patient education, appropriate use of physical and occupational therapy services, and psychological and social supports, should all be elements of the comprehensive management plan.

A new era in the treatment of MS emerged in the 1990s with the demonstration of efficacy of four immunomodulating therapies that affect the course of RRMS. These are, in order of approval in the United States, recombinant IFN-b1b (rhIFN-b1b, Betaseron/Betaferon), recombinant IFN-b1a (rhIFN-b1a, Avonex and Rebif, the latter available only in Europe and Canada at present), and glatiramer acetate (GA/copolymer-1/Copaxone). The ability of these agents to affect both clinical and MRI activity parameters in RRMS provides support for the role of immune mechanisms in the pathogenesis of RRMS. Studies of the mode of action of these therapies have further contributed to our understanding of the immunopathology of the disease and are discussed later.

Because of differences in study design and outcome-measure definitions, direct efficacy comparisons among the approved therapies is problematic. Compared with placebo-treated RRMS control subjects, treatment with alternate-day subcutaneous injections of 8 million units of rhIFN-b1b (Betaseron) was shown to decrease the primary efficacy outcome measure of frequency of relapses by 34% after 2 years (413). A significant decrease in the accumulation of MRI lesions was observed with treatment (413), although no beneficial effect on clinical disability was seen over the study period. More recent 5-year follow-up data reported that disease progression in the rhIFN-b1b–treated group was 35%, compared with 46% progression in the placebo group (122). A 30% decrease in the annual exacerbation rate in the treated group was maintained, and MRI data showed significantly less accumulation of lesion burden in the rhIFN-b1b–treated group (3.6%) compared with the placebo-treated patients (30.2%) over 5 years.

rhIFN-b1a (Avonex, weekly intramuscular injections), a glycosylated recombinant IFN-b, was evaluated in a 2-year study of weekly intramuscular injections of 6 million units (30 µg). The primary efficacy outcome measure was time to the onset of a sustained deterioration in disability. The proportion of patients progressing by the end of the trial was 21.9% in the treated group compared with 34.9% in the placebo group. The annual exacerbation rate was decreased by 32% in the treated group versus the placebo group. Treatment was also associated with a 40% reduction in mean MRI lesion load (414,415). The mean number of new enhancing lesions on MRI over the 2 years was 0.80 in the rhIFN-b1a–treated group versus 1.65 in the placebo group. In the PRISMS trial, a double-blinded, placebo-controlled, phase III trial of rhINF-b1a (Rebif, subcutaneous, three times weekly) in RRMS, treatment was associated with significant improvement in MRI parameters, which extended the prior evidence of benefit on relapse rate and disease progression (416). Common side effects of the IFN-b include flulike symptoms and injection site reactions (rarely necrosis with IFN-b1b). Headache and depression are less common, and, rarely, hepatotoxicity or bone marrow suppression may occur. Neutralizing antibodies (NABs) have been reported for both IFN-b and remain a contentious issue. Some reduction in the biologic effect and clinical benefit of rhINF b in NAB-positive patients has been suggested (417). The relative antigenicity of rhINF-b1b (alternate-day subcutaneous administration) may be greater than that of rhINF-b1a (weekly

intramuscular administration); however, a prospective comparison has not been made. The effect of dose on the generation of NAB is also not established.

Treatment with glatiramer acetate/copolymer 1 (Copaxone, GA), a daily 20-mg subcutaneous injectable synthetic polymer, was associated with a 2-year relapse rate reduction (primary outcome measure) of 29%, compared with placebo control (418). A subsequent MRI study revealed a beneficial effect of GA on newly forming gadolinium-enhancing lesions (419), and a follow-up study not yet published suggests that the beneficial impact on MRI is maintained for several years. GA is relatively well tolerated, with typically mild injection site reactions, infrequent urticarial responses, and an occasional stereotypical vasomotor reaction. Laboratory abnormalities have not been reported, and NABs are not an issue.

The biologic mechanisms of action by which the approved immunomodulators mediate their significant, although limited, beneficial effects on the course of RRMS are of great interest. As a class, IFN-b appears to exert its effects on the pathophysiology of MS at several sites. Converging studies have suggested that IFN-b may inhibit the migration of activated inflammatory cells across the BBB and into the CNS parenchyma. The abnormally increased cellularity often seen in CSF from active patients with MS is diminished after therapy with IFN-b1a (420). IFN-b may exert this effect at the level of adhesion molecules or MMPs. As noted earlier, the adhesion molecule VCAM-1 has been identified on both microglia and endothelial cells in chronic-active MS lesions, and its ligand, VLA-4, has been identified on the perivascular inflammatory cells of MS lesions. Treatment with IFN-b1b was associated with decreased levels of VLA-4 on inflammatory cells in patients with MS, a finding that correlated with a decrease in the MRI lesion burden. (321,323). IFN-b1b has also been shown to suppress T-cell expression of MMPs and to inhibit T-cell migration across the BBB (348,349). The rapid effect of IFN-b on the gadolinium-enhancing lesions of MS (350,421) represents a promising biologic marker of treatment response and establishes the BBB as an important site of action of IFN-b therapy. A possible role for IFN-b treatment in shifting cytokines in MS from a proinflammatory profile to an antiinflammatory profile is suggested by reports that treatment may decrease levels of TNF-a while enhancing levels of IL-6 (422) and of immunosuppressive cytokines (423). Finally, a possible role in limiting T-cell activation is suggested by the observation that the increased levels of the costimulatory molecule B7-1, reported on mononuclear cells in MS blood and CSF, are reversed in response to treatment with IFN-b1a (181,424).

The differences in the side effect profiles of IFN-b and GA suggest that these treatments have distinct mechanisms of action. GA is a random-sequence polypeptide of the four amino acids alanine (A), lysine (K), glutamate (E), and tyrosine (Y). Initial reports on the putative mode of action of GA suggested that GA may interfere with myelin antigen binding to the MHC class II molecule, thereby inhibiting presentation to autoreactive T cells (425). In subsequent studies, TCR antagonism was suggested to occur in addition to competition for MHC class II binding (426). GA was found to bind selectively, and with high affinity, to MHC class II DR molecules HLA-DR1, HLA-DR2, and HLA-DR4, but not to HLA-DQ or MHC class I molecules (427,428). These studies suggested that GA contains multiple epitopes that enable it to bind promiscuously to a range of MHC class II molecules, conferring the potential to activate $CD4^+$ T cells broadly. Consistent with this finding, limiting dilution analysis revealed a surprisingly high frequency of GA-reactive T cells in the normal circulation, ranging from 1 in 5,000 to 1 in 100,000 PBMCs, and demonstrated that GA indeed represents a highly cross-reactive, MHC class II–restricted antigen (429). Duda et al. examined the effects of daily subcutaneous administration of GA in patients with RRMS on the profile of the T-cell immune response. *In vitro* T-cell responses to GA, to the immunodominant MBP epitope 84-102 (as a model myelin antigen), and to combinatorial peptide libraries derived from the MBP 84-102 sequence and from a completely random 13mer sequence were measured before and during a year of therapy with GA. T-cell responses to the combinatorial peptide libraries were examined as a measure of TCR degeneracy. The authors demonstrated that GA-reactive T-cell lines generated *in vitro* were deviated toward a Th2 cytokine profile after treatment with GA. Furthermore, the cross-reactivity of GA-reactive T-cell lines was increased after GA treatment, although the T-cell reactivity to the immunodominant MBP epitope 84-102 or to tetanus toxoid was not significantly altered during therapy. In combination, these results show that GA does not appear to have a direct effect on preexisting memory T cells, nor is it selectively cross-reactive with MBP (429). Rather, GA treatment appears to induce GA-reactive Th2-polarized T cells that are more degenerate in their antigen recognition. These activated Th2 T cells may enter the CNS, where they may mediate bystander suppression at sites of inflammation and lead to decreased disease activity and the observed efficacy of GA on both clinical and MRI parameters. Overall, the currently approved immunomodulating agents provide a measurable, although only partial, benefit in the treatment of RRMS. The suggestion that the mode of action of IFNs differs from that of GA raises the possibility that together these therapies may provide additive benefit, and such a combination trial is currently under way.

Corticosteroids are used widely in the treatment of MS, although data supporting their use are sparse. Two follow-up studies of patients with isolated optic neuritis (a common presenting episode of MS) suggested that treatment with a short course of high-dose intravenous methylprednisolone was associated with a lower risk of developing subsequent demyelinating episodes (430). Based on these results, intravenous methylprednisolone became the primary treatment used for optic neuritis and for debilitating attacks of MS. Five-year follow-up data have shown that the development of clinically definite MS after optic neuritis did not differ by treatment group. In general, treatment of acute relapses with intravenous methylprednisolone may lead to more rapid improvement in symptoms, but it does not appear to change the ultimate outcome of the flare or the subsequent disease course. Pulse steroid treatment has also been used for patients with primary and secondary progressive MS. The mode of action of corticosteroids is not established. Relatively nonspecific suppression of T-cell responses is suspected, although differential effects on T-cell subsets have been reported (431). Steroids have been shown to reduce the activity of MMPs, and MRI evidence of decrease in gadolinium-enhancing lesions after treatment with steroids supports a possible therapeutic role at the BBB (432).

Treatment of patients in the progressive phase of MS with immunomodulating therapies has been less successful. In early studies, IFN treatment appeared to improve the outcome in patients with SPMS. This was not reproduced in subsequent trials, probably because the initial observations reflected the favorable impact of the therapy on the relapse component of relapsing-progressive patients in the study. The potential efficacy of Copaxone in SPMS is currently under investigation.

Cyclophosphamide is an alkylating agent with potent cytotoxic and immunosuppressive effects. A randomized, triple-arm study demonstrated a positive effect in patients with SPMS treated with a 2-week course of cyclophosphamide/adrenocorticotropic hormone (ACTH) as compared with patients who received ACTH alone (433). These findings led to a randomized single-blind trial by the Northeast Cooperative Treatment Group that tested the efficacy of outpatient intravenous cyclophosphamide pulses every 2 months in 236 patients who had initially received an intravenous cyclophosphamide/ACTH induction (434). Patients with disease for shorter durations had a more beneficial effect from cyclophosphamide treatment as compared with patients not receiving the pulse cyclophosphamide treatment. The current cyclophosphamide protocol used at the Partners' Harvard Multiple Sclerosis Center had not yet undergone a randomized clinical trial, until now, when it is being investigated in b-IFN treatment failures. Moreover, there is great difficulty in using the present statistical tools necessary to analyze response to therapy over 5 to 10 years. The present treatment regimen recommended calls for monthly infusions of cyclophosphamide with methylprednisolone for 1 year, followed by a second year of infusions at 6-week intervals, a third year of infusions at 8-week intervals, a fourth year of infusions at 10-week intervals, and a fifth year of four infusions at 12-week intervals (see Website) http://neuro-oas.mgh.harvard.edu/ms/. The dose of cyclophosphamide is based on body surface area, and it is increased such that 6 to 12 days after infusion, the total white blood cell count is brought to less than 2,000 cells/mm^3. The clinical effect of this treatment regimen in patients with more severe, relapsing-remitting progressive disease can be dramatic, and patients stable over the taper from every 10 weeks to every 12 weeks most often remain stable on cyclophosphamide withdrawal. Nevertheless, the toxicities of cyclophosphamide, including infertility and a potential increase in the risk of bladder cancer, preclude the routine use of the drug in patients early in the disease course, except in instances of multiple accumulated disabilities at the disease onset.

Several laboratory observations have suggested that cyclophosphamide may positively affect the immune response in patients with MS. Elevated IL-12 levels identified in patients with SPMS were shown to correlate with disease activity and were normalized by pulse cyclophosphamide therapy (206). A follow-up study treating patients with SPMS with pulse cyclophosphamide and methylprednisolone suggested that response to therapy was linked to the duration of progressive disease (435).

Mitoxantrone is an antineoplastic agent that intercalates with DNA and potently suppresses cellular and humoral immune responses. It has demonstrated clinical benefit on relapse rate and disability progression in patients with relapsing-progressive MS with a concomitant decrease in the development of new enhancing lesions and cumulative total lesion burden. It has been approved in the United States for the treatment of SPMS. Although the drug is generally well tolerated, it has a limited use of 2 years because of cardiotoxicity. Other immunosuppressive agents that have shown some limited benefit in the treatment of SPMS include methotrexate (436), azathioprine (437), cladribine (2-CdA, Leustatin) (438), and intravenous Igs (IVIGs) (439).

It appears likely that immunomodulating and immunosuppressive therapies provide their greatest benefit early in the disease, during a period in which inflammatory responses are major contributors to tissue injury. As progressive disease sets in and irreversible axonal injury becomes the major process underlying clinical deterioration, the relative contribution of inflammation to ongoing injury diminishes and with it the role of antiinflammatory therapies. This finding underscores the importance of initiating therapy early in the disease and developing, in parallel, neuroprotective strategies.

CONCLUSIONS AND FUTURE DIRECTIONS

Major strides have been made in our understanding of the immunopathogenic processes underlying the development and course of MS. Classic themes have been revisited with the application of modern techniques. The demonstration of distinct pathologic patterns in MS plaques suggests that several different mechanisms may contribute to tissue injury, and the prevailing mechanism may not be the same across patients. In part, this may reflect the remarkable genetic diversity and broad range of environmental exposures that together define the human experience. Differences in pathogenic substrates may explain the observed heterogeneity of clinical disease course and severity and the differential response to therapy. A more meaningful classification of MS that is based on defining the underlying process in individual patients may lead to more effective therapeutic approaches.

Various imaging modalities have combined with pathologic studies to highlight the substantial degree of axonal injury involved in both MS lesions and in normal-appearing white matter. These abnormalities are detected early in the course of MS and may be better predictors of subsequent disability than clinical measures of disease activity. Indeed, neurologic disability in MS is likely to have at least two pathologic underpinnings: inflammatory demyelination and axonal transection. Episodic demyelination involving infiltration of inflammatory cells is the likely substrate of clinical flares. Recovery from these probably reflects resolution of

local inflammation and edema in the short term. Axonal transection may remain largely silent for extended periods, partly because of the redundancy and functional compensation potential of CNS pathways. The process of axonal loss, however, is irreversible, and beyond a certain threshold further degeneration translates into a slow progressive clinical decline (129,373,440). This underscores the importance of early diagnosis and intervention in MS.

Although proven effective, current treatment approaches have only a modest impact on disease progression. The development of more effective therapies will be predicated on advancing our understanding of the underlying disease process. Many animal and human studies have contributed to a model of MS immunopathogenesis in which activated autoreactive T cells are likely to play an important role. By a process of molecular mimicry, exposure to an infectious agent may activate cross-reactive autoaggressive T cells that may then enter the CNS and mediate injury. Alternatively, direct insult to the CNS, such as by a neurotropic virus or trauma, may expose CNS epitopes in the setting of local inflammation and may trigger autoreactive T cells in a susceptible host. Migration of proinflammatory immune cells across the BBB in MS may reflect dysregulation of the complex molecular interactions involving adhesion molecules, chemokines, and MMPs. It remains to be determined whether the abnormalities in expression levels of these molecules associated with MS reflect a true pathogenic role or a mere epiphenomenon of the inflamed state. The microenvironment in which autoreactive T cells become activated both in the periphery and in the CNS is defined in part by the local profile of cytokines and costimulatory molecules, and it influences the effector profile of the T cells that may adopt an inflammatory or antiinflammatory character. A proinflammatory environment is likely to promote epitope spreading and propagation of disease. In contrast, antiinflammatory cells may mitigate the injurious responses by bystander suppression. The roles of B cells and humoral responses as well as the contributions of innate immunity are receiving renewed attention. In some settings, antibodies have been implicated in augmenting or even inducing disease. In others, they may play a protective role. Studies into the neurobiology of MS and, in particular, the roles of microglia and astrocytes and the interactions of neural elements, endothelia/ and inflammatory cells have been revealing. Neural-immune interactions occur in the normal physiologic state and may represent important homeostatic mechanisms, capable of promoting repair and regeneration. Indeed, these principles must be incorporated in any serious attempt to understand MS comprehensively.

Finally, ongoing studies into the mechanisms of action of the available immunomodulators are contributing to our understanding of the immunopathology and neurobiology of MS, and these insights will guide the development of the next generation of safe and effective therapies. In addition to targeting inflammation, future approaches will undoubtedly explore neuroprotective strategies designed to prevent oligodendrocyte death and axonal transection and complementary approaches that promote remyelination and recovery.

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57 AUTOIMMUNE HEMOLYTIC ANEMIA

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Although it is clear that immunologic mechanisms play a significant role in the pathophysiology of many disease processes, there are relatively few situations where it is possible to gain a detailed understanding of the ongoing mechanisms of immune damage taking place *in vivo* in humans. Autoimmune hemolytic anemia (AIHA) is of particular interest in this regard. Because it is possible to sample the red cell compartment repeatedly, it is possible to define many of the immunopathologic processes that occur in this disease in molecular and cellular terms. AIHA represents a group of disorders in which individuals produce antibodies directed toward one or more of their own erythrocyte membrane antigens. This leads to destruction of the antibody-coated erythrocytes. The pathophysiology of the decreased erythrocyte survival in this disorder has been examined with increasing sophistication for many years. Although more recently quantitative immunologic techniques have been brought to bear on these pathophysiologic studies, the early work of Dacie, Mollison, Jandl, and their colleagues (1,2,3 and 4) led to several important conclusions. These investigators observed that the hemolysis in AIHA is most commonly extravascular rather than intravascular and that the liver and spleen play a major role in the clearance of the antibody-coated cells. The importance of complement in the destruction of IgM-coated cells also was recognized.

This chapter first discusses the underlying mechanisms responsible for the immune clearance of red blood cells. Next, it focuses on the clinical syndrome of AIHA and then considers the clinical syndrome of paroxysmal nocturnal hemoglobinuria (PNH), a complement-mediated hemolytic anemia characterized by intravascular hemolysis caused by a red cell membrane defect. The membrane proteins that are responsible for the defect and the biochemistry of those proteins are considered in other text. We contrast the clinical findings to those in AIHA, where extravascular destruction of red blood cells predominates. Finally, we discuss drug-induced immune hemolytic anemia.

EXPERIMENTAL MODELS

Some years ago, we established an experimental model of immune hemolytic anemia in the guinea pig, one approach that permits the dissection of the various steps involved in erythrocyte destruction (5,6). As with human erythrocytes, guinea pig erythrocytes are relatively resistant to the lytic action of complement, and their hemolysis, mediated by antibody and complement, is primarily extravascular. Each of the factors important in erythrocyte destruction defined in this model has been observed to be of importance in the disease as it occurs in humans. We focused on the role of antibody class, the role of complement, and the significance of the reticuloendothelial (mononuclear phagocyte) system in the pathophysiology of the hemolysis. For these studies, we isolated rabbit immunoglobulin G (IgG) and IgM antiguinea pig erythrocyte antibody. We then used this antibody to sensitize chromium 51 (^{51}Cr)-labeled guinea pig erythrocytes. The radiolabeled, antibody-coated erythrocytes were injected intravenously into the guinea pigs, and the rate and pattern of clearance as well as the site of organ sequestration of the antibody-sensitized cells were followed.

In these studies, we quantitated the number of antibody molecules per erythrocyte by both radiolabeling the antibody with iodine 125 so that we could directly assess the number of antibody molecules that attached to the red cells and by using a sensitive complement fixation method: the C1 fixation and transfer test. With the latter test, antibody per erythrocyte could be expressed in terms of the number of complement or C1 (the first component of complement)-activating sites generated by the antibody. A single molecule of IgM antibody bound to an erythrocyte by several of its binding sites will bind and activate one molecule of C1 to initiate the classical complement pathway. In the case of IgG, two molecules of antibody side by side are required for C1 binding and initiation of the classical pathway. With antigens widely distributed on the erythrocyte surface, such as the antigens recognized by the rabbit antiguinea pig erythrocyte antibody, many hundreds or thousands of antibody molecules must be deposited on the erythrocyte membrane before two bind sufficiently close to one another to permit complement activation. We studied erythrocyte survival curves in both normal guinea pigs and in guinea pigs congenitally deficient in the fourth component of complement. C4-deficient guinea pigs have a complete block in their classical complement pathway, and complement is not activated beyond the C1 step. A comparison of C4-deficient and normal animals enabled us to assess the role of antibody versus the role of antibody plus complement in altering erythrocyte survival. In addition, we depleted guinea pigs of the third component of complement, C3, as well as the later-acting components by treating the animals with cobra venom factor. In both animals genetically deficient in C4 or depleted of C3 through C9 by cobra venom factor, the complement activation sequence does not proceed through C3, and erythrocytes do not become coated with C3 *in vivo*.

In this model, IgG-sensitized erythrocytes were removed progressively from the circulation and sequestered predominately in the spleen (Fig. 57.1). Approximately 2,000 IgG molecules per erythrocyte were necessary to generate a single complement or C1-fixing site. Similar numbers of molecules were required to decrease erythrocyte survival. Erythrocyte survival was influenced by the number of antibody molecules per cell: Increasing the number of IgG molecules per cell progressively increased the splenic sequestration of these cells.

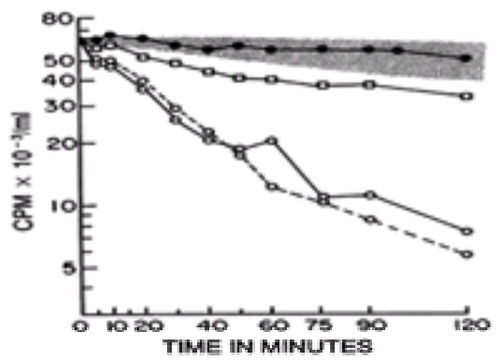

Figure 57.1. Survival of chromium 51 (^{51}Cr)-labeled guinea pig erythrocytes coated with immunoglobulin G (IgG) antibody in normal (*open circles*) and C4-deficient (*open squares*) guinea pigs. The survival of IgG-coated erythrocytes in C3-depleted guinea pigs was similar to that observed in C4-deficient guinea pigs. The *closed circles* represent the survival of ^{51}Cr-unsensitized erythrocytes. (*Shaded area*, 95% confidence limits.)

The IgG-coated erythrocytes were cleared from the circulation in an accelerated fashion in the absence of complement activation. This was evident from the studies with IgG-coated erythrocytes performed both in C4-deficient guinea pigs and in C3-depleted guinea pigs (Fig. 57.1). Complement-independent clearance of IgG-coated erythrocytes was predominately by macrophages in the spleen (by splenic macrophage Fc receptors). Larger numbers of antibody molecules per cell were required to

decrease erythrocyte survival in the absence of complement than in the presence of complement. As shown in Fig. 57.1, IgG-coated red cells were cleared more rapidly in normal guinea pigs compared with C4-deficient or C3- through C9-depleted animals.

We observed that the clearance of IgG-coated erythrocytes in the C3-depleted guinea pigs was identical to that in the C4-deficient animals. Animals treated with cobra venom factor have normal amounts of C1, C4, and C2, whereas C4-deficient guinea pigs have a normal alternative complement pathway. We believe the defect in the clearance of IgG-coated erythrocytes in both C4-deficient and C3- through C9-depleted animals resides in the failure of C3 to bind to the erythrocyte surface. The similar survival of IgG-coated erythrocytes in C4-deficient and C3- through C9-depleted guinea pigs suggests that the classical, rather than the alternative, complement pathway is of prime importance in the clearance of IgG-coated erythrocytes.

These studies also demonstrated that IgG-coated erythrocytes are cleared predominantly in the spleen regardless of whether complement activation occurs. When large amounts of IgG are bound to the erythrocytes or large numbers of complement-activating sites are generated, the liver becomes progressively more predominantly the organ of clearance. *In vitro* studies showed that macrophages of the reticuloendothelial system have several types of surface receptors for the Fc fragment of IgG (Fc receptors). These receptors are responsible for the binding and phagocytosis of IgG-coated erythrocytes. One of the Fc receptors is a high-affinity receptor present on macrophages and monocytes: FcRI. This receptor is more easily inhibited by plasma concentrations of IgG. There are two lower-affinity receptors on macrophages, FcRII and FcRIII (7,8). These receptors are likely important in the clearance of IgG-coated cells. Erythrocytes coated with multiple IgG molecules can interact with macrophages with multiple IgG Fc receptors, even in the presence of plasma concentrations of IgG. The multiple low-affinity Fc receptor binding sites may in aggregate lead to high-affinity binding of the erythrocytes to the macrophage surface, which in turn facilitates phagocytosis.

Macrophages also can alter IgG- or C3b/iC3b-coated erythrocytes in a manner that causes the red blood cells to form microspherocytes (9). These spherocytes are less able to pass through the splenic cords and sinuses and therefore have decreased survival. Their presence in the circulation is an indication of ongoing immune hemolysis. Macrophages also have receptors, designated CRI and CR3, for the activated third component of complement. These receptors recognize C3b and iC3b, respectively, and are capable of binding C3-coated erythrocytes. These receptors are discussed in greater detail in the chapters on complement. The receptors for the various C3 fragments do not recognize native C3; they only recognize fragments of C3 after C3 has undergone activation. Therefore, they are capable of efficient function in the presence of normal plasma concentrations of C3. The C3b- and IgG-coated cells can bind simultaneously to two different macrophage membrane receptors (10).

When erythrocytes are coated with IgM antibody and injected intravenously into guinea pigs, the pattern of clearance and site of organ sequestration are different from those of IgG-coated erythrocytes (Fig. 57.2). IgM-coated cells are cleared rapidly within the liver. Splenic clearance is secondary and usually amounts to less than 20% of the infused cells. In fact, no dose of IgM antibody leads predominantly to splenic sequestration. Erythrocyte survival is proportional to the number of IgM molecules per erythrocyte. With increasing numbers of IgM per red blood cell, erythrocyte clearance is progressively accelerated. About 60 IgM molecules (which generate 60 complement- or C1-fixing sites) are necessary to decrease the survival of IgM-coated cells. This finding contrasts with the single complement-fixing site generated by approximately 2,000 IgG molecules required to decrease the survival of IgG-coated cells. These studies established the absolute requirement for complement in the clearance of IgM-coated cells. This requirement was ascertained by examining the erythrocyte survival in C4-deficient and C3-depleted guinea pigs. IgM-coated erythrocytes survived normally in the complement deficient animals, even when agglutinating concentrations of IgM antibody were employed. *In vitro* studies showed that guinea pig macrophages neither detected nor bound IgM-coated erythrocytes in the absence of complement. Activation of the complement sequence by IgM resulted in the deposition of C3b on the erythrocyte surface. Erythrocyte-bound C3b and iC3b led to an interaction with hepatic macrophage C3b and iC3b receptors. This interaction was largely responsible for the clearance of IgM-coated erythrocytes.

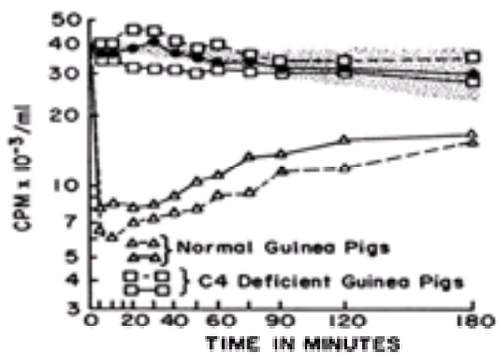

Figure 57.2. Survival of chromium 51-labeled guinea pig erythrocytes coated with immunoglobulin M (IgM) antibody in normal (*open triangles*) and C4-deficient (*open squares*) guinea pigs. The survival of IgM-coated erythrocytes in C3-depleted guinea pigs was similar to that observed in C4-deficient guinea pigs. (*Shaded area,* 95% confidence limits.) (From Frank MM, et al. Pathophysiology of autoimmune hemolytic anemia. *Ann Intern Med* 1977;87:210, with permission.)

Within the liver, IgM-coated erythrocytes are rapidly sequestered. Subsequently, they are either phagocytized and destroyed, or they are released from their C3 receptor attachment site back into the circulation, where they then survive normally, even though they still are coated with IgM and antigenically detectable C3. Extensive *in vitro* and *in vivo* studies showed that this release of IgM- and C3-coated erythrocytes from the macrophage C3 receptor attachment site is not due to elution of the antibody from the surface. Rather, the C3b/iC3b inactivator system, which involves several circulating plasma proteins, including factors I and H and a series of integral membrane proteins, for example, CR1 (see other text), causes the release of C3-coated erythrocytes from the macrophage C3b and iC3b receptor attachment sites (5,11). The C3-coated cells have on their surface an antigenically altered form of C3 that is no longer recognized by the macrophage C3 receptors. Thus, these erythrocytes survive normally. Increasing the concentration of IgM per erythrocyte accelerates the sequestration and also decreases the number of erythrocytes released from the hepatic macrophage receptor binding sites. Pretreatment of IgM- and C3b-coated erythrocytes with a source of serum C3 inactivator system proteins alters the erythrocyte cell-bound C3 and improves erythrocyte survival.

Therefore, these studies demonstrated that the two major classes of antibody that cause AIHA, that is, IgG and IgM, differ markedly in their biologic effects. IgG-coated erythrocytes are cleared predominantly in the spleen, whereas IgM-coated erythrocytes are sequestered predominantly within the liver. Splenic macrophage Fc receptors and C3 receptors are responsible for the clearance of IgG-coated cells. IgG-coated erythrocytes do not require complement for clearance; however, complement accelerates the clearance of IgG-coated erythrocytes in the spleen. The pattern of clearance of IgM-coated erythrocytes is entirely different. IgM-coated cells are cleared rapidly by the hepatic macrophage C3 receptors. The clearance is entirely complement dependent, and in the absence of complement activation, these cells survive normally. In areas of relatively rapid blood flow, that is, the liver, Fc receptor activity is likely at a relative disadvantage because FcRII and FcRIII, the predominant receptors, have extremely low affinity and the multiple interactions required for adherence are less likely to occur. Blood flow in the spleen is much slower and involves closer contact between sinusoidal phagocytes and circulating cells. This is believed to facilitate IgG-mediated splenic red cell clearance. The C3 inactivator system serves as an important control mechanism for the clearance of IgM-coated cells, mediating the release of IgM- and C3-coated cells from their hepatic macrophage C3 receptor attachment sites. Furthermore, exposure of IgM- and C3-coated erythrocytes to C3 inactivator system proteins can attenuate the clearance of these C3-coated cells by hepatic macrophages. In additional studies with cells coated *in vitro* with both IgG and IgM antibody, we observed that the addition of a small amount of IgM antibody to IgG-coated erythrocytes could shift the pattern of the sequestration from one predominantly involving the spleen to one predominantly involving the liver.

The effect of splenectomy and steroid hormones on the clearance of antibody- and complement-coated erythrocytes also was studied in the experimental model (12). Splenectomy markedly decreased the sequestration of IgG-sensitized cells. As the antibody concentration was increased, however, splenectomy became less effective in preventing the clearance of IgG-coated cells because the liver became the dominant organ in erythrocyte clearance. As expected, splenectomy did not alter the clearance of IgM-coated cells.

The effect of glucocorticoids and other steroid hormones on the clearance of IgG- and IgM-coated erythrocytes was examined (12,13,14 and 15). Pretreatment of guinea pigs with cortisone acetate or cortisol for 5 to 7 days impaired the splenic clearance of IgG-coated erythrocytes. Pretreatment of guinea pigs was necessary to observe this effect. Similar to the experience in humans, we observed that not all animals respond to steroids equally well, although the vast majority responded to steroids (13).

Several clinical observations suggest that glucocorticoids have a similar effect in humans. Patients with IgG antibody-mediated AIHA or immune thrombocytopenic purpura (ITP) treated with glucocorticoids often respond within days of the onset of therapy. At the time of response, the cells remain antibody coated, and there may be no decrease in antibody synthesis. Furthermore, some patients with IgG-mediated destruction of erythrocytes and platelets remained in clinical remission on steroid therapy, even when their cells remained antibody coated. These observations suggest that glucocorticoids affect the clearance mechanism in humans. Glucocorticoids were effective in inhibiting the clearance of IgG-coated erythrocytes, in both the presence and the absence of complement activation as shown by studies performed in normal and C4-deficient guinea pigs. In both types of animals, clearance decreased (16); however, the differences between the control groups and the

corticosteroid-treated animals were most marked when the studies were conducted in C4-deficient animals in whom IgG alone and Fc receptors alone were responsible for clearance. In each of these studies, glucocorticoid therapy decreased the extent, without altering the pattern, of erythrocyte clearance. One explanation of such findings is that glucocorticoids decrease either the number or the affinity of macrophage membrane receptors for Fc (Fc receptors). Further studies have established that this is the case (17).

Glucocorticoids also impaired the clearance of IgM-coated erythrocytes by the liver (12). This finding was not expected because these drugs are usually ineffective in patients with IgM-induced immune hemolytic anemia. These data suggested that high doses of corticosteroids might be effective in improving erythrocyte survival in some patients with IgM-induced immune hemolytic anemia whose red cells are coated with limited amounts of IgM and C3. This observation led to further clinical studies. Patients with a low-titer IgM (cold agglutinin)-induced immune hemolytic anemia, whose cells were coated with limited amounts of C3, may respond to corticosteroids (14), although extremely high concentrations of glucocorticoids were required to impair the clearance of IgM- and C3-coated cells in these patients. Thus, these studies indicated that glucocorticoids are most effective in reducing the clearance of IgG-coated cells. Glucocorticoids are least effective in preventing the clearance of IgM- and complement-coated cells. These observations were consistent with previous observations in humans with regard to the efficacy of steroids on the clearance of antibody and or complement-coated cells in immune hemolytic anemia.

We also examined the effect of agents that augment macrophage function. Guinea pigs were infected with the bacille Calmette–Guérin (BCG) vaccine strain of *Mycobacterium tuberculosis*. Such animals displayed markedly augmented macrophage clearance. These animals removed IgG-sensitized erythrocytes more rapidly from the circulation, both in the presence and in the absence of complement activity. The site of sequestration remained the spleen. The sensitized red cells were cleared from the circulation as if they were coated with large quantities of antibody. We postulated that the fixed macrophages of the reticuloendothelial system had an augmented number of Fc or C3 receptors on their surface, secondary to the infectious process. *In vitro* studies suggested that such is the case. In the case of IgM-sensitized erythrocytes, not only the extent of sequestration but also the pattern of sequestration changed. The IgM-sensitized cells were rapidly cleared in the liver as they are in the uninfected animal; however, the cells never were returned to the circulation. They were phagocytosed by hepatic macrophages. These studies may explain the accentuation of hemolysis in individuals with a compensated immune hemolytic anemia who develop an infection.

An additional series of experiments examined the effect of treatment with the cytotoxic agent cyclophosphamide on the clearance of IgG-coated cells. Cyclophosphamide treatment of guinea pigs did not delay the clearance of antibody-sensitized erythrocytes, suggesting that the therapeutic effect of these agents is due to their effect on synthesis of antibody by B-lymphocytes rather than to any effect on macrophage-mediated erythrocyte clearance.

More recently, we examined the capacity of other steroids and their analogs to modulate the clearance of the IgG-coated erythrocytes by splenic macrophages (13). We observed that estradiol, in contrast to cortisol, enhances the clearance of IgG-coated erythrocytes by splenic macrophages in a dose-dependent manner. On the other hand, estradiol does not alter the splenic macrophage clearance of heat-altered erythrocytes or the hepatic macrophage clearance of IgM- and C3b-coated erythrocytes (18). This finding suggests that the effect of estradiol is on the splenic macrophage Fc receptors responsible for the clearance of the IgG-coated cells. Neither deoxycorticosterone (a precursor in steroid synthesis) nor tetrahydrocortisone (an inactive steroid metabolite) influenced clearance. These studies suggesting that the macrophage Fc receptors may be modulated *in vivo* by hormonal mechanisms were supported by studies demonstrating that splenic macrophages isolated from estradiol-treated animals exhibited remarkably enhanced Fc receptor expression, but not C3 receptor expression, when compared with control animals. These data may explain the alteration in the clinical status of patients with immune hemolytic anemia and immune thrombocytopenia during changes in hormonal states, such as pregnancy. During pregnancy, estrogen levels rise to a level similar to that necessary to accelerate the clearance of IgG-coated erythrocytes in our guinea pig model (13). Similarly, during pregnancy, the course of IgG-induced AIHA is known to accelerate. Further studies showed that other steroid analogs also can alter the clearance of IgG-coated cells by affecting splenic macrophage Fc receptor expression.

More recent studies in knockout and transgenic mice further elucidated the pathophysiology of the clearance of IgG-coated cells (19,20,21 and 22). Studies in mice transgenic for the human Fc receptor FcRIIA suggest that this receptor plays a significant role in the clearance of IgG-coated cells. Extensive studies in knockout mice have demonstrated the critical importance of Fc receptors in the clearance of IgG coated cells. At the concentrations of IgG antibody employed in these studies, complement did not play a role in the clearance of IgG coated cells. Further studies showed that another Fc receptor, FcRIIB, may serve a regulatory role by inhibiting the phagocytosis of IgG-coated cells.

The data derived from the preceding studies help to elucidate the pathophysiology of immune hemolytic anemia in humans. The following section summarizes the application of these concepts to AIHA in humans.

IMMUNE HEMOLYTIC ANEMIA IN HUMANS

The occurrence of anemia was first described in ancient times, but until the modem era, it was difficult or impossible to ascertain whether the anemia was due to the shortened survival of erythrocytes. With the onset of transfusion therapy, it became clear that transfused cells had a limited survival in some anemic individuals. In some cases, the presence of hemoglobinuria could be related to the onset of intravascular hemolysis; it was impossible, however, to relate shortened survival, in the absence of hemoglobinuria, to an immunologic reaction to the red cells until the development of the antiglobulin test by Coombs and colleagues in 1945. This test allowed the detection of antibody and complement on circulating cells. Within a year after the first report of the antiglobulin test, the presence of immunoreactive material on the surface of erythrocytes in a patient with hemolytic anemia was noted, and the concept of AIHA was born. With the onset of methods for tagging erythrocytes with radioactive tracers, survival studies could be performed conveniently, and many studies of the pathophysiology of hemolytic anemia were performed. It is clear now that AIHA is most commonly caused by IgG antibody (2,23). The antigen to which the IgG antibody is directed is usually one of the Rh erythrocyte antigens, although often its precise specificity is not easily defined. This antibody usually has its maximal activity at 37°C, and thus this entity has been termed *warm antibody–induced hemolytic anemia*.

Immunoglobulin G-induced (warm antibody–mediated) immune hemolytic anemia can occur without an apparent underlying disease (idiopathic AIHA); however, it also can occur with an underlying immunoproliferative disorder, either malignant or nonmalignant, such as chronic lymphocytic leukemia, non-Hodgkin lymphoma, and systemic lupus erythematosus. Certain patients with immunodeficiency can develop AIHA as well. Rarely, IgG-induced immune hemolytic anemia has been observed in patients with an underlying malignant disease that is not an immunoproliferative disorder (Table 57.1). Such malignant disorders include ovarian tumors and myelofibrosis with myeloid hyperplasia. Additionally, bacterial infections, such as tuberculosis; viral infections, such as cytomegalovirus disease; and chronic inflammatory conditions, such as ulcerative colitis have been described as associated conditions.

Infections
Viral infections, especially respiratory infections
Infectious mononucleosis and cytomegalovirus
Mycoplasma, especially pneumonia
Tuberculosis
Disorders associated with autoantibody production
Systemic lupus erythematosus
Rheumatoid arthritis
Thyroid disorders
Ulcerative colitis
Chronic active hepatitis
Immunodeficiency syndromes
X-linked agammaglobulinemia
Dysgammaglobulinemia
Common variable hypogammaglobulinemia
IgA deficiency
Wiskott-Aldrich syndrome
Malignancies
Non-Hodgkin's lymphoma
Hodgkin disease
Acute lymphocytic leukemia
Carcinoma
Thymoma
Ovarian cysts and tumors

IgG, immunoglobulin.

TABLE 57.1. Diseases Associated with Autoimmune Hemolytic Anemia in Childhood

The incidence of idiopathic IgG-induced AIHA varies among different series; however, overall about half the patients with IgG-induced immune hemolysis do not have a detectable underlying disease at diagnosis. The other half have an underlying disease, such as those mentioned, or have a drug-induced immune hemolytic anemia (24). Some patients, both those with the “idiopathic” disease and those with disease associated with an underlying immunoproliferative disorder, have ITP in conjunction with IgG-induced AIHA (Evan syndrome) (25). Patients have been described with immune hemolytic anemia, immune thrombocytopenia, and immune granulocytopenia (D.B. Cines, D.S. August, and A.D. Schreiber, personal communication), with antibodies directed toward erythrocytes, platelets, and granulocytes. It is not clear whether such IgG antibodies directed against each blood cell line recognize a common blood cell antigen or represent antibodies with different specificities.

Although the destruction of red blood cells on exposure of a patient to cold was first reported in the mid nineteenth century, it was not until the turn of the century that Landsteiner first demonstrated the agglutination of blood by a patient's serum in the cold (26). Later, it was observed that IgM autoantibodies most commonly are found in patients who have cold agglutinin disease and that they are generally responsible for the clinical symptoms of the disease (27). The IgM antibody in cold hemagglutinin disease usually is directed against the I antigen or related antigens on the human erythrocyte membrane. As with all IgM antibodies, agglutinating

activity is particularly efficient because of the multiple antigen-combining sites on the IgM molecule. In this disorder, the IgM antibody has a particular affinity for its red cell antigen in the cold (0–10°C), and the affinity is lower at higher temperatures. Like warm antibody (i.e, IgG-mediated) AIHA, cold agglutinin disease can be divided into those cases considered primary or idiopathic or those associated with the presence of an underlying disease (i.e., secondary).

Chronic cold hemagglutinin is due to a clonal expansion of lymphocytes in which a monoclonal antibody that recognizes a polysaccharide antigen on red cells, termed I or i, is produced. The most common form of chronic cold agglutinin disease is the primary or idiopathic form. Usually a disease of older persons, its peak incidence is in the fifties and sixties (28). Most often, it presents as fatigue, anemia, and occasionally jaundice in an elderly patient, but it may be associated with the development of acrocyanosis as a result of sludging of blood in peripheral vessels on exposure to cold or with acute hemolysis. This disease is associated with the presence of a monoclonal IgM antibody, usually exhibiting a high cold agglutinin titer (>1:1,000). This IgM binds to erythrocytes avidly in the cold but shows no binding activity at 37°C. In most, but not all, patients, the antibody is of the klight-chain type and has specificity for the I antigen present on the erythrocytes of most adults. The I-antigenic determinants are closely related to the ABO core antigenic determinants. Although present on the erythrocytes of more than 99% of adults, the antigenic groupings recognized by the antibody develop during childhood and are not present on blood taken from the umbilical vein of the newborn. Thus, operationally, I-specificity is established by the ability of the antibody to agglutinate the blood of almost all adults but an inability to agglutinate the erythrocytes of newborns. The antibody responsible for the development of the cold agglutinin syndrome appears to reflect the expansion of a highly restricted or single clone of cells. The antibody appears to represent a highly restricted clonal response to the I-antigen. Antibodies among patients tend to share idiotypic determinants consistent with their uniform recognition of the I-antigen.

Secondary cold hemagglutinin disease, or IgM-induced immune hemolytic anemia, is associated most commonly with an underlying mycoplasma infection, particularly *Mycoplasma pneumoniae*, in which antibody with typical anti-I-specificity is produced. It can occur with other infections, however, such as infectious mononucleosis, cytomegalovirus, and mumps. With infectious mononucleosis anti-i (antibody to an antigen related to I but present on cord blood cells), cold agglutinins are produced, but overt hemolysis is unusual. Under most circumstances, with an underlying infection, the cold agglutinin (IgM antibody) is polyclonal, that is, immunochemically heterogeneous.

Cold hemagglutinin disease also can be seen in patients who have an underlying immunoproliferative disorder. The plasma of healthy adults and children contains low levels of IgM antierythrocyte antibodies, that is, low levels of IgM cold agglutinins. Rarely, cold-reacting autoantibodies have been observed with specificity directed against red blood cell antigens other than I or i, that is, the P and the PR antigens. Also, rarely, IgA cold agglutinins have been observed.

The reason for the preferential reaction of cold agglutinin with the human red blood cell membrane in the cold is not completely understood. Most cold agglutinins have no measurable activity above 30°C. Although it has been postulated that either the antibody or the antigen may undergo a structural change on exposure to cold, most data suggest that the antigen on the erythrocyte surface is altered in the cold. This may represent a cold-dependent conformational change in the antigen recognized by the antibody-combining site or a cold-induced change in the erythrocyte surface increasing antigen availability. When intact erythrocytes are studied, IgM anti-I interactions occur only in the cold; however, reactivity at 37°C is noted when the I-antigen is isolated from the erythrocyte membrane. In addition, polysaccharide antigens in general react more strongly with antibodies in the cold than do protein antigens. Presumably, the weak, nonionic interactions with polysaccharides are more easily disrupted as the reaction temperature is raised and brownian motion is increased than are the ionic reactions with proteins.

As in all patients with AIHA, erythrocyte survival is generally proportional to the amount of antibody on the erythrocyte surface. In cold hemagglutinin disease, the extent of hemolysis is a function of the titer of the antibody (cold agglutinin titer), the thermal amplitude of the IgM antibody (the highest temperature at which the antibody is active), and likely the level and activity of the regulatory proteins of the complement system.

Autoimmune Hemolytic Anemia in Children

Viral and respiratory infections are the diseases most commonly associated with AIHA in childhood (29). The anemia is generally mild in these cases, but occasionally there is brisk hemolysis. Associated infections are listed in Table 57.1, as are other disorders, such as systemic lupus erythematosus, also commonly associated with childhood AIHA. The hemolytic anemia may be the initial manifestation or may accompany other symptoms. AIHA also has been reported with a number of immunoregulatory disorders, including immunodeficiency. In some patients, the immunodeficiency is well defined (30), but other patients have the pattern of dysgammaglobulinemia (31). Many of these patients have chronic lymphadenopathy. Chronic hemolysis and immune pancytopenia are common in this group of patients, and there is an increased risk of malignancy. AIHA is rare in childhood malignancy, but it has been reported with acute lymphocytic leukemia, Hodgkin's disease, and non-Hodgkin lymphoma (32,33), in some cases preceding the appearance of the malignancy. Red cell autoantibodies may also be passively transferred from a mother with AIHA to the fetus. Although most reported cases in infants have described little or no intravascular hemolysis, severe anemia may result. Exchange transfusion has been successful in some cases (34).

Pathophysiologic Features

The factors that govern the survival of antibody- and complement-coated erythrocytes in humans are much the same as those that govern the clearance of these cells in animals. In cold hemagglutinin disease, the IgM antibody in the circulation of patients with the disease interacts with the erythrocyte surface, where the cells circulate to areas below core body temperatures, and activates the early steps of the classical complement pathway (35,36). Once C1 is bound to the IgM molecule and activated, it sequentially binds and activates the fourth and second components of complement. The first of these two steps clearly takes place at temperatures as low as 0°C. When the cells return to body temperature, activation proceeds, even though the cold agglutinin antibody can dissociate from the erythrocyte. The C3 convertase generated cleaves C3 into two antigenic fragments, one of which, C3b, may bind to the erythrocyte surface. At this step, there is considerable amplification of the IgM effect with a single classical pathway convertase capable of cleaving many C3 molecules and depositing many C3b molecules on the erythrocyte surface. In some cases, the complement sequence of reactions may be completed with resulting hemolysis, but this is unusual because of the presence of membrane-bound proteins that restrict complement action, the regulation of complement activation (RCA) gene cluster proteins, including DAF and CD59 (see chapter on complement). In the presence of factors H and I, the C3b may be converted to iC3b. These C3-coated erythrocytes are cleared via the complement receptor by mechanisms discussed earlier, even though antibody is no longer present on the red cell surface. The macrophage C3b and iC3b receptors bind, sphere, and may mediate phagocytosis of the C3-coated erythrocytes (14,15,37). Extravascular sequestration usually predominates in patients with this disease. In humans, as in the guinea pig model, there are no receptors on macrophages capable of interacting with IgM-coated cells in the absence of complement; thus, in the absence of an intact classical complement pathway, IgM-coated red cells have a normal survival.

Spherocytosis of erythrocytes is a clinical hallmark of AIHA. It is not possible to induce spherocytosis of erythrocytes *in vitro* by the addition of antibody and complement. Spherocytes will form only if macrophages are present in the reaction mixture. Whereas spherocytes may be due to the partial ingestion of opsonized red cell membranes by phagocytic cells, the exact mechanism for macrophage-induced spherocytosis is unclear (9,38). The erythrocyte can be released from its macrophage membrane attachment site and circulate until it is trapped within the splenic cords. The decreased ability of these microspherocytes to alter their shape and traverse the splenic cords and sinuses results in splenic entrapment and eventual macrophage-mediated destruction.

In humans, the clearance of IgM plus complement coated cells is rapid and takes place primarily in the liver (6,39). The human erythrocyte membrane, in contrast to the sheep erythrocyte membrane, is relatively resistant to the lytic action of complement. When large numbers of IgM molecules are present on the erythrocyte surface, however, complement activation is extensive. The terminal complement components then can place sufficient membrane attack complexes on the erythrocyte surface to lyse the erythrocytes in the intravascular space. The specificity of the IgM antibody is also important because some IgM antibodies appear to be quite efficient at inducing hemolysis.

Complement regulatory proteins, as discussed earlier, are likely particularly important in cold hemagglutinin disease (11) because cell destruction is mediated entirely by C3 and the later complement components. The complement regulatory proteins regulate the number of active C3 fragments on the cell surface. The C3-coated erythrocytes interacting with CRI and factor I are degraded to C3dg or C3d. These C3dg- or C3d-coated erythrocytes are not bound by the macrophage C3b receptors and have a normal survival (5,40,41). Thus, the presence of C3 (C3dg or C3d)-coated erythrocytes in cold hemagglutinin disease explains the observations of a normal erythrocyte survival in patients who still have C3, as detected by the antiglobulin test, on their erythrocyte membranes (42).

The thermal amplitude of the IgM cold agglutinin is important in determining the extent of hemolysis in cold hemagglutinin disease. At a relatively low level of cold agglutinin sensitization, patients with higher thermal amplitude antibodies (those antibodies that possess activity at temperatures approaching 37°C) still may have considerable hemolysis. Such patients have been described as having a low-titer cold hemagglutinin syndrome with a high thermal amplitude antibody. The correct diagnosis in such patients is important because they appear to respond to glucocorticoid therapy in a manner different from that in the usual patient with high-titer cold hemagglutinin disease (14). Furthermore, some unusual patients have an IgG cold agglutinin. The presence of such an IgG antibody is potentially important because it appears to indicate responsiveness to steroids or splenectomy (28).

In humans, as in guinea pigs, IgG antibody produces effects that are quite different from those of IgM antibody. Although complement activation is not necessary for the clearance of IgG-coated cells, complement can accelerate the clearance of IgG-coated erythrocytes. Generally, more IgG molecules on the erythrocyte surface are needed to bind and activate a single molecule of C1 (35) because two IgG molecules in close proximity to each other (a doublet) are required. Once C1 is bound and activated, C4 and C2 activation occurs in a manner similar to that described for IgM antibody, and C3 convertase is formed. C3 cleavage results, and C3b is deposited

on the erythrocyte surface. Macrophages within the reticuloendothelial system have Fc receptors for IgG (37,44). These macrophage Fc receptors bind IgG-coated erythrocytes and mediate spherocyte formation or phagocytosis. Once sufficient IgG is present on the erythrocyte surface so that C1 activation occurs, however, erythrocyte clearance is further accelerated. In such a circumstance, clearance represents the combined effect of the macrophage Fc receptors and the macrophage C3b/iC3b receptors. These receptors interact synergistically to induce the binding of erythrocytes coated with IgG and C3 (15). As in the animal models, IgG-coated erythrocytes are cleared progressively from the circulation, primarily in the spleen (77), and hemolysis is almost always extravascular.

NATURAL HISTORY

There appears to be little genetic predisposition to the development of AIHA (45). The occasional rare familial association of cases may be secondary to the familial predisposition to systemic lupus erythematosus or a similar underlying connective tissue disease. Thus, there are patients with AIHA who have a family history of other autoimmune diseases, such as autoimmune thrombocytopenia, rheumatoid arthritis, and glomerulonephritis. AIHA is not an uncommon disease. In large centers, 15 to 30 cases are seen yearly (46), with an annual incidence of approximately one case per 75,000 to 80,000 persons in the general population. As with any disease that may require careful serologic study for diagnosis, the level of sophistication and diagnostic capability of the institution influence the reported incidence. Nevertheless, AIHA occurs considerably less commonly than does autoimmune thrombocytopenia.

Autoimmune hemolytic anemia caused by either IgG or IgM antibody does not appear to be more prevalent in any particular racial group and can affect persons of any age. There is a general impression that AIHA occurs more commonly in women, although in most series the incidence is roughly equivalent between sexes (23). Any increased incidence in female patients may be due to the increased incidence of systemic lupus erythematosus in women. Although warm antibody (IgG-induced) immune hemolytic anemia can occur at any age, there appears to be a peak incidence in the 50-year-old age group. In contrast, idiopathic cold agglutinin disease is a disease predominantly of elderly persons.

The peak incidence of AIHA in childhood is in the first 4 years of life. Children older than 10 years at onset are most likely to have a chronic course and most likely to have an underlying disorder. In a study of the prevalence of the disease in childhood, the incidence in persons younger than 20 years of age was slightly less than 0.2 per 100,000 (47). In contrast to the situation in adults, in the reported series in children, there is a male preponderance, especially in acute cases and in patients younger than 10 years of age (48,49,50,51,52 and 53). Familial cases also have been reported (45).

Patients with AIHA vary considerably in their mode of clinical manifestation, and manifestations may be more acute in children than in adults. In children, the fall in hemoglobin may occur over a period of hours to days, with resolution of the disease often within months. In several large series of children with AIHA, a mean of 48% of cases were acute in onset and short-lived. Some cases are chronic, however, with intermittent relapses, as often seen in adults (48,49,50,51,52 and 53). The range in different series is considerable, with 25% to 70% of children having a chronic course. Children with chronic AIHA recover fully, whereas others have persistence of hemolysis or intermittent relapses. These series emanate from different geographic locations and from centers with different referral patterns. In the experience at the Children's Hospital of the University of Pennsylvania and the Duke University Children's Hospital, most cases of AIHA in the pediatric population have been acute and not associated with an underlying disorder.

Immunoglobulin M–induced immune hemolytic anemia, usually cold agglutinin disease, may be associated with a respiratory infection. Cases resulting from *M. pneumoniae* infections are the most common and have anti-I-specificity. Those IgM antibodies observed in infectious mononucleosis usually are directed against the I antigen. The Donath–Landersteiner cold hemolysin is an unusual IgG antibody with anti-P specificity that was originally noted in cases of congenital or acquired syphilis. The disease it causes is termed *paroxysmal cold hemoglobinuria*. Hemolysis in this syndrome most commonly occurs intravascularly, after the antibody has passed through a cell attachment phase in the lower temperatures of the peripheral circulation. The extravascular hemolysis is due to the unusual complement-activating efficiency of this IgG antibody. As its name implies, this antibody is associated with cold hemoglobinuria. At present, this antibody, though uncommon, is found most frequently in children with viral infections (54,55 and 56). Hemolysis, although sometimes severe, is usually mild, and tends to resolve as the infection clears.

Mortality in the pediatric age group has ranged from 9% to 29% (45,48,49,50 and 51,53,58). Death during the acute stage is usually due to severe anemia or to hemorrhage from associated thrombocytopenia. Mortality in chronic cases or in adults is higher and usually occurs because of an underlying serious disorder, such as Hodgkin disease or non-Hodgkin lymphoma or as a complication of therapy. Fatal sepsis, of course, has been observed following splenectomy (51).

CLINICAL AND LABORATORY FINDINGS

Many of the symptoms of AIHA, such as weakness, malaise, and light-headedness, are caused by the presence of anemia. Patients who have underlying cardiovascular disease may have significant dyspnea on exertion and peripheral edema as well as angina pectoris. If hemolysis is significant, mild jaundice may be noted, particularly in the presence of hepatic dysfunction. In addition, patients with an underlying disease often have symptoms associated with that disease, for example, fever and weight loss with an underlying malignant disease or joint symptoms secondary to an underlying systemic vasculitis. Physical findings are also generally referable to the underlying disease. For example, in patients with an underlying non-Hodgkin lymphoma, hepatosplenomegaly and lymphadenopathy are common. Mild splenomegaly may be present in patients with severe AIHA. Massive splenomegaly suggests an underlying disorder such as a lymphoma. Other signs that may result from the anemic state include those caused by congestive heart failure (edema, ascites, or pulmonary congestion). Severe jaundice is uncommon. Thus, the common presenting symptoms are pallor, jaundice, dark urine, abdominal pain, and fever. Pallor may precede the appearance of jaundice. The clinical status depends on the rapidity of the hemolysis and the severity of the anemia. In mild cases, fatigue may be the only symptom. In severe cases, the patient may appear acutely ill or even moribund, with tachycardia, tachypnea, signs of hypoxia, and cardiovascular collapse. In severe IgM-induced cold agglutinin disease, the skin may have a livedo reticularis pattern and the patient may demonstrate acrocyanosis on exposure to the cold.

Laboratory data reveal the presence of anemia and, if bone marrow function is adequate, reticulocytosis. Diagnosis rests on the presence of anemia, reticulocytosis, and a positive result on direct Coombs test. Examination of the peripheral smear may show spherocytes, polychromasia, nucleated red blood cells, and erythrophagocytosis. Rosettes of red cells around white cells may be visible in a buffy-coat preparation. Agglutination of the red cells may be evident in cold agglutinin disease. In severe cases, macroagglutination is visible on the microscope slide or in a capillary tube. The white cell count is usually normal or elevated. Autoimmune hemolysis also is associated with thrombocytopenia or leukopenia in a small number of patients. Indirect hyperbilirubinemia is common. It is the positive result on direct Coombs test, however, that alerts the clinician to the correct diagnosis.

Reticulocytopenia may be observed, especially in children in the first days of the anemia. In a small percentage of patients, the reticulocytopenia may persist for weeks to months (58,59 and 60). Bone marrow aspiration usually shows erythroid hyperplasia, but hypoplasia is present in a few patients (60). Autoantibodies directed against burst-forming unit, erythroid (BFU-E) colonies are believed to be responsible for the reticulocytopenia in some patients (60), although antibody directed at a blood cell antigen present primarily on reticulocytes is another possibility.

The diagnosis of AIHA is established most effectively by directly examining the patient's circulating red blood cells for the presence of antibody or complement components on their surface, which is done most easily by performing a direct Coombs antiglobulin test. Classically, in this test, the patient's red blood cells are made to interact with a rabbit or goat antihuman serum globulin reagent, and agglutination of the patient's red blood cells is assessed. It is also possible to use antibody to human immunoglobulin or complement components as a more specific test reagent. In this case, agglutination induced by anti-IgG indicates the presence of IgG on the surface of the red blood cells, whereas agglutination with an anti-C3 or anti-C4 (a positive result on nongamma Coombs test) is used to test for the presence of C4 and C3. In IgG-induced hemolytic anemia, IgG or IgG plus complement components are found on the surface of erythrocytes. Therefore, such patients usually have a positive result for IgG on the Coombs test but may have a positive result on the nongamma Coombs test as well. In IgM-induced hemolytic anemia (cold agglutinin disease), IgG is not found on the red cells, and the IgM cold agglutinin, because of its low affinity for red cell antigens at 37°C, is not found either; C3, stably bound at 37°C, is detected on the red cell membrane. Therefore, in cold agglutinin disease, usually only a positive result on nongamma (C3) Coombs test is observed.

Rarely, patients with IgG-induced immune hemolysis have levels of IgG per erythrocyte that are undetectable by the standard Coombs test, which requires the presence of hundreds of molecules of IgG on the erythrocyte surface for the result to be positive. When this phenomenon was originally described, the small amounts of red cell–bound IgG antibody were detected by using a complex antiglobulin consumption test (61,62). Now, however, a Coombs test using radiolabeled anti-IgG (63), which is ten times more sensitive than the standard Coombs test, also may be used to detect the antibody.

Thus, testing with Coombs antisera shows several patterns of reactivity (58). The red cells may be coated with IgG in the presence or absence of detectable complement (warm antibody IgG-mediated AIHA) or with complement protein alone (IgM-induced hemolysis, i.e., cold hemagglutinin disease). Rarely, IgM is detected as well. In one large series of patients, IgG with or without complement was found on the red cells in 85% to 95% of patients with chronic disease but in fewer (about 30%) of those with acute disease (50,58). Cold agglutinins (IgM-induced AIHA) and the coating of red blood cells with complement alone were more common in acute disease. Early studies suggested that the finding of IgG plus complement indicated a more guarded prognosis; however, it is now believed that it is not possible to predict the chronicity or severity of AIHA from the Coombs testing pattern.

A cold agglutinin titer is also diagnostically helpful. This test is performed by examining the patient's plasma for agglutinating activity at 0°C directed against normal ABO-compatible eurythrocytes containing the I-antigen. The cold agglutinin titer is the highest dilution of antibody that still agglutinates normal red blood cells in the

cold. Most patients with immune hemolysis secondary to cold hemagglutinin disease have cold agglutinin titers greater than 1 to 1,000.

THERAPY

In many patients with IgG- or IgM-induced immune hemolytic anemia, no therapeutic intervention is necessary because the hemolysis is mild. If an underlying disease is present, control of this disease often brings the hemolytic anemia under control as well. If the patient is having significant anemia secondary to hemolysis, however, therapeutic intervention is in order.

Glucocorticoids

Patients with IgG-induced immune hemolytic anemia respond, in general, to glucocorticoid therapy in dosages equivalent to 1 to 2 mg of prednisone per kilogram of body weight daily. These drugs are believed to decrease hemolysis in IgG-induced hemolytic anemia by three major mechanisms (12,64). First, they decrease the production of the abnormal IgG antibody. This is a common effect and can be expected to produce a gradual decrease in the strength of the Coombs test result and a rise in hemoglobin within 2 to 6 weeks. Second, glucocorticoids are reported to be associated with a fall in the amount of antibody detected by the direct Coombs test, as if they induced a decrease in antibody affinity. This has been associated with improved erythrocyte survival; it is probably an uncommon effect of glucocorticoid therapy. Third, glucocorticoids have been shown *in vitro* and *in vivo* to interfere with the macrophage Fc receptors responsible for clearance from the circulation of IgG-coated cells (12,15,64). The effect is to improve erythrocyte survival despite the continued presence of IgG and C3b on the erythrocyte surface. Thus, the Coombs test in some patients may remain positive in the face of an improved erythrocyte survival and rising hemoglobin. This effect of glucocorticoids may be rapid and may be responsible for the rise in hemoglobin noted in some patients to occur with 1 to 4 days of glucocorticoid therapy. In a number of animal studies, however, it was shown that glucocorticoids have no effect on erythrocyte survival until therapy has been continued for 5 to 7 days. Similarly, most patients will respond to glucocorticoid therapy within 2 to 3 weeks (and some within days). In some patients, 4 to 6 weeks of therapy may be required for a response to be evident.

Once a therapeutic response is achieved and the patient stabilizes, tapering of steroids should begin. This may take several weeks to many months. Alternate-day steroid therapy can be effective in some patients after the clinical course stabilizes. Interestingly, alternate-day therapy may be less effective in AIHA than in some of the inflammatory autoimmune diseases, and patients should be monitored carefully for an exacerbation. Care should be taken in stopping glucocorticoids if the patient continues to demonstrate a positive result on the direct Coombs test. Approximately 70% to 80% of patients have an initial response to high-dose glucocorticoids. In a small proportion of patients with chronic AIHA in adults, the steroids can be tapered and stopped with the patient remaining in remission. Some patients have control of their hemolytic process on continued low- to medium-dose steroid therapy. For patients who are steroid dependent, the initial and long-term side effects of these drugs restrict their long-term use. These effects include diabetes and hypertension, electrolyte imbalance, increased appetite and weight gain, moonlike facies, osteoporosis, myopathy, and increased susceptibility to infection. The severity of these side effects relates both to the duration of therapy and to dosage. Splenectomy should be considered in patients who are steroid unresponsive or require more than 10 to 20 mg of prednisone per day or substantial doses of steroids every other day for maintenance. Each patient requires individual evaluation of underlying diseases, surgical risk, extent of anemia, and steroid intolerance. In some patients, the presence of a mild hemolytic anemia may be preferable to splenectomy or other treatment options. The initial goal of therapy is to return the patient to normal hematologic values and nontoxic levels of glucocorticoid therapy, but for some patients, a modified goal of improvement in hemolysis to a clinically asymptomatic state with minimum glucocorticoid side effects is more realistic.

Glucocorticoids usually are not effective in cold hemagglutinin disease (27,64). This is probably due to the fact that these patients generally have large amounts of IgM antierythrocyte antibody and large numbers of C3 molecules deposited on their red cells. In addition, some of the hemolysis may be intravascular, and glucocorticoids do not inhibit complement-mediated lysis. A few patients with a low-titer cold hemagglutinin disease syndrome, in which the antierythrocyte antibody has activity at temperatures approaching 37°C, do respond to steroid therapy (14). In addition, the few patients described with an IgG cold agglutinin appear to be both steroid and splenectomy responsive (43). Patients with cold hemagglutinin disease respond best to the avoidance of cold and control of their underlying disease. Fortunately, in many patients, hemolytic anemia is mild.

Splenectomy

The red pulp of the spleen, with its resident macrophages, is the major site for sequestration of IgG-coated blood cells in humans as in animals. This appears to be due to the unique circulatory pathways in the spleen whereby hemoconcentration occurs in the splenic cords and erythrocytes make their way through fine fenestrations between macrophages. This results in intimate contact between macrophages (with their membrane Fc receptors) and IgG-coated blood cells possibly in the presence of a minimal amount of plasma IgG.

Removal of this major site of red cell destruction is an effective therapeutic strategy in IgG-induced immune hemolytic anemia. The response rate to splenectomy is approximately 50% to 70%; however, the vast majority of the responses are partial remissions. Interestingly, before glucocorticoid therapy became available for the treatment of AIHA, splenectomy was performed commonly, but the patient usually had a relapse. Presumably, as the sensitized erythrocytes continued to circulate, they bound more and more antibody. They finally achieved a degree of sensitization where the liver was able to mediate clearance. Probably, patients who are least responsive to splenectomy are those whose erythrocytes are coated with large amounts of IgG. In this circumstance, the liver plays a larger role in clearance. The partial remissions that occur with splenectomy are often quite helpful in that they result in a lessening of the hemolytic rate, with a rise in the hemoglobin value, and allow a reduction in the amount of glucocorticoid needed to control the hemolytic anemia. Because of the increased risk of sepsis (36), patients should be carefully selected. Patients who are unresponsive to glucocorticoids, who require moderate to high maintenance doses, or who have developed glucocorticoid intolerance can be considered for splenectomy.^{51}Cr-labeled red cell kinetic studies are probably not helpful because the procedure is time-consuming, expensive, and not a reliable indicator of response to splenectomy in most cases.

A second effect of splenectomy also has been suggested in AIHA and was shown to be important in autoimmune thrombocytopenia. Splenectomy may lead to a decrease in the production of the IgG antierythrocyte antibody because the spleen contains a large B-cell pool. Splenectomy, like corticosteroid therapy, is usually not effective for patients with cold hemagglutinin disease because IgM-coated erythrocytes are cleared predominantly in the liver. An occasional case in which a patient with an apparent IgM-induced hemolytic anemia responded to splenectomy was reported and may have been due to decreased production of IgM antibody by the spleen in these few patients or to the presence of an IgG cold agglutinin (28). Immunization with pneumococcal vaccine should be performed prior to splenectomy to decrease the likelihood of postsplenectomy pneumococcal infection.

Immunosuppressive Agents

Several immunosuppressive agents have been used in the treatment of AIHA. Commonly used agents include the thiopurines (6-mercaptopurine, azathioprine, and thioguanine) and alkylating agents (cyclophosphamide and chlorambucil). Immunosuppressive agents act to decrease the production of antibody, and therefore it generally takes at least 2 weeks before any therapeutic result is observed. A reasonable clinical trial consists of 3 to 4 months of therapy. These drugs are rarely needed in the treatment of childhood AIHA.

Patients are selected for immunosuppressive therapy when a clinically unacceptable degree of hemolytic anemia persists following treatment with corticosteroids and splenectomy. Altenatively, they may be corticosteroid resistant or intolerant and a poor surgical risk for splenectomy. Clinical benefit has been noted in about 50% of patients. Dosage of drug should be adjusted to maintain the leukocyte count greater than 4,000, the granulocyte count greater than 2,000, and the platelet count greater than 50,000 to 100,000 per microliter. The use of alkylating agents, such as cyclophosphamide, also may have long-term potential for increasing the incidence of malignancy, particularly acute leukemia. Such side effects require that the clinical indications for an immunosuppressive trial be strong and that patient exposure to the drug be limited.

Immunosuppressive therapy has been effective therapy in cold agglutinin disease. Alkylating agents (cyclophosphamide or chlorambucil) have been used and appear to have a beneficial effect in 50% to 60% of patients.

Transfusion Therapy

Most patients with AIHA do not require transfusion therapy because the anemia is developed gradually and physiologic compensation has occurred (65). Occasional patients experience acute or severe anemia, however, and require transfusions for support until other treatment reduces the hemolysis. Transfusion therapy is complicated by the fact that the blood bank may be unable to find “compatible” blood, usually because of the presence of an autoantibody directed at a core component of the Rh locus, which is present on the erythrocytes of essentially all potential donors, regardless of Rh subtype. The usual recommendation is for the blood bank to identify the most compatible units of blood of the patient's own major blood group and Rh type and to transfuse the patient with the most compatible units available. With this approach, it is unlikely that the donor blood will have a dramatically shortened red blood cell survival.

In cold hemagglutinin disease, it is important to warm all intravenous infusions, including whole blood, to 37°C because a decrease in temperature locally in a vein can enhance the binding of the IgM antibody to red cells and accelerate the hemolytic process. Furthermore, agglutination of the transfused chilled or even room

temperature cells in small peripheral blood vessels can result in severe ischemic changes and vascular compromise.

Miscellaneous Therapy

Intravenous gamma globulin, which has been used extensively in the treatment of ITP, may be effective in treating patients with AIHA, probably by interfering with the clearance of the IgG-coated cells (66). Treatment regimens vary from 400 mg to 2 g per kilogram of body weight daily for 5 days, with additional treatment as needed to maintain the effect. Intravenous gamma globulin appears less effective in AIHA than in ITP (67).

Plasmapheresis or exchange transfusion has been used to treat patients with severe IgG-induced immune hemolytic anemia but has met with limited success, possibly because more than half of the IgG is extravascular and the plasma contains only small amounts of the antibody (most of the antibody being on the red blood cell surface). Plasmapheresis has been effective, however, in IgM-induced hemolytic anemia (cold agglutinin disease) because IgM is a high-molecular-weight molecule that remains predominantly within the intravascular space, and at 37°C, most of the IgM is in the plasma fraction. Plasmapheresis is useful only as short-term therapy, but it may be lifesaving in the rare patient with severe uncontrollable hemolysis. Other measures that have been used effectively in some patients with IgG-induced immune hemolysis are vincristine, vinblastine infusions, and hormonal therapy. For example, there has been interest in the use of the synthetic weak or impeded androgen danazol (68). Interferon therapy and monoclonal antibodies directed at select lymphocyte antigens are in experimental use.

IMMUNE PANCYTOPENIA

Evans syndrome refers to AIHA accompanied by thrombocytopenia (25). It occurs in a small percentage of adults and children with acute AIHA. In an even smaller percentage of patients, it is also associated with marked neutropenia (D.B. Cines, D.S. August, and A.D. Schreiber, personal communication). AIHA in the presence of thrombocytopenia or neutropenia is more commonly associated with a chronic or relapsing course. Many patients have associated disorders, such as chronic lymphadenopathy or dysgammaglobulinemia. Some patients are hematologically normal between relapses, which may involve depressions in any of the three cell lines. Usually, prednisone therapy is effective in controlling the acute episodes and is not needed between relapses. Some patients, however, have persistent immune cytopenia and require prolonged steroid treatment or more aggressive therapy. Splenectomy may result in improvement, but the risk of infection is probably higher in children and adults with pancytopenia than in those with AIHA alone and relapses are more common (25,30,31,60,61,62,63,64,65,66,67,68,69,70,7172,73 and 74). Antibodies directed against red cells, leukocytes, and platelets have been demonstrated in some patients with immune pancytopenia (70,75). Suppression of hematopoietic cell maturation by T cells was demonstrated in one patient and circulating autoantibodies in another (69).

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

Paroxysmal nocturnal hemoglobinuria was first described in the late nineteenth century. It is an acquired disorder initially thought to consist of paroxysms of intravascular hemolysis reflected in nocturnal hemoglobinuria (44,77). It is now recognized that chronic intravascular hemolysis is the more frequent clinical presentation. PNH is a primary bone marrow disorder that not only affects the red cell lineage but also affects the platelet, leukocyte, and pluripotent hematopoietic stem cell lines. It is believed to be a disorder of stem cells of a clonal nature (78,79) and can arise from or evolve into other dysplastic bone marrow diseases, including aplastic anemia, sideroblastic anemia, and myelofibrosis. Rarely, PNH evolves into acute leukemia. A major clue to the cause of this disease was provided by the finding that patients have a somatic mutation in the DNA of the X chromosome coding for a protein (phosphatidylinositol 1 glycan class A, or PIGA) that is important in the pathway that controls the formation of the phosphatidylinositol anchor of various membrane proteins, including the complement control proteins (80). Many different mutations of the *PIGA* gene have been reported.

Although PNH is often a disease of young adults, it can occur in any age group and in persons of either sex (81). Chronic intravascular hemolysis of varying severity is the most common presentation. The severity of the hemolysis and the degree of hemoglobinuria depend on the number of circulating abnormal red cells and the degree of expression of the membrane abnormality among these cells. Two to three populations of abnormal red cells, termed PNH type I, II, and III cells, may be present simultaneously and differ in their expression of GPI-linked proteins and their lytic susceptibility. Patients commonly have iron deficiency anemia as well because of the large amount of iron lost in the urine during intravascular hemolysis with persistent hemoglobinuria and hemosiderinuria. Other frequent clinical complaints include abdominal, back, and musculoskeletal pain. Such pain may be associated with intravascular hemolysis and hemoglobinuria, or it may be ischemic, secondary to the complication of venous thrombosis of major or minor vessels. Thromboses of the hepatic veins (Budd-Chiari syndrome) and of portal, splenic, mesenteric, cerebral, and other veins may occur and are common causes of death. Acute intestinal infarction requiring surgical resection has been reported (82), and thrombotic episodes may require anticoagulant therapy (83). Platelets and leukocytes also appear to have unusual susceptibility to lysis, and thrombocytopenia or granulocytopenia or both may be the initial manifestation(s) of the disease; these are commonly present. The bone marrow is usually hyperplastic, but it may be hypocellular, consistent with aplastic anemia. The clinical course is variable and depends on the occurrence of the life-threatening complications of progressive bone marrow disease or venous thrombosis.

Paroxysmal nocturnal hemoglobinuria should be considered in anyone who has aplastic anemia (84). In general, patients are not predisposed to the development of infection. At least half of these patients live for many years. Diagnosis rests on the clinical picture and the clinical laboratory measurement of a population of circulating cells with unusual sensitivity to complement-mediated lysis. This sensitivity is most readily demonstrated by fluorescence-activated cell sorter (FACS) analysis of DAF, CD59, or other GPI anchored molecules that are defincient in PNH. The deficiency of GPI linked proteins is determined by flow cytometry using monoclonal antibodies. The size of the affected clonal population and the extent of GPI protein deficiency are important determinants of clinical symptoms. PNH also can be diagnosed by positivity in the sugar-water test and in the Ham acidified serum test. In the sugar-water test, the patient's serum is mixed with 5% dextrose in water and incubated with the patient's cells. In PNH, hemolysis ensues. Everyone has antibody molecules that recognize their own cells under conditions of low ionic strength. These antibodies activate the classical complement pathway. Normal erythrocytes resist lysis, but PNH erythrocytes are susceptible to lytic attack. In the Ham test, the patient's cells are incubated in acidified serum. Under these conditions, the alternative complement pathway is triggered and lysis of PNH, but not normal cells, follows. The Ham test result also is positive with some, but not all, normal sera from patients with a syndrome of congenital dyserythropoietic anemia (hereditary erythroblastic multinuclearity with a positive acid-hemolysis test, or HEMPAS).

Pathogenesis

Patients with PNH have an unusual sensitivity of their erythrocytes and often granulocytes and platelets to the lytic action of complement. Activation of complement by either the classical or alternative pathway results in the deposition of larger numbers of complement molecules on the PNH blood cell surface than on normal cells. The excessive binding of complement to blood cells in PNH is due to more efficient alternative-pathway C3-convertase activity on the cell surface (85). The surface of a PNH erythrocyte is a better acceptor for C3 than is the surface of a normal cell. This results in greater activation of the terminal complement components C5 to C9, causing more cell lysis than with normal cells. Furthermore, PNH cells are more effectively damaged by the C5 through C9 complex generated on the erythrocyte surface (86,87) because the C5b through C9 lytic complex penetrates the PNH cell membrane more efficiently than the normal cell membrane.

Patients with PNH lack the complement regulatory proteins present on the membranes of all normal blood cells, thereby causing the increased susceptibility to complement lysis of PNH erythrocytes. The fundamental feature of blood cells in PNH patients is that they are deficient in proteins linked to the membrane by a GPI anchor. DAF and CD59 are the complement regulatory proteins that are GPI linked and that are deficient on hematopoietic cells in patients with PNH. Many patients with PNH have several populations of abnormal erythrocytes. The complement lysis sensitivity test, which examines the susceptibility of antibody-sensitized erythrocytes to complement-mediated lysis, can be used to define the various PNH cell populations. PNH type 1 cells have a moderate increase in susceptibility to complement attack. They commonly express close to normal levels of GPI linked proteins. PNH type 2 cells exhibit decreased expression of GPI linked proteins. These erythrocytes appear to have decreased levels of DAF, but they do not have the membrane deficit (CD59) that leads to sensitivity to attack by the C5b complex. PNH type 3 cells are highly susceptible to complement attack. They appear to lack phosphatidylinositol-linked control proteins completely, lacking both DAF and CD59. As noted, the platelets and leukocytes in PNH are also abnormally sensitive to complement-mediated lysis, and this abnormality is likely to have the same cause (76,88). This may relate to the pathogenesis of the venous thromboses.

Therapy

Hemolysis is controlled with the use of prednisone in some patients. A dose of 15 to 40 mg every other day has been reported to decrease the rate of hemolysis in some adult patients (83), but a response is by no means certain. During acute episodes, a higher dose given daily for a short period may help to control the hemolysis. In patients with anemia, androgens, including the anabolic steroid danazol, may be effective (68,83). Several patients have been able to attain a modest increase in their platelet count while on androgen therapy. Bone marrow transplantation has been successful in some patients, but in general the treatment of PNH has not been satisfactory. Bone marrow transplantation is the only curative therapy for PNH. As discussed earlier, PNH patients may be iron deficient. Acutely, iron replacement may result in increased hemolysis because of the formation and release of a new cohort of sensitive red cells, and hemolysis on iron replacement has been noted. Oral replacement should be used if possible, but parenteral iron therapy may be necessary when iron losses are very large.

DRUG-INDUCED IMMUNE HEMOLYSIS

Drug-induced immune hemolytic anemia may be divided into three primary pathophysiologic entities. The clinical signs and symptoms are identical to those of AIHA. Patients may present with chronic hemolytic anemia or occasionally with catastrophic intravascular hemolysis (quinidine type). The diagnosis is established primarily by history and *in vitro* assay.

a-Methyldopa Type

a-methyldopa and its derivatives (such as levodopa) produce a clinical syndrome virtually identical to IgG-induced immune hemolytic anemia (2,89). This is the most common type of drug-induced immune hemolytic anemia. The mechanisms of the IgG antibody formation are poorly understood. This drug stimulates the production of IgG warm-reactive antibodies with anti-Rh specificity; it also may inhibit the splenic macrophage clearance of the IgG-coated cells. A primary mode of action of the drug in this disorder may be an alteration of immunoregulation, allowing B-lymphocytes that produce Rh antibodies to escape from suppression.

It is of interest that 15% of patients on a-methyldopa therapy develop antinuclear antibodies. Many patients, up to 25%, exposed to methyldopa develop a positive result on Coombs test for IgG. Of diagnostic importance is that almost all patients have IgG antierythrocyte antibodies present in their plasma as well. Most patients do not develop sufficient IgG coating for hemolysis; however, about 0.8% of patients exposed to a-methyldopa do develop significant hemolysis and hemolytic anemia 3 to 37 months after the onset of therapy (2). Diagnosis can be made by examining the patient's red blood cells and plasma. *In vitro*, it is not necessary to have the drug present for the patient's plasma to deposit IgG antibody on donor erythrocytes. The Coombs test result can remain positive in some patients up to 2 years after withdrawal of the drug (90). A similar syndrome has been reported with mefenamic acid.

As stated, many patients receiving a-methyldopa therapy develop a positive result on Coombs test (both direct and indirect) for IgG, but few patients develop significant hemolysis. It appears that the level of IgG per erythrocyte accounts at least in part for this observation because patients with the highest amount of erythrocyte-associated IgG appear to have the most significant hemolysis (2). A second feature, in my view, that may explain the high incidence of Coombs positivity without hemolysis in this syndrome is a low avidity antibody. The striking finding that almost all patients have antibody present in their plasma, as well as on the erythrocyte surface, contrasts with the finding in most other patients with IgG-induced AIHA, in which a positive result on indirect Coombs test (plasma antibody) is less common. Second, it appears that the IgG antierythrocyte antibody can be eluted easily from the erythrocyte surface (90). These observations suggest that a-methyldopa-induced IgG antierythrocyte antibody may be an antibody that has low avidity for its erythrocyte Rh antigen, which may partially explain its inefficiency in producing hemolysis.

Hapten Type

The hapten type of drug-induced immune hemolysis classically develops in patients exposed to high doses of penicillin. A portion of the penicillin molecule or its active metabolites combines with the erythrocyte surface, acting as a hapten. This induces an antibody response directed against the penicillin-coated erythrocyte membrane. This is usually an IgG response, and complement activation is common. The erythrocytes become coated with IgG and often with C3. This syndrome rarely develops unless patients have received 10 to 20 million units of penicillin per day. The diagnosis can be established by incubating the patient's serum with donor erythrocytes preincubated with penicillin. The deposition of IgG antibody will occur only in the presence of penicillin and can be detected by the Coombs test.

Quinidine Type

The quinidine type of AIHA usually occurs with quinidine, but it has been reported with quinine, stibophen, chlorpromazine, and sulfonamides (90). Commonly called an “innocent bystander” reaction, it is thought to be due to an antibody directed against quinidine having a low affinity for the red cell surface. Presumably, the drug binds weakly to the cell glycoprotein. The antibody recognizes the complex (91). This interaction results in activation of the classical complement pathway and deposition of C3 on the erythrocyte surface. It is believed that the immune complex transiently adheres to the red blood cell surface, activates complement, and then dissociates. With quinidine, an IgM antiquinidine antibody appears to be involved. The diagnosis can be established *in vitro* by examining the complement deposition on donor erythrocytes by patient serum, which occurs only in the presence of the drug, for example, quinidine. The Coombs test is used to detect the complement deposition on the erythrocyte surface.

Nonspecific coating of the erythrocyte surface has been observed with the antibiotic cephalothin, in which cephalothin becomes bound to the erythrocyte membrane and causes the red blood cell to be coated by many plasma proteins. The Coombs test result is positive. Hemolytic anemia does not occur. Cephalothin, however, can cause hemolytic anemia by acting as a hapten by a mechanism similar to that of penicillin. In all these drug-induced processes, patients respond to withdrawal of the offending drug. If necessary, a brief course of corticosteroid therapy can be administered effectively.

Many autoimmune or drug-related hemolytic anemias are accompanied by thrombocytopenia or neutropenia as a result of similar pathophysiologic processes. Postinfectious ITP also may result from similar mechanisms. The role of complement in antibody-mediated destruction of platelets or neutrophils seems to be analogous to its role in erythrocyte destruction. Thus, at low levels of antibody, complement accelerates clearance by the reticuloendothelial system, whereas at high levels of antibody, complement may cause direct intravascular cell lysis.

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58 IMMUNE THROMBOCYTOPENIA

Russell E. Ware, M.D., Ph.D.

Circulating blood platelets play a critical role in the body's ability to achieve overall hemostasis. Platelets contribute to hemostasis after vascular injury in several ways, including adhesion to the subendothelium and extracellular matrix; aggregation at the site of vessel injury; participation in the coagulation cascade via receptors for coagulation factors; and involvement in the final clot that contains fibrin, erythrocytes, and platelets. Because platelets are integrally important for overall hemostasis, pathologic conditions that reduce their number or function are often clinically significant.

In contrast to thrombocytopenia that results from underproduction of platelets, immune-mediated thrombocytopenia is characterized by normal or increased thrombopoiesis but accelerated platelet destruction. Antiplatelet antibodies bind to platelets, which then are cleared by the reticuloendothelial system (RES). This disorder was formerly known as *idiopathic thrombocytopenic purpura*, but it is now better named *immune thrombocytopenic purpura (ITP)* in recognition of the antibody-mediated platelet destruction. In most cases, ITP is an autoimmune disorder characterized by isolated immune-mediated thrombocytopenia. ITP also can be associated with other clinical conditions, however, including pregnancy, systemic autoimmune disorders, immunodeficiency states, or drug exposure.

HISTORICAL FEATURES

Fifty years ago, Harrington et al. provided the first evidence for antibody-mediated platelet destruction by describing a circulating antiplatelet factor in adults with ITP (1). This factor, isolated from the gamma-globulin fraction of serum in most of the patients tested, was infused into normal volunteers (including Dr. Harrington himself) and induced severe but reversible thrombocytopenia. Subsequent work demonstrated that this factor was in the 7S immunoglobulin G (IgG) serum fraction, was species specific, and affected both autologous and homologous platelets (2). Twenty-five years later, antibodies were first quantitated accurately on the platelets of patients with ITP (3), an event that represented a great advance in our understanding of the pathophysiology of this disorder. Based on these results, ITP could be considered the platelet equivalent of antibody-mediated erythrocyte destruction that was well described in the setting of autoimmune hemolytic anemia. Over the past 25 years, we have begun to unravel slowly the humoral and cellular responses that lead to antiplatelet antibody formation.

Therapy for ITP was available long before immunopathologic mechanisms involved in antibody-mediated platelet destruction were understood. Splenectomy was first used successfully in ITP almost a century ago. After the immune basis of the disorder was established, corticosteroid therapy was introduced in the 1950s with success. Intravenous immunoglobulin (IVIG) became popular in the 1980s for the treatment of ITP following serendipitous observations in the setting of immunodeficient children with ITP (4). More recently, anti-D immunotherapy has emerged as a novel and effective therapeutic option for children and adults with ITP.

IMMUNOLOGIC HIGHLIGHTS

The primary immunologic defect in ITP involves autoreactive B-lymphocytes that secrete antiplatelet antibodies, but the role of T-lymphocytes (and other immune cells that orchestrate the B-cell immune response) in the pathophysiology of ITP has begun to emerge. The observation that the clinical course of ITP can range from brief and self-limited to long-term and unremitting, coupled with the widely variable responses to therapy, suggests that ITP may in fact be several heterogeneous immunologic disorders that culminate in a final common pathway of immune-mediated thrombocytopenia.

Antiplatelet antibodies, which are typically IgG, bind to budding platelets in the bone marrow and to circulating platelets in the peripheral blood. Antibody coating of platelets is not immediately cytolytic, but it leads to immune clearance of these “opsonized” platelets by the RES. Therapy can be directed against autoantibody synthesis, antibody–platelet interactions, or platelet clearance by immune effector cells.

EPIDEMIOLOGY

Acute

The patient with ITP will have either a brief self-limited illness known as *acute* ITP or will develop a long-term disorder termed *chronic* ITP. Acute ITP occurs primarily in childhood and can be defined as resolution of thrombocytopenia within 6 to 12 months of onset, although most patients improve within 3 months (5). About 80% to 90% of children who develop ITP will have an acute clinical course compared with only 5% to 10% of adults. The clinical presentation of acute ITP often begins with a virallike prodrome, followed several weeks later by the abrupt onset of hemorrhagic symptoms. Patients and their families are often extremely anxious and frightened because spontaneous large bruises (purpura) can develop on all parts of the body, along with showers of petechiae and mucosal bleeding. The peripheral platelet count is typically extremely low, frequently below 10×10^9 per liter, but the hemoglobin concentration, white blood cell count, differential, and peripheral blood smear are within normal limits except for decreased numbers of platelets. There is usually no family history of autoimmune disease.

Chronic

In contrast, patients with chronic ITP usually have a more insidious clinical presentation. The preceding history does not include a viral-like prodrome, but often several months of easy bruising and sporadic petechiae; dramatic spontaneous hemorrhagic signs and symptoms are atypical. The peripheral platelet count is modestly low, usually between 20 and 50×10^9 per liter, and the remainder of the blood count is normal. Most patients with chronic ITP are adults, and the incidence increases with age (6), although chronic ITP is also seen in children of all ages, even toddlers. Many patients with chronic ITP have a family history of autoimmune disease. Despite these apparent clinical and laboratory differences, there are frequently overlapping presentations between acute and chronic ITP. At the time of clinical presentation, no single parameter can reliably and accurately predict the clinical course of a patient with ITP. Table 58.1 summarizes some of the typical clinical and laboratory findings in patients with acute versus chronic ITP.

Parameter	Acute ITP	Chronic ITP
Age (yr)	<10	>10
Sex	M = F	F > M
Onset of symptoms	Abrupt	Insidious
Viral prodrome	Yes	No
Platelet count	<20 x 10^9/L	20-50 x 10^9/L
Platelet associated IgG	Markedly elevated	Modestly elevated
Family history of autoimmune disease	No	Occasionally
Other immunologic abnormalities	No	Occasionally

IgG, immunoglobulin G.

TABLE 58.1. Differentiation of Acute and Chronic Immune Thrombocytopenic Purpura (ITP) at Clinical Presentation

CLINICAL PRESENTATION

A variety of pathologic conditions result in thrombocytopenia, and each must be excluded before establishing the diagnosis of ITP. Sepsis, immunodeficiency, drug reactions, malignancy, disseminated intravascular coagulation, bone marrow failure, and systemic inflammatory or collagen-vascular disorders can have concurrent thrombocytopenia. ITP is therefore often a diagnosis of exclusion, which is based frequently on a compatible clinical history, appropriate physical examination, supportive laboratory tests, and the absence of a systemic illness. Queries regarding medication use, rheumatologic signs and symptoms, family history, and past medical history are necessary to establish the diagnosis of ITP.

Signs and Symptoms

The hallmark of ITP is a low platelet count, and typical signs and symptoms reflect the degree and duration of thrombocytopenia. Most patients with acute ITP describe the abrupt onset of bruises that seem “spontaneous” or inappropriately large for any preceding contact or trauma. Patients also describe 1- to 2-mm evanescent “red dots” (*petechiae*) that develop on the skin at pressure locations, such as those touched by elastic bands in clothing. Mucosal bleeding also can occur; nasopharyngeal bleeding (nares, gingiva) is frequently described, and for menstruating female patients, metromenorrhagia is a common presentation. More severe bleeding, such as gastrointestinal blood loss or intracranial hemorrhage, is rare in ITP.

Physical Findings

A careful physical examination is an important part of the initial evaluation of a patient with presumed ITP. Hemorrhagic symptoms in ITP are primarily mucocutaneous, characteristic of thrombocytopenic bleeding, rather than muscle and joint bleeding more typical of coagulation factor deficiency. Bruises can develop on all parts of the body in ITP, and large ecchymoses on the trunk of small children can lead to the suspicion of child abuse (Fig. 58.1). Petechiae are commonly found on the trunk, extremities, and even in the oropharynx. Mucosal bleeding usually is limited to the nasopharynx and gingiva; frank bleeding from the genitourinary or gastrointestinal tract is much less common. No lymphadenopathy or organomegaly suggestive of malignancy should be found; specifically, palpable splenomegaly is almost never found in ITP and should suggest an alternative diagnosis.

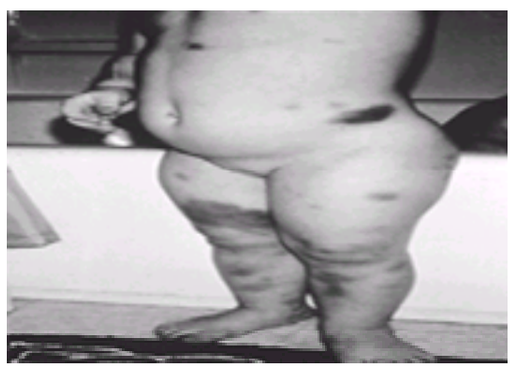

Figure 58.1. Large bruises (purpura) on the trunk and extremities in a young child with immune thrombocytopenia. (See Color Figure 58.1)

Laboratory Tests

There is no true consensus regarding the optimal initial laboratory evaluation for a patient with ITP (7), although most clinicians agree that testing need not be extensive. A complete blood count is necessary to document thrombocytopenia and to exclude abnormalities in other hematopoietic cell lines. The peripheral blood smear should reveal platelets that are larger than normal (Fig. 58.2), which is indicative of their young age and is due to rapid turnover. Extremely small platelets are more suggestive of the Wiskott–Aldrich syndrome. Leukocytes must be inspected carefully to exclude circulating malignant cells. Erythrocytes should be normal-sized and hemoglobin content; macrocytosis [e.g., mean cell volume (MCV) >100 fL] is more consistent with bone marrow failure syndromes such as aplastic anemia, Fanconi anemia, or paroxysmal nocturnal hemoglobinuria. In addition, the red cells should not show evidence of traumatic rupture (schistocytes) characteristic of thrombotic thrombocytopenic purpura (TTP).

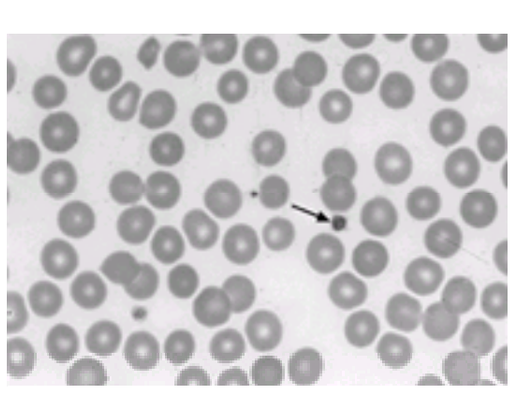

Figure 58.2. Peripheral blood smear of a patient with immune thrombocytopenia. Few platelets are present, but those that are newly released into the circulation (*arrow*) are larger than the normal diameter of 1 to 2 µ. (See Color Figure 58.2.)

Measurement of the erythrocyte sedimentation rate or antinuclear antibody (ANA) titer is often recommended in the initial laboratory evaluation as a screen for a systemic inflammatory or autoimmune disorder. In our experience, testing for antibodies to dsDNA or extractable nuclear antigens is indicated only if the ANA is positive (8). Quantitation of serum immunoglobulins and testing for exposure to the human immunodeficiency virus should be considered if there is a concern about immunodeficiency. Such testing always should be performed before treatment with immunoglobulin products.

The importance and clinical utility of measuring platelet antibodies is a subject of debate. Quantitation of platelet antibodies is frequently available only at selected institutions, and the technique varies among investigators. Unlike the analogous red blood cell system, in which autoantibodies can be identified readily using

agglutinating antisera, platelets often undergo spontaneous and nonspecific agglutination and aggregation. Direct assays using murine monoclonal antibodies that react with human IgG should be used to detect platelet-associated immunoglobulin (PAIgG). These direct-binding assays demonstrate that normal platelets have approximately 500 to 1,000 molecules of IgG on their surface (9). Using accurate direct-binding assays, the presence of surface-bound antiplatelet antibodies appears to be a sensitive but nonspecific marker for ITP (3,10). About 90% of patients with ITP will have elevated PAIgG (high sensitivity), but no more than 50% of patients with elevated platelet antibodies will have ITP (low specificity). Patients with other pathologic conditions such as collagen-vascular disease, malignancy, and infection also can have elevated amounts of PAIgG, which is consistent with the hypothesis that platelet-bound antibodies are at best a marker for immune-mediated platelet destruction of any type, and are clearly not specific for ITP.

The necessity of performing a bone marrow evaluation to assist in the diagnosis of ITP is sometimes an area of controversy, especially in children with a classic presentation. Examination of aspirated cells should reveal normal to increased numbers of megakaryocytes, reflecting increased marrow thrombopoiesis. The megakaryocytes are often young in nuclear appearance and maturation, however, and may not demonstrate budding platelets because of the destruction of platelets within the marrow. The primary reason for bone marrow evaluation in ITP is to exclude other diagnoses, such as acute leukemia, bone marrow failure syndromes, or myelodysplasia. Clues for these other diagnoses are often apparent on history and physical examination as well as on the peripheral blood smear, such as macrocytosis, neutropenia, or leukemic blasts. Although it can be argued that bone marrow aspiration is an unnecessary adjunct for the patient with a history, physical examination, and blood smear compatible with a diagnosis of ITP, many clinicians perform this relatively innocuous procedure to provide assurance for the patient and family that cancer is not present and to allow the therapeutic use of corticosteroids. In addition, bone marrow biopsy in selected cases can help distinguish ITP from myelodysplasia, aplastic anemia, or other platelet-production problems.

Pathology

Figure 58.1 shows large bruises on the trunk and extremities in a young child with ITP, and Fig. 58.2 shows a peripheral blood smear of a patient with ITP. Few platelets are present, but those that are newly released into the circulation are larger than the normal 1- to 2-μ diameter.

Therapy and Prognosis

Patients with acute ITP by definition have a brief, self-limited illness that spontaneously resolves within several months. Although patients may have severe thrombocytopenia and substantial mucocutaneous bleeding that is cosmetically displeasing, clinically significant or life-threatening hemorrhage is quite rare. Intracranial hemorrhage is the most serious bleeding complication of ITP, but it occurs in fewer than 1% of patients. The likely explanation for this apparent paradox is that, compared with normal platelets, circulating platelets in ITP are younger, larger, and more efficient at repairing vessel injury. Therapy for acute ITP should therefore either be limited to observation alone or designed to prevent severe hemorrhagic events when the platelet count is extremely low ($<10 \times 10^9$/L). Therapy for chronic ITP, in contrast, should be designed not only to prevent severe hemorrhage but also to induce immunologic remission, if possible. Many treatment modalities are effective in the treatment of ITP, although each has potential risks and benefits (11). Table 58.2 summarizes the most frequently used therapeutic options for the management of ITP.

Treatment	Dose	Comments
Corticosteroids	2 mg/kg/d p.o.	Short-term use preferred Sometimes useful in chronic ITP
Intravenous immunoglobulin	0.5–1.0 g/kg/d i.v. x 2	Rapid rise in platelet count Expensive, inconvenient
Splenectomy	—	Curative in 70% of patients Risk of postsplenectomy sepsis
Anti-D	50 μg/kg/d i.v. x 1	Rh-positive nonsplenectomized patients Less expensive, more convenient than IVIG
Azathioprine	50–200 mg/d p.o.	Immunosuppression
Cyclophosphamide	50–150 mg/d p.o.	Carcinogenesis
Danazol	200–800 mg/d p.o.	Hepatic dysfunction Androgenic side effects
Vincristine	1 mg/m² wk i.v.	Neurotoxicity
Platelet transfusions	Pooled or pheresis	Emergent, life-threatening bleeds Short responses, alloimmunization

i.v., intravenous; IVIG, intravenous immunoglobulins; p.o., orally.

TABLE 58.2. Treatment Modalities for Immune Thrombocytopenic Purpura (ITP)

Corticosteroids frequently are prescribed for the treatment of patients with ITP, although great controversy surrounds their routine or prolonged use. Randomized prospective trials demonstrated that a short oral course (2–3 weeks) of prednisone or prednisolone at 2 mg per kilogram of body weight daily will increase the platelet count in children with ITP faster than no treatment (12,13), and “megadoses” of intravenous methylprednisolone (30 mg/kg/dose) also have been reported to be beneficial (14). Corticosteroids should not be prescribed for more than 4 to 6 weeks' duration, however, because of the many well-described complications of long-term steroid therapy. Low-dose alternate-day corticosteroid usage is typically unable to sustain a reasonable platelet count in patients with ITP. Initial excitement about high-dose pulse dexamethasone for ITP (15) has faded, however, with additional reports of only modest efficacy. Possible mechanisms of action of corticosteroids in ITP include interference with antibody–platelet interactions with decreased antibody binding and increased serum antibody levels (Fig. 58.3), decreased platelet phagocytosis by the RES (5,16), improved capillary integrity with less vascular endothelial leakage (17), and diminution of platelet autoantibody production by splenic and bone marrow lymphocytes (18).

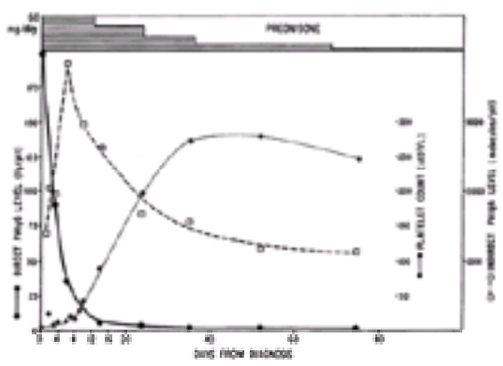

Figure 58.3. Serial platelet counts and platelet-associated immunoglobulin G (PAIgG) levels in a young male patient with acute immune thrombocytopenia purpura. He was treated with oral corticosteroids at high dose for 2 weeks and then slowly weaned. His platelet count exceeded 100×10^9 per liter after 3 weeks of therapy. Serial measurements of PAIgG levels demonstrated that the direct PAIgG rapidly normalized, whereas the indirect PAIgG was initially normal, temporarily increased in the first week, then returned to normal levels.

Since the initial report almost two decades ago by Imbach and co-workers (4), intravenous immunoglobulin (IVIG) has become a popular therapeutic option for ITP. IVIG will increase the platelet count in either acute or chronic ITP (19,20), typically within several days of treatment. Comparisons of IVIG with corticosteroids in acute ITP suggest similar response rates, although IVIG may increase the platelet count to >30 x 10^9/L slightly faster than corticosteroids or observation alone (21). In chronic ITP, IVIG can be given every 3-4 weeks as a maintenance therapy (20). The typical therapeutic dose of IVIG is 1 g per kilogram of body weight, given either as a single dose or on two consecutive days (22). Mechanisms of action for IVIG in ITP include RES blockade with saturation of macrophage Fc receptors and survival of antibody-coated platelets (23), interference with platelet clearance by decreasing Fc receptors and their affinity for IgG (24), interference of antibody binding to platelets (25), and suppression of autoantibody synthesis by antiidiotype antibodies (26). Despite its efficacy, IVIG has several drawbacks, including its expense, long infusion time, frequent side effects (headache, nausea, and fever), and risk of transmission of an infectious agent. Neurologic complications have been recognized as frequent side effects of IVIG therapy (27); renal failure also may occur, perhaps related to the sucrose load and osmolality of the product (28,29). As of September 1999, the United States Food and Drug Administration MEDWATCH program has received 114 adverse event reports of renal dysfunction or acute renal failure associated with IVIG administration, including 17 deaths.

More recently, anti-D therapy has become available for the treatment of ITP. Anti-D antibodies recognize the Rhesus (Rh) D erythrocyte antigen and are commonly used to prevent erythrocyte sensitization following pregnancy in an Rh-negative mother. In ITP, anti-D is used to coat the patient's own D-positive erythrocytes, thereby

inducing temporary RES blockade similar to IVIG. Unlike IVIG, however, anti-D does not appear to have other mechanisms of action on the immune system (30). The use of anti-D is limited to ITP patients who are Rh positive (85% of the population) and those who have not undergone prior splenectomy; antibody-coated erythrocytes evidently require functioning splenic tissue to induce RES blockade (31). Commercially available anti-D licensed for the treatment of ITP (WinRho SDF manufacturer: Cangene Corp., Winnipeg, Canada, distributor: Nabi, Boca Raton, Florida) is produced from a limited number of hyperimmunized human volunteers. Anti-D is administered intravenously at 50 µ per kilogram of body weight per dose over 2 to 5 minutes. Platelet response is maximal about 5 days postinfusion and lasts for 3 to 4 weeks (32). The primary side effect of anti-D infusion, which is both predictable and necessary, is mild erythrocyte hemolysis that manifests as hemoglobinuria for 12 to 36 hours and an eventual 1 to 2 g per deciliter drop in hemoglobin concentration. The MEDWATCH program, however, has collected 15 cases of intravascular hemolysis following anti-D infusion that caused clinically compromising anemia and renal insufficiency, including four deaths.

Surgical splenectomy in ITP has the theoretical benefit of eliminating both the main site of autoantibody production as well as the primary site of platelet clearance and destruction. Great care must be taken during splenectomy to ensure that all accessory spleens are removed; this is a particularly important consideration when a laparoscopic procedure is performed. Splenectomy has no role in the management of the child with acute ITP unless an intracranial hemorrhage has occurred; in this setting, emergency splenectomy should be performed with platelets transfused after the splenic pedicle is clamped. In chronic ITP, splenectomy should be considered when other therapeutic alternatives have become unsafe, inconvenient, or ineffective. Splenectomy is curative for only about 70% of children with ITP, however, and it leaves the recipient at a low but theoretical risk for postsplenectomy sepsis. For this reason, children are generally prescribed twice-daily penicillin following splenectomy. Although there is currently no accurate method of predicting response to splenectomy, identification of the spleen as the site of sequestration may predict the efficacy of splenectomy (33). The recent observation that persons who respond to IVIG are more likely to benefit from splenectomy (34) has now been called into question (35). The appropriate timing of splenectomy, especially for adults with ITP, should be determined on an individual basis with consideration of therapeutic responses to IVIG and anti-D therapies.

A variety of alternative therapeutic agents usually are reserved for patients with ITP refractory to conventional therapy, particularly those who have no benefit from splenectomy. Azathioprine (daily oral dose, 50–200 mg) can decrease autoantibody production and perhaps interfere with macrophage function. Because the beneficial effects of azathioprine may require 3 to 6 months of therapy, it is often given in combination with other agents. Cyclophosphamide is prescribed at 50 to 150 mg orally per day, with dose adjustments based on bone marrow suppression. Danazol, a semisynthetic androgen, can be effective when oral doses of 200 to 800 mg per day are used; limiting toxicities include hepatic dysfunction and masculinizing side effects. Vincristine (1 mg/m^2/week) can be used for rapid but short-lived responses, especially when waiting for other chemotherapy to take effect. Because vinca alkaloids bind to tubulin within the platelets, they can be incubated with allogeneic platelets prior to a transfusion. Such “vinca-loaded” platelets are effective presumably by delivering the cytotoxic drug directly to the RES. The use of multiagent chemotherapy should be reserved for severe refractory cases. Additional agents that have limited or anecdotal reports of efficacy include dapsone, cyclosporin A, and a-interferon. In certain settings, splenic irradiation may be beneficial (36).

Platelet transfusions in ITP should be limited to severe hemorrhage (e.g., intracranial hemorrhage) and other life-threatening clinical situations but can be effective in increasing the platelet count temporarily (37). Circulating antiplatelet antibodies will bind to transfused platelets and lead to their rapid clearance by the RES but may provide temporary protection against bleeding. A dose of IVIG (0.5–1.0 g/kg) prior to the platelet transfusion can blockade the RES and lead to improved survival of the transfused platelets (22). In addition, administration by continuous infusion may prolong the *in vivo* life span of the transfused platelets.

The prognosis for patients with ITP is generally good. For children with ITP, the vast majority will have a self-limited illness with no sequelae. Clinical relapses following resolution of acute ITP are extremely rare and suggest an underlying immunodeficiency or systemic autoimmune disorder. Direct PAIgG levels typically return to normal following acute ITP (38), and women with previous childhood ITP only rarely deliver infants with thrombocytopenia (39). Some patients with chronic ITP have gradual improvement in their platelet count to levels that do not require therapy. For these patients as well as those who achieve remission after successful splenectomy, the PAIgG level also normalizes (38). Long-term follow-up data are scant, but one series of adults with ITP reported a disease-free survival of 83% following splenectomy (40). Some patients believed to be in “remission” actually may have a well-compensated state of antiplatelet antibody production, accelerated platelet destruction, but increased platelet production.

IMMUNOPATHOLOGY

Humoral Response

The platelet surface contains numerous glycoproteins (gp) that can act as autoantigens, but the absence of circulating antiplatelet antibodies in many cases of ITP has hindered their identification and characterization. Some patients have plasma antibodies that react with the gp IIb/IIIa complex (41), and others have circulating antibodies against the gp Ib/IX complex (42), whereas a few have antibodies that react with both glycoproteins (43). Using platelet eluates to release platelet-bound IgG, however, van Leeuwen et al. found that 35 of 42 patients with ITP had antibodies reactive with normal platelets but not with platelets lacking IIb/IIIa (44), confirming that the IIb/IIIa complex is the most common autoantigen in ITP. Epitope mapping of anti-IIb/IIIa antibodies in chronic ITP has suggested reactivity primarily against the cytoplasmic (intracellular) domain (45) as well as a requirement for the cation-dependent intact conformation of IIb/IIIa (46). Newer methods of antiplatelet antibody detection may improve the identification of specific platelet autoantigens in ITP and help our understanding of how plasma antiplatelet antibodies bind to platelet antigens and effect immune clearance.

Antiplatelet antibody production in ITP occurs primarily within the spleen. Splenic leukocytes from ITP patients produce more than five times the IgG of normal persons, with sufficient platelet-directed antibody to coat all estimated antigen sites (47). The frequently curative effect of splenectomy provides additional evidence for splenic antibody production because the PAIgG level normalizes in pediatric patients who enter remission following splenectomy (38). Not all patients respond to splenectomy, however, and so alternative sites of antibody production must exist, such as the liver, bone marrow, and perhaps even peripheral lymphoid tissue. Accessory spleens, which are present in 10% to 20% of persons, can cause a relapse of ITP.

In addition to surface-bound IgG, increased amounts of complement proteins C3 and C4 also have been identified on platelets in ITP (48). IgG synthesized *in vitro* by splenic leukocytes from patients with ITP can fix complement (49); so presumably antiplatelet antibodies can bind to the platelet surface and fix complement through the classic pathway of complement activation. Platelet complement is sometimes identified without elevated PAIgG, however, and also in nonimmune thrombocytopenic states (50), making the origin of the platelet-bound complement still open to debate. Once bound, it is possible that complement contributes to platelet destruction because macrophages possess C3b receptors that can accelerate platelet clearance. In the analogous system of immune-mediated erythrocyte destruction, the presence of surface-bound complement markedly increases phagocytosis of IgG-coated erythrocytes. Platelet-bound complement also could lead to clearance by hepatic or marrow macrophages, perhaps accounting for the failure of splenectomy to be curative in all cases.

Similarly, the role of immune complexes in ITP has not been clearly delineated. Antigen–antibody complexes can attach to platelets nonspecifically and lead to immune-mediated clearance of platelets as “innocent bystanders” (51). Particularly in acute ITP, which often has a preceding virallike prodrome, a normal immune response to the pathogen could inadvertently damage the platelet either directly or through immune complex deposition. Alternatively, antibodies against the pathogen may cross-react with a platelet antigen (molecular mimicry) and lead to immune-mediated clearance.

Cellular Response

Although the primary immunologic defect in ITP likely involves autoreactive B-lymphocytes that secrete platelet antibodies, a lack of self-tolerance by T-lymphocytes and other cells that orchestrate the antibody response also may be important in the pathogenesis of this disorder. A variety of immunoregulatory defects involving cellular immunity have been described in ITP, including abnormal cell-mediated proliferative responses to mitogens and platelets, a diminished autologous mixed lymphocyte reaction, and decreased suppressor cell number and activity (52). Abnormal T-lymphocyte subpopulations in some patients with ITP also have been reported, including an inverted CD4/CD8 ratio, the presence of circulating $CD4^+/CD8^+$ T-lymphocytes, and elevated numbers of class II major histocompatibility complex (MHC)-positive activated T-lymphocytes (53). Increased numbers of oligoclonal T-lymphocytes have been identified in patients with ITP (54), suggesting a dysregulation of lymphocyte responses. Finally, the significant overrepresentation of human leukocyte antigen (HLA) DRB1*0410 in Japanese patients with ITP (55) supports the hypothesis that genetically determined immune responses are important in this disorder.

A possible mechanism by which T-lymphocytes could mediate platelet destruction is by enhancing autoantibody synthesis. Antiplatelet reactivity mediated by $CD4^+$ T-helper lymphocytes in adult patients with chronic ITP has been reported (56). Furthermore, the subpopulation of $CD4^+$ lymphocytes known as the *suppressor-inducer subset* was found to be quantitatively reduced in those ITP patients who demonstrated antiplatelet T-cell proliferative responses. Reduced numbers of suppressor-inducer T-lymphocytes were previously reported in other autoimmune diseases, including systemic lupus erythematosus, Sjögren syndrome, rheumatoid arthritis, and progressive multiple sclerosis. We established T-cell clones from patients with ITP that demonstrated proliferative reactivity against allogeneic platelets (57); six clones were $CD4^+$ helper inducer and two were $CD8^+$ lines.

Other cellular responses that may play an important role in the platelet destruction of ITP relate to cytokine production. Children with chronic ITP have been reported to have increased serum levels of interleukin-2 (58) as well as interferon-g and interleukin-10 (59). Cytokine-mediated g-interferon production can lead to class II MHC expression on platelets (59,60), thereby contributing to the autoimmune process. Elevated levels of macrophage colony-stimulating factor in ITP patients also have been reported (61), suggesting that soluble cytokines may influence macrophage-mediated platelet destruction. Taken together, these data suggest that cellular

responses may be important in the pathogenesis and pathophysiology of ITP.

Immune-mediated Platelet Destruction

Platelet clearance in ITP occurs following an interaction between the IgG- or complement-coated platelets and the RES. Macrophages within the RES recognize the coated platelets through either their Fc or C3b receptors and presumably destroy the platelets by direct phagocytosis. Although radiolabeled platelet studies in ITP have documented uptake by the spleen, thorax (representing bone marrow and pulmonary macrophages), liver, and indeterminate areas (possibly representing peripheral lymphoid tissue), the spleen is believed to be the most important site of platelet clearance. The large numbers of platelets that normally reside within and circulate through the spleen are in intimate contact with a high concentration of antiplatelet antibody and splenic macrophages. Coupled with the slow blood flow within splenic tissue, these factors make the spleen an extremely potent milieu for immune-mediated platelet clearance and destruction.

Immunopathogenesis of ITP: Summary

Patients with acute ITP have a transient disturbance of the immune system, possibly involving molecular mimicry, characterized by the synthesis of antibodies that bind to autologous platelets. These antibodies may be in the form of immune complexes, but they appear to recognize platelet glycoprotein epitopes. In chronic ITP, there is a fundamental abnormality in the humoral and cellular compartments that allows auto reactive T-lymphocytes to proliferate and B-lymphocytes to produce antiplatelet antibodies. In both acute and chronic ITP, the presence of IgG on the platelet surface (with or without complement) leads to rapid platelet clearance by macrophages within the spleen and other parts of the RES.

IMMUNE THROMBOCYTOPENIA IN OTHER CLINICAL SETTINGS

Although ITP is, by definition, a hematologic disorder that occurs in the absence of other systemic illnesses or identifiable etiologies, several clinical conditions are frequently associated with immune-mediated thrombocytopenia. These variants of ITP are listed in Table 58.3 and are discussed in the following sections.

Pregnancy
Maternal vs gestational ITP
Neonatal alloimmune thrombocytopenia
Autoimmune disorders
Systemic lupus erythematosus
Thyroiditis
Inflammatory bowel disease
Immunodeficiency
Congenital
Acquired
Drug exposure
Antibiotics
Anticonvulsants
Quinidine/quinine
Heparin
Alloimmunization
Platelet transfusions
Posttransfusion purpura
Miscellaneous
Sepsis
Malignancy
Preeclampsia

TABLE 58.3. Clinical Settings Frequently Associated with Immune-Mediated Thrombocytopenia (ITP)

Pregnancy

The diagnosis and management of immune-mediated thrombocytopenia during pregnancy can be challenging. In many cases, a mother has no previous history of ITP but develops thrombocytopenia in the second or third trimester of pregnancy. Termed *gestational thrombocytopenia* or *gestational ITP*, this condition is benign. Gestational ITP is only rarely associated with severe fetal thrombocytopenia (62) and should be managed conservatively.

The term *maternal ITP* usually is reserved for women with previously documented ITP who become pregnant. The mother is a potential source of antiplatelet antibodies, which can cross the placenta and induce immune-mediated thrombocytopenia in the developing fetus. A mother with previous acute ITP (perhaps as a child with complete resolution) has low risk of problems during pregnancy (39). As noted earlier, circulating antiplatelet antibodies do not persist in women with a history of acute ITP (38).

A woman with chronic ITP who becomes pregnant has a higher risk of fetal thrombocytopenia. Unfortunately, the maternal platelet count is not always predictive of the fetal platelet count. High circulating levels of maternal antiplatelet antibodies (not platelet-bound) may identify infants at risk for transplacental passage of antibody (63,64). Additional risk factors for fetal thrombocytopenia include previous splenectomy, severe maternal thrombocytopenia during pregnancy, and a previous child born with thrombocytopenia (65,66). Direct measurement of the fetal platelet count by scalp-vein sampling is not always possible before the onset of labor, and percutaneous umbilical blood sampling carries a risk of fetal bleeding and induction of labor. The clinical management of maternal ITP remains controversial, therefore, although most clinicians no longer recommend routine elective cesarean section to avoid fetal head trauma during vaginal delivery. If maternal thrombocytopenia is severe, antenatal corticosteroid therapy and even splenectomy have been used, as has IVIG, which crosses the placenta and presumably induces fetal RES blockade. Following delivery of an affected infant, therapy is typically unnecessary but can include corticosteroids or IVIG.

A related condition known as *neonatal alloimmune thrombocytopenia* occurs when maternal and paternal platelet antigens differ substantially at Br (gpIa), Bak (gpIIb), or PL^A (gpIIIa) epitopes. Presumably as a result of small fetomaternal hemorrhages, the mother becomes sensitized to “foreign” paternal antigens expressed on fetal platelets and develops antiplatelet alloantibodies that can cross the placenta and destroy fetal platelets. Severe thrombocytopenia can result in intracranial hemorrhage, and, unlike the analogous erythrocyte Rh antigen incompatability and sensitization, first-born infants can be affected (67). Effective therapy traditionally includes the postnatal transfusion of washed maternal platelets into the infant, but the efficacy of antenatal or postnatal IVIG in this setting also has been described.

Systemic Autoimmune Disorders

Mild thrombocytopenia (50–100 × 10^9/L) in patients with systemic autoimmune or inflammatory diseases such as thyroiditis, primary biliary cirrhosis, or inflammatory bowel disease is well recognized but usually is not associated with hemorrhagic symptoms. The precise prevalence of thrombocytopenia in these clinical settings is not known, but one study found 21 of 295 patients with systemic lupus erythematosus (SLE) to be affected (68).

The pathophysiology of thrombocytopenia in patients with systemic autoimmune disorders typically has been ascribed to generalized immune dysregulation because multiple polyclonal autoantibodies against platelets, leukocytes, and erythrocytes can be detected. Platelet autoantigens may differ in SLE or ITP compared with classic ITP (69), with a higher prevalence of antibodies reactive with the platelet protein vinculin (70). Therapy specific for the thrombocytopenia is not usually necessary, although IVIG can be beneficial in this setting. Treatment should be reserved for severe thrombocytopenia or active bleeding.

Immunodeficiency

Patients with congenital immunodeficiency are at risk for the development of immune-mediated thrombocytopenia, presumably resulting from improper regulation of their immune system. We recently described two children with unsuspected common variable immune deficiency who developed ITP characterized by an acute but relapsing course (71). Treatment of ITP in immunodeficient patients is complicated by the presumed need to avoid immunosuppressive therapy.

Acquired immunodeficiency also is associated with thrombocytopenia and can occur in the settings of antineoplastic chemotherapy, chronic lymphocytic leukemia, bone marrow transplantation, or infection with the human immunodeficiency virus (HIV). Immune mechanisms of platelet destruction in HIV and ITP may differ substantially from those described for classic ITP in that higher levels of PAIgG and complement components are present on the platelets of patients with HIV–ITP (72). Autoantibody specificity is not different in HIV–ITP, but the presence of many platelet-bound immune complexes may contribute to the pathogenesis of this disorder. Direct infection of megakaryocytes by HIV may also be important in the pathogenesis of thrombocytopenia (73). Treatment for HIV–ITP usually includes corticosteroids and IVIG but has not been entirely satisfactory; the thrombocytopenia of HIV–ITP may respond best to antiretroviral therapy such as zidovudine (74). Beneficial reports of ainterferon, anti-D, anti-CD16 monoclonal antibody, and splenectomy for HIV–ITP may lead to improved therapy for this condition.

Drug-Induced Thrombocytopenia

Numerous medications induce immune-mediated platelet destruction, characterized by severe thrombocytopenia and elevation of PAIgG levels (75). In most cases, the thrombocytopenia is dose independent and idiosyncratic and occurs several weeks after the first dose. Platelet destruction may result from the medication itself, a metabolite, or the drug delivery vehicle. Many drugs have been associated with immune-mediated thrombocytopenia, including a variety of antibiotics and anticonvulsants.

Quinidine and its analog quinine represent a prototype for drug-induced thrombocytopenia. Quinidine-dependent antibodies initially were thought to form an immune complex with the drug, which then fixed to platelets and led to RES clearance and destruction. Quinine- and quinidine-dependent platelet antibodies have been reported to react with several platelet glycoprotein antigens, however (76). Quinine actually causes widespread but specific conformational changes in platelet membrane surface antigens, thereby exposing neoantigens that can induce drug-specific antibodies (77).

Heparin is another important cause of drug-induced thrombocytopenia that results from frequent use of the drug and an observed incidence of about 5%. A mild dose-dependent form of heparin-induced thrombocytopenia (type 1 HIT) occurs within the first few days of therapy, is characterized by only mild thrombocytopenia, and resolves spontaneously (78). Type 2 HIT is an immunologically mediated form of thrombocytopenia that persists unless heparin is discontinued. In type II HIT, thrombocytopenia typically develops after 1 week of intravenous standard heparin therapy, although it can also occur following subcutaneous administration or even intravenous flushes. The pathophysiology of type 2 HIT initially involves the binding of heparin with platelet factor 4 (PF4), a secreted platelet a granule protein, to form large complexes. Heparin-dependent antibodies, usually IgG, then interact with the heparin–PF4 complexes to activate platelets and endothelial cells (78). Arterial or venous thrombosis develops in a small proportion of affected patients, presumably because of thrombin formation in the circulation. Therapy involves cessation of heparin as well as alternative antithrombotic agents.

Gold and methyl-dopa (Aldomet) appear to induce changes in platelet antigens that are cross-reactive with antidrug antibodies. Other drugs that induce immune-mediated thrombocytopenia have not been investigated in full detail. Therapy typically includes the discontinuation of the drug, if possible, or the use of immunomodulating agents such as corticosteroids or IVIG.

Alloimmunization

Immune-mediated clearance of platelets also can occur following exposure to allogeneic platelets, with development of alloantibodies against “foreign” platelet antigens. Alloantibodies are by definition directed against surface antigens not expressed on autologous platelets and therefore should lead to destruction only of allogeneic platelets. The most common clinical settings of platelet alloimmunization are pregnancy and multiple platelet transfusions. In the latter condition, the patient develops antiplatelet alloantibodies directed against HLA class I antigens, although ABO blood group antigens and many platelet-specific antigens also can be immunogenic. The use of leukocyte-depleted or ultraviolet B–irradiated platelets can greatly reduce the incidence of platelet alloimmunization (79).

Once a patient is alloimmunized, transfused platelets are quickly bound by alloantibodies and rapidly removed by the RES. Matching donor and recipient HLA types often improve platelet transfusion outcome by avoiding the alloantigens. Platelet cross-matching techniques (80) can aid the transfusion center in identifying compatible platelets for transfusion, and laboratory advances have made this procedure more cost-effective. The use of IVIG for alloimmunized patients remains controversial because conflicting results have been reported.

A related condition, known as *posttransfusion purpura* (PTP), is a rare but serious syndrome characterized by the development of platelet alloantibodies approximately 1 week after a platelet transfusion. Unlike typical alloimmunization, however, the patient's own platelets (which lack the alloantigen) are also destroyed by the antibodies. The classic clinical scenario involves a PL^{A1}(–) patient who is transfused with PL^{A1}(+) platelets, although other antigen systems have been described. Following development of anti-PL^{A1} alloantibodies and clearance of the transfused platelets, autologous platelets are also destroyed. In PTP, the thrombocytopenia is often severe and may be associated with clinical hemorrhagic symptoms.

The immunologic events that unfold in PTP have not been clearly delineated but may involve the deposition of circulating immune complexes (consisting of the alloantibody bound to soluble antigen) on autologous platelets. Alternatively, autologous platelets may bind soluble antigen and become transiently (+) for surface expression, and, therefore, susceptible to alloantibody binding and RES-mediated clearance. A third explanation is that alloimmunization somehow lifts suppression and allows the emergence of autoreactive clones. Therapy includes the avoidance of platelet transfusions and either plasmapheresis or exchange transfusion. The use of IVIG has also been used successfully in this disorder.

Miscellaneous

In addition to the previously described conditions, various other clinical settings occasionally are associated with immune-mediated thrombocytopenia. Septicemia, malignancy, solid-organ transplantation, cardiopulmonary disease, and preeclampsia can cause shortened platelet survival, in part as a result of immune mechanisms.

FUTURE DIRECTIONS

Over the past century, some important advances have been made in our understanding of immune-mediated thrombocytopenia. The identification and quantification of specific antiplatelet antibodies, recognition of the important role played by the RES in autoantibody synthesis and platelet destruction, and the emergence of several effective therapies have improved our ability to diagnose and manage patients with ITP. Challenges remain, however, in our understanding of the immunologic processes by which platelet autoantibodies develop, especially why certain persons manifest autoreactive antibodies that interact specifically with autologous platelets. A better understanding of the immune cell interactions that normally restrict autoantibody synthesis and expression are necessary to advance our knowledge in the coming years.

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59 BLOOD GROUPS, IMMUNOLOGIC HAZARDS OF TRANSFUSION, AND HEMOLYTIC DISEASE OF THE NEWBORN

Marilyn J. Telen, M.D.

Although the first blood transfusions were recorded in the seventeenth century, the modern era of transfusions was born with the discovery of the ABO blood group by Karl Landsteiner in 1900 (47). The first pretransfusion ABO typing and cross-matching were performed in New York in 1907 (87), but problems with anticoagulation hampered development of transfusion therapy until World War I, when sodium citrate began to be used as an anticoagulant. By World War II, Loutit and Mollison (58) had pioneered the use of acid–citrate–dextrose, a solution that allowed a higher and clinically more useful blood-to-anticoagulant ratio.

The 1940s saw the beginning of a rapid increase in the knowledge of red cell antigens, beginning with the Rhesus (Rh) system and the relationship of the D antigen to hemolytic disease of the newborn (HDN) (53). Since then, hundreds of red cell antigens have been discovered (42). Other advances during the ensuing 50 years included improvements in sterile collection and storage as a result of the advent of plastics and the institution and expansion of infectious disease testing, which now renders blood transfusion an extremely safe procedure.

As the ability to provide safe blood products has grown, so too has the clinical need for them. Complex surgery and especially organ transplantation have created large demands for blood products, as has the increasing use of myelosuppressive chemotherapy for oncologic diseases. Thus, whereas whole blood is now rarely transfused, blood components, such as red cells, platelets, plasma, plasma components, and occasionally leukocytes, are frequently used to support complex medical and surgical therapies. Transfusion is also now used to enhance immune-mediated resistance to malignant disease, such as in the infusion of donor lymphocytes for their antileukemic effect after allogeneic hematopoietic stem cell transplantation for leukemia (32).

Although blood transfusion is only rarely associated with major morbidity and mortality, we have learned much about the less evident effects of transfusion. In addition to the ability of various blood products to stimulate alloimmune responses, we now know that blood transfusion can have other types of immunologic effects. Some of these effects are mediated by transfused immunocompetent cells, such as lymphocytes and monocytes, whereas others are mediated by soluble factors, such as cytokines.

IMMUNOCHEMISTRY AND GENETICS OF ERYTHROCYTE BLOOD GROUP ANTIGENS

The red cell membrane is a lipid bilayer, undergirded by a highly regular membrane cytoskeleton composed primarily of spectrin and actin (7). A complex of membrane proteins assortment is attached to and permeates the lipid bilayer (96). Integral membrane proteins traverse the bilayer one or more times and have both extracellular as well as cytoplasmic domains. Other surface proteins, such as those attached by glycophosphatidylinositol anchors, are peripheral membrane proteins whose peptide portions are entirely extramembranous and extracellular. The cytoplasmic peripheral proteins are attached to the membrane by fatty acylation or interaction with integral or cytoskeletal proteins.

Although several hundred blood group antigens have been identified on erythrocytes, many of these antigens are of only rare clinical importance because of factors such as low levels of expression, low rates of polymorphism, and low immunogenicity. A few dozen, however, are clinically important because antibodies to them frequently are encountered and account for substantial reduction in antigen-positive cell survival. The antigen systems most frequently identified in transfusion medicine, along with phenotypic frequencies, are listed in Table 59.1.

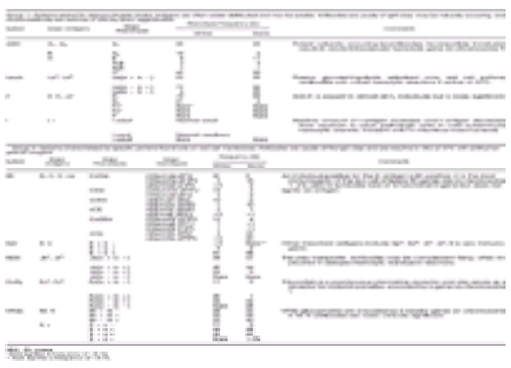

TABLE 59.1. Major Blood Group Systems

ABO, Lewis, and P Systems

The ABO system is clearly the most important alloantigen system for transfusion medicine, although one should remember that A and B antigens are really histocompatibility antigens with wide tissue distribution. The ABO blood group system is unique because of the fact that healthy individuals who lack either the A or the B antigen invariably have antibodies to these antigens from early infancy onward. These antibodies, which are primarily immunoglobulin M (IgM) but may comprise a substantial amount of IgG in group O persons, probably develop in response to A- and B-like immunogens that are present ubiquitously in nature. Most fatal hemolytic transfusion reactions result from ABO incompatibility.

The ABO blood group system represents the interaction of two unrelated gene loci. The H (*FUT1*) gene on chromosome 19q13.3 encodes a fucosyltransferase that adds fucose in an a1–2 linkage to a terminal galactose residue (48). The resulting H antigen is expressed by group O cells and is further modified in group A and B individuals. When no active H enzyme is present, the genotype is *hh*, and the cells express no H antigen, exhibiting the O_h, or Bombay, phenotype. In this situation, no A and B antigens can be formed, even when there are functional genes at the ABO locus.

The genetic locus responsible for formation of the A and B antigens is located on chromosome 9q34.1–q34.2, in linkage with the *NP* (nail-patella hereditary onychoosteodysplasia) and *AK* (adenylate kinase) loci (84). The *A* and *B* genes act as codominant alleles. The group O phenotype results from the presence of two recessive, inactive (*amorph*) alleles at the *ABO* locus. In the North American white population, distribution of the various ABO phenotypes in the population is approximately as follows: group A, 42%; group B, 9%; group O, 45%; and group AB, 3%. The group B antigen is considerably more common in Asian and African

populations, in whom it is found with frequencies of 27% and 20%, respectively.

The A and B antigens are present when an *N*-acetylgalactosamine or galactose, respectively, is attached to a substrate consisting of type 2 paragloboside to which a fucose has been attached by the product of the *H* gene (74) (Fig. 59.1). Thus, the *A* and *B* genes encode glycosyltransferases that modify the H structure. The complementary DNAs (cDNAs) encoding the A and B transferases differ from each other because of four nucleotide substitutions that account for the differing specificity of A and B transferases (113,114). Occasionally, members of a family are observed to have the *cis-AB* phenotype. In such families, the *A* and *B* genes appear to be transmitted to progeny as linked genes; however, the genetic basis for this phenomenon is usually the unusual existence of a single gene encoding a variant transferase with both A and B transferase activity (115).

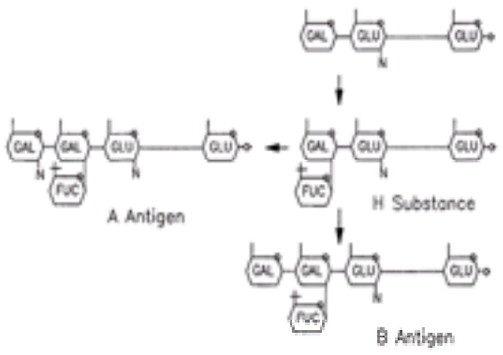

Figure 59.1. The formation of A and B antigens from H substance. (*Arrows* indicate action of the H, A, and B enzymes.) GAL, galactose; GLU, glucose; FUC, fucose; N-GLU, *N*-acetylglucosamine. (From WF Rosse. Clinical immunohematology: basic concepts and clinical applications. Boston: Blackwell, 1990, p. 197, with permission.)

The Lewis blood group system is another example of interaction between two separate genes. The Lewis antigens reside on soluble substances (glycosphingolipids) that are not an intrinsic part of the red cell membrane but are adsorbed onto cells from the plasma (89). The *Le* gene (*FUT3*) encodes a transferase that adds a fucose to the penultimate sugar (*N*-acetyl-galactosamine) of soluble ABH precursor chains (type 1 paragloboside), forming Le^a substance. When the terminal sugar of type 1 paragloboside is also fucosylated by the transferase that is the product of the *Se* (*FUT2*)gene], the antigen formed is Le^b. Thus, in the genotype *lele*, in which the *Le* gene does not encode a functional transferase, neither Le^a nor Le^b is expressed. If neither *Se* gene is functional (genotype *sese*), then the phenotype will be Le(a + b–) in the presence of a functional *Le* gene or Le(a–b–) in the presence of the *lele* genotype.

The P antigens likewise represent the stepwise action of several glycosyltransferases (89). The three P system antigens, P^k, P, and P_1, are produced by the action of three glycosyltransferases. Seventy-five percent of individuals have P, P_1, and P^k antigens on their red cells, whereas about 25% express P and P^k but not P_1. Rarely, red cells may be positive for P^k and negative for P and P1 or negative for all three antigens. This latter condition, designated the *Tj(a–) phenotype*, often leads to the production of a broadly reactive alloantibody and carries with it an increased risk of spontaneous abortion (54).

The P system is important in two clinical situations. The P antigen, or globoside, serves as the receptor for B19 human parvovirus, the agent responsible for most aplastic crises in patients with hemolytic anemias and human immunodeficiency virus (HIV) infection as well as for erythema infectiosum (fifth disease) (116). The P antigen is also typically the target of the autoantibody produced in paroxysmal cold hemoglobinuria (55).

Rhesus Blood Group System

Outside the ABO system, the Rh blood group system is clinically the most important in transfusion medicine. The Rh system is quite complex and encompasses at least 45 antigens. The five major antigens are encoded by two genes residing close to one another on chromosome 1 (12,13 and 14): One gene encodes the D antigen, and the other encodes the protein that bears the antithetical antigens C or c and E or e. The proteins that bear these antigens are quite unusual; they are all about 30-kd, nonglycosylated integral membrane proteins with multiple membrane spanning domains and relatively small peptide domains exposed at the cell surface (24,30). Nevertheless, the D antigen is highly immunogenic; about 80% of Rh-negative recipients of 1 U of D-positive red cells will form anti-D. The C/c and E/e antigens are also quite immunogenic. Together, antibodies to these five antigens account for most cases of HDN, even since inception of the use of Rh immune globulin to prevent maternal alloimmunization to D.

The Rh proteins appear to exist in the membrane as a macromolecular complex of about 170,000 Da (34) that includes the nonglycosylated Rh polypeptides as well as the Rh-associated glycoprotein (RhAG) encoded by a gene on chromosome 6 (83). Other membrane glycoproteins, such as glycophorin B (GPB), LW, CD47, the Duffy (Fy) protein, and band 3, also may associate with the Rh complex in the red cell membrane. The evidence for interaction among these proteins is provided by immunochemical studies using a variety of murine monoclonal as well as human antibodies (59,109) as well as by studies of protein expression (6) and studies of the molecular basis of the Rhnull phenotype (40). Expression of the Rh genes on chromosome 1 is limited to erythroid and early megakaryocytic lineage, whereas RhAG is expressed by leukocytes as well as erythrocytes. The function of the membrane Rh complex is uncertain. The configuration of the Rh polypeptide is similar to that of membrane transport proteins, and the Rh peptides have about 20% homology to methylamine permease and ammonium transporters of yeast, bacteria, and some plants. Nevertheless, the function of the Rh proteins in red cells remains undefined.

Rh_{null} red cells are totally deficient in Rh polypeptides and RhAG and express no Rh antigens. They have a number of physiologic abnormalities, including their stomatocytic shape, increased osmotic fragility, increased activity of Na, K-adenosine triphosphatase (ATPase), reduced intracellular cation and water content, and relative deficiency of membrane cholesterol (2). Their decreased membrane surface area, combined with intracellular dehydration, predisposes them to osmotic lysis. Most Rh_{null} phenotypes (*regulator type*) are due to lack of expression of RhAG caused by a variety of genetic alterations, whereas a few (*amorph type*) are due to mutations in the *RhCE* gene in the absence of a functional *D* gene (i.e., in an Rh-negative background).

The RhD and RhCE proteins are highly homologous, as are the structures of the genes encoding them. These two genes have been found to give rise to an extremely rare degree of diversity of haplotypes and variant antigens in the Rh blood group system.Although some phenotypes arise through point mutations, small deletions, or sets of mutations that occur in concert, many Rh genes have been described to contain transposed segments (i.e., *D* genes that contain one or more exons and introns from the *RhCE* gene and vice versa (Fig. 59.2).

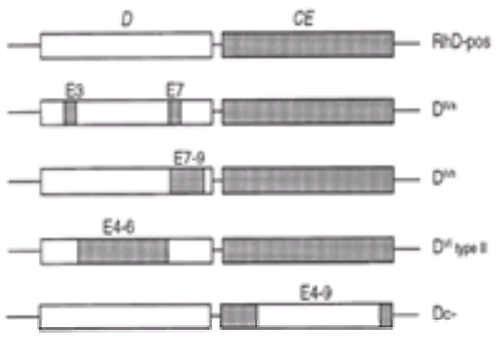

Figure 59.2. The *RHD* and *RHCE* genes have given rise to numerous variants. As shown here, some *RhD* genes contain coding sequences derived from the *RhCE* genes (shown in gray), as in the D^{IVa}, D^{IVb}, and D^{VI} type II phenotypes. The *RhCE* genes also may contain sequences derived from the *RhD* gene (shown in white), as in the Dc– phenotype. E, exon.

Sialoglycoprotein (Glycophorin) Antigens

The MNSs antigens are determined by variations in the amino acid sequences of two highly related glycoproteins, glycophorin A (GPA, MN glycophorin) and GPB (NSs glycophorin). These two proteins account for 95% of the periodic-acid Schiff (PAS)-staining proteins seen in gel electrophoresis of erythrocyte membrane proteins and are highly glycosylated proteins encoded by neighboring genes on chromosome 4 (15,79). The M and N antigens of GPA (M-GPA and N-GPA, respectively) depend on paired polymorphisms at amino acid residues 1 and 5 (Fig. 59.3): serine and glycine in M-GPA, and leucine and glutamine in N-GPA (16,106). The N-terminal 26 amino acids of GPB are homologous to N-GPA; thus, GPB also carries the N antigen. The S and scantigens of GPB are determined by a methionine/threonine polymorphism at residue 29 (17). Although the M and N antigens depend on amino acid polymorphisms, human antibodies to these antigens often require the presence of normal glycosylation of the serine and threonine residues at positions 2, 3, and 4 (Fig. 59.3) (86).

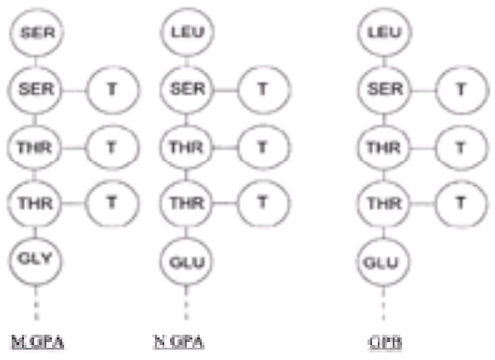

Figure 59.3. Terminal sequences of MN and Ss glycophorins (glycophorins A [GPA] and B [GPB], respectively). The S and s antigens are determined by a methionine/threonine polymorphism at amino acid position 29 on GPB. SER, serine; THR, threonine; GLY, glycine; LEU, leucine; GLU, glutamine; T, tetrasaccharide.

Lack of GPA expression is denoted En(a–) and is associated with clinically important anti-En^a (anti-GPA) alloantibodies. Autoantibodies seen in autoimmune hemolytic anemia also may have En^a specificity. Lack of GPB is denoted the S-s-U- phenotype; the U antigen is lacking, although the biochemical basis for this antigen is uncertain and may involve interaction with another membrane protein. When both GPA and GPB are absent (the MkMk phenotype), cells are M-N-S-s-U-. Lack of GPA, GPB, or both entails no physiologically important cellular changes.

The genes that encode GPA and GPB are highly homologous, a fact that apparently has been responsible for frequent uneven crossing-over and gene conversion events and consequently a large number of variant phenotypes. Some variants are due to creation of hybrid molecules by unequal crossing over, such as is seen in hemoglobin, whereas others are due to complex mutations or gene conversion events.

A third set of “glycophorin” antigens constitute the Gerbich blood group system, which resides on molecules called glycophorins C (GPC) and D (GPD) (10). These two proteins are unrelated to GPA and GPB and derive from a single gene on chromosome 2 through alternative translation start sites. Unlike GPA and GPB, however, the absence of Gerbich antigens and the total deficiency of GPC/D are associated with a rare form of hereditary elliptocytosis (3,82).

Other Major Protein Antigens

The Kell, Kidd, and Duffy blood group antigens are frequently polymorphic and immunologically important barriers to blood transfusion. All antigens in these systems thus far have been identified only on red cells, and all antigens reside on membrane proteins.

The Kell antigens reside on a 93,000-molecular-weight glycoprotein (81) whose cDNA exhibits considerable homology with a number of other neutral zinc-binding endopeptidases, including the common acute lymphoblastic leukemia antigen (CALLA) (50). The Kell gene resides on chromosome 7 (117). The most important antigens in this system are K1 (K) and K2 (k). The K1 antigen occurs in about 9% of the white population and 2% of the black population; the K2 antigen occurs in more than 99% of both blacks and whites. The K1 antigen is extremely antigenic, causing antibody production in about 5% of K1-negative recipients of K1-positive blood. After D, K1 and E are the two most commonly identified blood group alloantibodies. Although it has now been shown that the Kell protein retains endopeptidase activity, the physiologic importance of this activity is unclear (51).

Two rare phenotypes occur in the Kell system. The K_0 phenotype is associated with lack of all Kell system antigens and increased expression of an antigen encoded by the X chromosome, Kx. The McLeod phenotype, on the other hand, is associated with absence of Kx antigen and weak expression of inherited Kell system antigens. The Kx antigen resides on a 34,000-molecular-weight protein whose expression appears necessary for normal expression of the Kell protein and its antigens on red cells. Lack of Kx is accompanied by acanthocytosis and mild hemolytic anemia (101). The disorder, known as *neuroacanthocytosis,* also is associated with deficiency or abnormality of Kx (112). Because the protein is encoded by the X chromosome, the female carrier state for the McLeod phenotype can be identified by analyzing peripheral blood for a subpopulation of acanthocytic, Kx-negative red cells. The McLeod phenotype is also sometimes linked to the X-linked form of chronic granulomatous disease and Duchenne muscular dystrophy, both of which may result from a partial deletion of the X chromosome.

The Duffy system comprises two antithetical antigens, Fy^a and Fy^b; a “null” Fy(a–b–) phenotype is relatively common in blacks. Interestingly, red cells with the Fy(a–b–) phenotype are resistant to invasion by *Plasmodium vivax* and *Plasmodium knowlesi* malarial parasites (60,61); however, they are not resistant to invasion by the common African parasite *Plasmodium falciparum*. Investigation of the molecular basis of the Duffy blood group led to the discovery that the Duffy protein is a promiscuous chemokine receptor capable of binding both C-C and C-X-C chemokines (11, 38). Its absence from the red cells of many people of African origin is due to a mutation in the GATA site that regulates expression in erythroid cells rather than to a mutation in the coding region (107); individuals with this mutation express the Duffy protein in nonerythroid tissues, where other transcription regulatory regions are more important (43). The Kidd system also comprises two antithetical antigens, Jk^a and Jk^b, and has a null Jk(a–b–) phenotype. The latter phenotype is associated with abnormal urea transport (28,36). Although Jk(a–b–) cells appear to survive normally *in vivo*, this defect is demonstrable *in vitro*. The 50-kd protein identified as the carrier of Kidd antigens (97) has now been shown to be a urea transporter (72). Antibodies to antigens of the Kell, Duffy, and Kidd systems are most often IgG antibodies that react optimally at 37°C and may fix complement. Kidd antibodies especially have been associated with unusually severe delayed hemolytic transfusion reactions. Antibodies to Jk^a and Jk^b often decline rapidly in titer following primary immune stimulation so that they are not detected prior to subsequent transfusions. Also, these antibodies almost uniformly activate complement well. Therefore, they may be responsible for severe anamnestic hemolytic transfusion reactions.

Blood Group Antigens on Other Functionally Interesting Proteins

The antigens Chido (Ch) and Rogers (Rg) reside on variants of the C4 component of complement encoded by chromosome 6 (73). These antigens are adsorbed from plasma and are weakly expressed by red cells. Antibodies to Ch and Rg most often react weakly in agglutination reactions, are able to be inhibited by plasma from the phenotypically appropriate donor, and rarely if ever cause decreased red cell survival.

The Knops (Kn), McCoy (McC), and York (Yk) antigens reside on the complement receptor type 1 (CR1, CD35) (65,80), whose gene lies within the regulation of complement activation (RCA) cluster on chromosome 1 (85). This receptor occurs in four isoforms distinguishable by molecular mass (190, 220, 250, or 280 kd under reducing conditions) (66). As yet, however, no clear linkage between any of these isoforms and specific Kn/McC/Yk antigens has been made. Antibodies to these antigens are relatively common but have little if any role in causing hemolytic transfusion reactions or HDN.

The Cromer antigens reside on another complement regulatory protein, decay accelerating factor (DAF, CD55) (103). This 70-kd protein is also encoded from within the RCA cluster on chromosome 1 and bears homology to several other complement regulatory proteins. Antibodies to Cromer antigens are usually IgG in nature and are of variable but sometimes significant clinical importance.

The Colton antigens reside on the water channel protein aquaporin-1 (98). This red cell water channel was the first protein of this family to be discovered. Interestingly, persons lacking this protein from their red cells and who are thus Co(a–b–) do not have a significant clinical disorder. Another red cell membrane transport protein that carries blood group antigens is band 3, also called AE-1 (anion exchanger-1); it carries both ABH and the Diego antigens (9). Although antibodies to the Colton and

Diego antigens can be clinically significant, they are rarely encountered.

Red cells also express a number of adhesion molecules on their surfaces. Although these red cell proteins are believed to be inactive under normal circumstances, they do bear blood group antigens. For example, the LW blood group antigens reside on the LW protein, a member of the Ig superfamily with homology to intercellular adhesion molecule-2 (ICAM-2); it is capable of binding leukocyte integrins (5). Lutheran antigens reside on another IgSF member, which can serve as a laminin receptor (108), and Indian antigens reside on CD44, which is a hyaluronan receptor (104).

PLATELET-SPECIFIC BLOOD GROUP ANTIGENS

Increasing use of myelosuppressive chemotherapy to treat many different types of malignancies, as well as growing use of stem cell transplantation, has markedly increased the number of platelet products that are transfused. Thus, changing medical practice, along with the technology of molecular genetics, led to the identification of protein polymorphisms that underlie platelet-specific blood groups. Although they are found almost exclusively on platelets among circulating blood cells, platelet-specific antigens are actually found in other tissues as well.

Five major human platelet blood groups have been identified (110) (Table 59.2). In all but one system (HPA-3), the high-frequency antigen is present in 85% or more of the white population. The most commonly encountered antibody to a platelet-specific antigen is directed against the PlA1 (HPA-la) antigen. Antibodies to this antigen are the most common cause of both neonatal alloimmune thrombocytopenia as well as posttransfusion thrombocytopenic purpura.

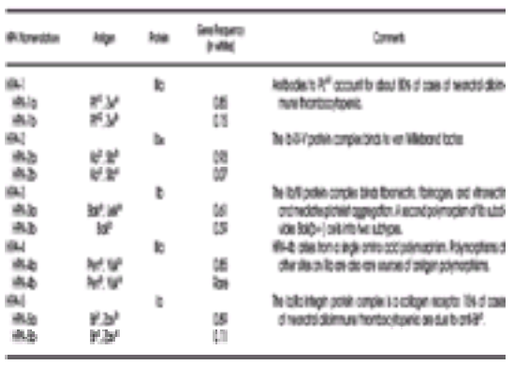

TABLE 59.2. Human Platelet Blood Group Antigens (HPA)

In the setting of platelet transfusion, immune clearance of transfused platelets is most commonly accounted for by antibodies directed against human leukocyte antigen (HLA) class I molecules rather than against platelet-specific antigens. HLA alloimmunization is largely preventable by reducing the leukocyte content of both red cell and platelet transfusions to fewer than 10^6 leukocytes per unit; this can be routinely accomplished using leukoreduction filtration. Platelets also bear antigens in common with red cells, including those of the ABO, P, Ii, and Lewis systems. The clinical importance of ABO incompatibility is variable; studies have shown that ABO incompatibility may, but will not always, negatively influence survival of transfused platelets (35,49).

NEUTROPHIL-SPECIFIC BLOOD GROUP ANTIGENS

Neutrophil-related blood group antigens are less well characterized than red cell and platelet antigens. One system, NA1/NA2, is well understood and of known clinical importance. These antigens represent two alleles of the FcRIII low-affinity IgG receptor (CD16 molecule) (41). Analysis of cDNA from NA1 and NA2 homozygotes predicts four amino acid substitutions differentiating NA1 from NA2. In addition, these substitutions modify glycosylation sites so that NA1 FcRIII has four potential N-linked glycosylation sites, and NA2 FcRIII has six. The FcRIII molecule is the most common target in neonatal alloimmune neutropenia. Maternal alloantibodies most often are directed against the NA1 polymorphic determinant; however, complete maternal deficiency of FcRIII with associated anti-FcRIII antibody also has been described as a cause of neonatal neutropenia (100).

IMMUNOLOGIC HAZARDS OF BLOOD TRANSFUSION

The transfusion of whole blood, blood components, or blood-derived products has a plethora of immunologic and other effects on their recipients. Described immunologic effects include stimulation of alloantibodies to both cellular and plasma protein antigens, passive transfer of antibody against similar antigens, passive transfer of cytokines and other effector molecules, passive transfer of immune effector cells (lymphocytes), and transmission of infectious agents that affect the immune system (e.g., HIV). Antigen-antibody interactions lead additionally to a variety of immune-mediated events, including hemolysis, pulmonary compromise, allergic reactions, and anaphylaxis. Transfusion also has been reported to cause immunosuppression, although the mechanism and clinical significance of this remain controversial.

Factors Associated with Immune Red Cell Destruction

Transfused red cells may be cleared from the recipient's circulation in an accelerated manner as a result of humoral factors, that is, isoantibodies or alloantibodies, or because of a combination of humoral and cellular immune mechanisms. The rate of clearance of cells bearing an alloantigen to which the recipient has been sensitized varies greatly, however. Some antibodies effect hemolysis or clearance by the reticuloendothelial system (RES) within minutes, whereas others cause only relatively slow immune clearance over days to weeks. Although the factors that influence this process are complex, the rate of clearance of transfused erythrocytes often can be predicted by knowledge of the target antigen involved. Antibody responses to other cell types, such as platelets, are less well studied and understood.

The factors that determine the rate at which transfused red cells are cleared from the circulation during an alloimmune response include the following:

Plasma concentration of antibody
Thermal range of antibody
Immunoglobulin class and subclass
Red cell antigen density
Red cell antigen biochemical characteristics (e.g., mobility in the membrane)
Extent of complement activation
Interactivity of RES macrophages
Amount of incompatible red cells transfused
Presence of plasma complement components

Antibody-Dependent Factors

Several factors integral to the antibody molecules involved dictate much about the course of destruction of antigen-positive red cells. *Thermal amplitude* refers to the temperature range within which an antibody will react with target cells. For most alloantibodies to blood group alloantigen systems, *in vitro* and presumably *in vivo* reactivity is optimal at 37°C. Some alloantibodies and many autoantibodies are “cold reacting” and bind much better at lower temperatures. In general, alloantibodies that react only at temperatures below 30°C are not clinically significant because they are usually IgM molecules that dissociate from red cells at body temperature (62). Cold-reacting autoantibodies occasionally have wide thermal ranges, however, reacting optimally at 0 to 4°C but maintaining activity at temperatures seen in the extremities (> 30 and < 37°C). These antibodies often fix complement readily and can cause *in vivo* hemolysis of both autologous and transfused red cells.

The number of available immunoglobulin molecules is also a key factor dictating the rate of clearance of transfused cells (75). Although the likelihood of an antibody causing significant hemolysis may be predicted by the strength of reactions observed *in vitro* and by the titer of the antibody, titer may change dramatically over days, and other factors also influence the rate of cell clearance. For example, red cells coated *in vitro* with varying amounts of anti-D will be cleared *in vivo* in direct proportion to the amount of antibody bound to the cells; but antibodies to the Kn^a, McC^a, Hy, and Yt^a antigens are typically present in relatively high titers and yet only rarely cause hemolytic transfusion reactions.

The immunoglobulin class and subclass to which most immunoglobulin molecules of a particular specificity belong also influence the mechanism and rapidity of cell destruction. IgM antibodies are often the first formed in a primary immune response, although IgG antibodies are typically the first detected antibodies to many antigens, including D and K1. Some alloantigens, such as M and N, Le, and P1, rarely stimulate significant IgG responses. Some autoantibodies, including those to H, I, and P, are also typically IgM, are low in titer, have low thermal amplitudes, and are usually of no clinical consequence.

The IgG alloantibodies are typically warm-reactive (optimal at 37°C) and are most commonly directed against antigens of the Rh, Kell, Duffy, and Kidd blood group systems. These antibodies occur almost exclusively after immune stimulation by transfusion or pregnancy and constitute the most common causes of delayed transfusion reactions resulting from anamnestic responses when they are not detected in pretransfusion testing. IgG antibody responses often include a mixture of IgG subclasses, and it is well recognized that different subclasses can mediate cell clearance to different degrees and by different mechanisms. RES macrophages have three types of immunoglobulin Fc receptors. All three types of receptors will bind immunoglobulin-coated red cells *in vitro* (45), but binding of FcRI appears to be necessary for lysis of sensitized red cells *in vivo* (45,56). FcRI appears to bind primarily IgG1 and IgG3. Moreover, when cells coated with anti-D are injected, *in vivo* clearance increases with the proportion of IgG3 in the antisera used (105). IgG2 and IgG4 antibodies are not thought to mediate cell destruction. Rare alloimmune antibodies to red cell antigens consist primarily of IgA.

Complement Activation

Complement activation plays a major role in the clearance of immunoglobulin-sensitized red cells. The complement system is discussed extensively elsewhere in this book, and its role in blood cell clearance has been well defined (90). Coating of red cells by antibody may permit binding of C1q and initiation of the complement cascade and ultimately will produce enough terminal complement complexes (C5b–9) to cause direct cell destruction. This mechanism typically accounts for the fulminant hemolysis seen in major ABO hemolytic transfusion reactions (90). IgG alloantibodies, however, rarely cause large-scale hemolysis by complement-mediated lysis. Unlike IgM, IgG must present a doublet configuration on the red cell membrane to fix C1q. Furthermore, whereas IgG1 and IgG3 can act strongly to activate complement, IgG2 does so only weakly, and IgG4 cannot activate complement. Antibodies to Kidd antigens typically activate complement well, whereas those directed against Rh antigens do so rarely if ever. Differences in the likelihood of complement activation by an immune response to a particular antigen depend also on the nature of the antigen, position of the epitope, and density and mobility of the antigen within the membrane.

Fixation of complement also plays a role in RES clearance of antibody-coated cells. In most instances, early complement components are bound to red cells coated with alloantibody, but the factors that downregulate complement activation succeed in forestalling formation of enough C5-9 complexes to lyse the cells directly. Such downregulation may occur at the amplification stage, in which C3/C5 convertases can be inactivated by spontaneous dissociation as well as through the action of specific complement regulatory proteins. Such regulatory proteins include the plasma proteins factor H (111) and C4-binding protein (29) as well as the membrane proteins CR1 (CD35) (88) and DAF (CD55) (71). Downregulation of complement activation also occurs at the stage of formation of the membrane attack complex (C5–9). The plasma protein vitronectin (also known as S protein) inactivates C5b–7 complexes formed in fluid phase (77), whereas the membrane protein membrane inhibitor of reactive lysis (MIRL, protectin, CD59) interferes with the generation and effectiveness of membrane-bound C5–9 complexes (37).

The complement components C3b and C4b, however, are capable of mediating immune adherence to many cell types, including polymorphonuclear leukocytes, monocytes, macrophages, and some lymphocytes, through the CR1 receptor. Other complement receptors, including CR2 and CR3, also are expressed on many hematopoietic cells. Immune adherence mediated by complement components stimulates phagocytosis poorly, however, whereas the simultaneous interaction of cell-bound IgG with macrophage Fc receptors is a more powerful stimulus to phagocytosis. Complement component-mediated immune adherence, however, can stimulate antibody-dependent cellular cytotoxicity (ADCC) (46, 76). The importance of ADCC *in vivo* during hemolytic transfusion reactions is not known.

In rare instances, deficiency of complement components or of complement regulatory factors has remarkable effects on complement-mediated immune destruction. For example, occasional ABO-mismatched transfusions elicit only slow hemolytic reactions when complement deficiency is present as a result of congenital or acquired conditions. In the acquired disease paroxysmal nocturnal hemoglobinuria, deficiency of the membrane complement regulatory proteins DAF and MIRL make cells more susceptible to complement-mediated lysis.

Antigen-Dependent Factors

Antigens differ from one another in many respects, including biochemical makeup, density, and mobility in the membrane. In addition, antibodies to different epitopes on the same molecule may cause significant differences in severity of transfusion reactions. For example, alloanti-M and anti-En^a both bind to the GPA protein, which is expressed at approximately 1×10^6 copies per cell. Anti-M is rarely clinically significant, however, whereas anti-En^a is usually so. Numerous factors may play a role in this dichotomy. Anti-M may be IgG or IgM, is usually relatively cold reacting, and binds to an epitope near the N-terminus of the protein; anti-En^a is usually warm-reacting IgG that binds considerably closer to the membrane. It is difficult to determine why these antibodies tend to differ in thermal amplitude and clinical significance, but antigen characteristics almost certainly play a role.

On a theoretic level, it seems reasonable to suppose that limitations in antigen mobility would hamper the ability of IgG antibodies to cluster and activate complement. Limitations in protein mobility most likely arise from the interaction of integral membrane proteins with the membrane skeleton or from interaction with each other.

Serologic Detection of Blood Group Antibodies

The technology used for the detection of red cell-directed alloantibodies traditionally relied on detection of antibody-mediated agglutination in the presence or absence of antiglobulin serum. Although these techniques are relatively simple, they have been adequate for the detection of hundreds of separable antigens and for the accumulation of an impressive amount of knowledge regarding the genetics of blood group antigen systems. More recently, new and, in some cases, mechanized techniques have been developed; these methods have not, however, proven clearly superior in the detection of clinically significant alloantibodies.

Agglutination Reactions

Normal red cells repulse each other because of the negative charge, called the z potential, that is derived from the millions of ionized sialic acid carboxyl groups on the erythrocyte surface. When antibody molecules bind to cell surface antigens, however, they can form “bridges” from one cell to another, thus forming agglutinates. Current agglutination testing methods are designed to maximize this process by a variety of means.

Detection of the isoagglutinins anti-A and anti-B and typing of cells by reagent anti-A and anti-B are usually performed at room temperature. Because the antibodies involved are primarily IgM, and the antigens are present in relatively high copy number, direct agglutination is usually obtainable after short incubation times. Some red cell alloantibodies can be detected with similar methods, but for most IgG alloantibodies, the Coombs, or antiglobulin, test is used.

When pretransfusion screening is performed to detect alloantibodies, serum from the prospective recipient is mixed with a small panel of group O reagent red cells whose antigenic phenotype in at least the Rh, MNSs, P, Kell, Kidd, and Duffy systems has been determined. In this manner, the serum is ensured to have been tested for antibodies to the major antithetical antigens within these systems. Incubation is done at 37°C, which is optimal for most of these alloantibodies, except P and MN. In addition, incubation may be performed in the presence of solutions designed to enhance antibody binding or agglutination.

In most instances, agglutination is not visible after incubation of cells with IgG alloantibodies. Thus, cells that have been incubated with serum are then washed with a saline solution to remove excess unbound immunoglobulins and incubated with an antiserum containing antibodies to the Fc portion of human immunoglobulins as well as to human complement, usually the C3 component. This procedure was pioneered by Moreschi in animal studies in 1908 (64) and later was applied to human red cell serology by Coombs (67). Although originally involving heterologous antisera, usually produced in rabbits, modern Coombs reagents are often blends of murine monoclonal antibodies to IgG heavy chains and human C3. Thus, these reagents may fail to detect binding of IgA alloantibodies as well as IgG4 immunoglobulins.

In Vivo Red Cell Survival Studies

Rarely, an antibody that produces *in vivo* cell destruction is not detectable by agglutination testing, and thus *in vitro* testing is not sufficient to determine which erythrocytes may be safely transfused. A second indication for *in vivo* testing arises when an antibody of uncertain clinical significance is identified. In such a case, *in vivo* testing can determine whether cells phenotypically negative for the target antigen are required for transfusion; when the target antigen is of high frequency or when the recipient has made multiple alloantibodies, documentation of a lack of clinical significance of an antibody can greatly facilitate the selection of blood for transfusion.

Although small volumes of blood may be transfused and the patient monitored for signs and symptoms of hemolysis, such transfusion “trials” are not generally as sensitive and are of greater risk than are red cell survival tests using small volumes (1–2 mL) of chromium 51 (^{51}Cr)-labeled red cells. After injection of such cells, blood is usually sampled first at 3 minutes; this value may be taken as 100% survival in most cases. Cells presensitized with antibody or cells subject to clearance by a

particularly strongly reactive antibody may undergo some clearance within 2 to 3 minutes. Blood then is sampled several times during the first 10 to 15 minutes and again at 1 hour and 24 hours; later samples are also sometimes appropriate. When there is normal cell survival, both the 1-hour and the 24-hour survival rate is greater than 95%. Rapid intravascular lysis, such as seen with ABO antibodies, may reduce cell half-life to less than 10 minutes (22). Less rapid cell destruction may occur with either one-phase or two-phase kinetics. Red cells sensitized by primarily noncomplement-fixing IgG antibodies usually are cleared gradually from the circulation with a destruction curve defined by a single exponential; the slope of the curve depends on the factors discussed previously, including concentration of antibody, antigen density, and RES macrophage function. Most red cell destruction takes place extravascularly, by phagocytosis and perhaps ADCC. The half-life of chromium-labeled cells cleared by this mechanism can be quite variable, from minutes to days. When complement-fixing IgM antibodies, such as alloanti-Le^a or autoanti-I, are involved in cell clearance, a two-phase process may be seen. At first, an initial amount of the transfused cells (usually up to 30%) is cleared, after which the remaining cells survive relatively normally. The initial rapid clearance is presumed to be due to fixation of complement and ensuing phagocytosis or ADCC. On the surviving cells, however, C3b is converted to inactive C3 fragments that are not recognized and bound by complement receptors. These fragments tend to protect the cells from further complement fixation, and thus the cells subsequently survive relatively normally.

Performing the appropriate sampling in a ^{51}Cr survival study is important, as a cold-reacting antibody that causes destruction of 30% of cells in a short period, followed by normal survival of the remaining cells, may sometimes be safely ignored. A one-phase curve in which 30% of the cells are destroyed in 24 hours by an IgG antibody may presage a significant delayed hemolytic transfusion reaction.

Hemolytic Transfusion Reactions

Accelerated immune destruction of transfused red cells is the cardinal feature of a hemolytic transfusion reaction. Acute reactions occur within minutes to hours of transfusion; delayed reactions are observed days to weeks after transfusion. In general, acute reactions involve a larger element of intravascular hemolysis, whereas delayed reactions predominantly involve extravascular red cell clearance. Much overlap occurs, however. Acute reactions most often are associated with preformed antibody, especially naturally occurring isoagglutinins against the A and B antigens. Delayed hemolysis most often occurs as part of an anamnestic response to an antigen to which the blood recipient has been previously exposed through transfusion or pregnancy. Most antibodies that cause delayed hemolytic transfusion reactions target antigens within just a few blood group systems, including Rh, Kell, Kidd, and Duffy. The RhD (D) antigen is especially immunogenic; 1 U of D-positive blood stimulates antibody production in about 80% of D-negative recipients. Because it is now common practice to transfuse group O D-positive blood to men and to women past childbearing age in emergency situations, anti-D remains a commonly encountered alloantibody, despite the fact that D-positive blood normally is not given to D-negative recipients once the recipient's blood type is known.

Intravascular Hemolytic Reactions

Intravascular hemolysis occurs when sufficient complement is fixed on transfused red cells to lead to formation of enough terminal attack complexes (C5–9) to lyse circulating transfused red cells. Again, it is the ABO system antibodies that are most often the culprits, but other antibodies (such as anti-Jk^a, anti-Vel, and anti-Tj^a) also can produce brisk intravascular hemolysis. When intravascular hemolysis is rapid, such as with ABO reactions, the physician will often observe hemoglobinemia, hemoglobinuria, vascular collapse, disseminated intravascular coagulation (DIC), and renal failure. During operative procedures, intractable bleeding may be the first sign of a hemolytic transfusion reaction.

The sequence of events that causes the clinical signs and symptoms of hemolytic transfusion reactions is quite complex. Activation and fixation of complement components lead both to red cell destruction and release of vasoactive substances (e.g., C5a) and procoagulant materials; large numbers of immune complexes also are formed. Leukocytes are activated, and multiple cytokines are released (18,20,21). Some of these cytokines are associated with expression of procoagulant molecules by both leukocytes and endothelial cells, and these changes may contribute to the DIC sometimes observed in acute hemolytic transfusion reactions (19). Renal failure probably results from immune complex deposition as well as hypoperfusion. If sufficient hemoglobin is released to overwhelm the transport mechanism of the proximal renal tubules, hemoglobin chains may precipitate in the renal tubules, where a combination of acidification and water reabsorption may make the hemoglobin insoluble; this process can also result in failure of tubular function. It is also known that, under other conditions, large amounts of free hemoglobin can be excreted by the kidney without untoward effects (78).

In vitro models of immunologic events associated with antibody-induced hemolysis have begun to be investigated. Among the cytokines produced by circulating leukocytes when exposed to red cells and ABO-incompatible plasma are interleukin (IL)-8 and tumor necrosis factor (TNF-a) (18,20). When IgG antibodies were involved, a similar *in vitro* model documented production of IL-1b, TNF, IL-6, and IL-8 by monocytes (21). The high-affinity immunoglobulin FcRI appeared to be involved in the pathway leading to production of IL-8 and erythrophagocytosis; however, neutralization of IL-1 and TNF did not prevent production of IL-8 (21).

If the clinician observes signs or symptoms frequently associated with a major hemolytic transfusion reaction (e.g., cyanosis, sensation of choking or substernal pressure, abdominal pain, hypotension, unusual bleeding, hemoglobinuria, and oliguria), the blood transfusion should be stopped immediately. Intravenous access should be maintained, however, and fluid support (with pressors when necessary) and diuresis, if possible, should be aggressively initiated. Long-term support may require dialysis if renal shutdown is complete or of long duration.

Investigation of such a reaction should include demonstration of signs of hemolysis, such as plasma-free hemoglobin and urine hemoglobin, as well as identification of the causal antibody and delineation of the circumstances leading to the transfusion reaction. Most major hemolytic transfusion reactions occur because of errors in patient sample labeling, clerical errors, and misidentification of the blood or patient at the time of transfusion. Thus, all records and labels should be carefully checked, and new samples should be drawn to reconfirm the recipient's ABO type, especially for a patient who has not been typed prior to the sample used for the implicated transfusion. Normally, all typing, antibody screening, and cross-matching should be repeated with both the blood sample previously obtained as well as a second sample obtained at the time of the reaction.

Extravascular Hemolytic Reactions

Extravascular hemolysis is most often caused by IgG antibodies produced after exposure to foreign antigens through transfusion and pregnancy. The antigens most often involved are those of the Rh, Kell, Kidd, and Duffy systems. These antibodies rarely fix complement adequately to cause predominantly intravascular hemolysis, but membrane-bound IgG and complement components bind to specific receptors on RES macrophages (91). Antibody-coated cells may be partially or completely phagocytized or destroyed by natural killer (NK) cells through ADCC lysis.

Although IgG alloantibodies largely cause extravascular hemolysis, the clearance induced by some antibodies can occur over a relatively short time and may be associated with constitutional symptoms, including fever, chills, and vague back, flank, or abdominal pain. Hemoglobinemia, hemoglobinuria, and hyperbilirubinemia are often present, and laboratory testing may demonstrate elevated lactate dehydrogenase (LDH), absent haptoglobin, and positive serologic findings, including a positive result on the direct antiglobulin test (DAT) and the presence of specific serum antibody.

In general, delayed hemolytic transfusion reactions usually involve extravascular hemolysis induced by antibody produced during an anamnestic response to an erythrocyte alloantigen. Although delayed hemolytic reactions can occur after a primary exposure to antigen, this is rare because of the length of time needed for a primary response (several weeks) and the initial IgM character of that response. Instead, delayed transfusion reactions occur because the antibody produced in a previous primary immune response has decreased to undetectable levels by the time of pretransfusion testing; antibodies may also be missed due to technical errors during testing or due to the specific type of testing performed.

More often than not, delayed alloimmune transfusion reactions are clinically insignificant (69). Accelerated red cell destruction is often unnoticed or inapparent. Thus, the blood bank may detect evidence of posttransfusion sensitization (“delayed serologic transfusion reaction”) when the clinician is unaware of signs or symptoms of hemolysis. As many as 1 in every 1,500 transfusions may be followed by serologic evidence of incompatibility between the transfused blood and the recipient's posttransfusion serum (102). The mortality rate of delayed hemolytic transfusion reactions is probably less than 1%, however, and major morbidity such as renal failure, although reported, is also exceedingly rare. Exceptions include a high morbidity and mortality of delayed hemolytic transfusion reactions in patients with sickle cell disease and an increased incidence of renal failure in patients with already compromised renal function, such as chronic renal insufficiency due to diabetes.

Expected laboratory findings in delayed hemolytic transfusion reactions include (a) positive results on the DAT with or without complement and (b) the presence of a previously unidentified alloantibody either in the serum or in the eluate made from circulating red cells. When a patient has received several units of blood, the circulating cells may not all be positive for the implicated antigen, and thus a mixed-field agglutination pattern in which not all red cells are involved in agglutinates might be expected. As the antigen-positive red cells are cleared from the circulation, the DAT result might be expected to become negative, whereas the titer of the responsible antibody would be expected to increase, at least over the short term.

Immunologic Hazards of Platelet Transfusions

Transfusion of stored packed red blood cells entails transfusion of all cellular blood elements, including leukocytes and platelets, unless the blood product is first

modified (e.g., by washing or filtration) to remove nonerythroid cells. Thus, many red cell recipients are also exposed to platelet and leukocyte antigens, although an increasing percent of red cell units are now processed through leukocyte-depletion filters, which reduce both the leukocyte and platelet content. Therapeutic use of platelet concentrates carries a significant risk of alloimmunization to HLA antigens and a somewhat lower risk of immunization to platelet-specific antigens. The rate of alloimmunization is dependent on the number of transfusions received, the underlying disease, and the type of concomitant therapy. Patients being treated for leukemia may become alloimmunized less often than patients being treated for aplastic anemia, especially when the latter are not receiving immunosuppressive therapy. As many as 60% to 70% of recipients of multiple unmodified platelet concentrates become alloimmunized to HLA antigens (39), whereas only a small percentage develop antibodies to platelet-specific blood group antigens. Platelets also bear ABH antigens but do not express antigens of the highly immunogenic Rh, Kell, Kidd, and Duffy blood groups. The identification of HLA alloantibodies in patients who appear alloimmunized and refractory to platelet transfusions is routinely accomplished using a standard lymphocyte cytotoxicity test, discussed elsewhere in this book.

The high rate of alloimmunization to HLA antigens after platelet transfusion led to intensive efforts focusing on prevention of HLA alloimmunization and provision of platelets that will survive despite HLA alloantibodies. At present, alloimmunization to HLA antigens appears best to be preventable by filtration of all red cell and platelet products with leukoreduction filters. When used appropriately, such filters can reduce the number of leukocytes per transfused unit to fewer than 5×10^6. This procedure alone reduces the incidence of HLA sensitization in a population that receives multiple transfusions to 30% or less.

For patients who become alloimmunized during therapy or who start transfusion therapy after having already become alloimmunized (e.g., via pregnancy) and who are thus “refractory” to transfusion with random donor platelets, several approaches have been taken in an effort to provide platelets with normal or near-normal survival. One straightforward method is to provide the patient with platelets from a donor whose platelets carry the same class I HLA antigens. Only about 1 in 4,000 donors will provide a perfect, four-antigen match for a random patient, however. The odds worsen if the patient is black or Hispanic because of the largely white donor population in the United States and the different frequencies of certain alleles in racially different populations. Platelet units that match two or three of the alloimmunized recipient's HLA antigens have been shown to survive better than non-HLA-matched platelets (57), but the success of such partially matched units is highly variable. One factor shown to be important is whether the “mismatched” antigens belong to the same “cross-reactive groups” as the recipient's antigens (25). When mismatched antigens belong to the same cross-reactive group, survival of transfused platelets is improved over that of platelets expressing non–cross-reactive antigens (25). Even with modified antigen matching, however, providing HLA-matched platelets requires a large, previously typed donor pool from which to draw, especially because the half-life of compatible transfused platelets dictates the need for two to three platelet transfusions weekly for many patients and even more for those with surgical or hemorrhagic problems.

Several investigators have looked into the value of “cross-matching” platelets to select non-HLA antigen–matched platelets that might survive markedly better than randomly chosen ones. Although such tests are not now standardized, one is commercially available. Studies of outcomes following transfusion of such “compatible” platelets to alloimmunized patients have shown at least some value for these procedures (27,44).

Although HLA alloimmunization is usually the primary cause of failure of platelet transfusions in multiply transfused recipients, several other factors also deserve mention. Because platelets carry ABH antigens, the role of ABO incompatibility has been examined (4,26,35,49). In at least some patients, ABO incompatibility significantly reduces platelet survival (35,49), whereas in other patients, the recipient's anti-A or anti-B titer may be at least initially insufficient to effect clearance of significant numbers of platelets. Thus, although ABO matching is not required for platelet transfusion, such matching should be the first step in dealing with a patient who is refractory to transfusion of platelets from random donors (49).

Finally, when both ABO and HLA matching are unsuccessful in identifying platelets that will survive in an alloimmunized recipient, the possible existence of alloantibodies against platelet-specific blood group antigens must be considered. Identification of such antibodies and testing for expression of such target antigens are not yet routine and widespread. Serum with which to type platelets is generally scarce because such antibodies are relatively rare. The molecular bases of many platelet blood group antigen polymorphisms are now known (for review, 70), and some laboratories are able to genotype patients for these antigens.

Posttransfusion Purpura

Occasionally, an alloimmune reaction directed against a platelet-specific blood group antigen gives rise to the syndrome known as *posttransfusion purpura*. This syndrome usually occurs 5 to 12 days (mean, 7 days) after transfusion, when the blood recipient experiences profound thrombocytopenia, with the platelet count usually falling precipitously to below 10,000 per microliter. Although the full mechanism of this syndrome is not understood, it clearly results from an anamnestic response to a platelet antigen. Usually, the affected patients are women who have been previously immunized during pregnancy. The inciting event may be a platelet transfusion but is much more often a product transfused for its red cell content (i.e., whole blood or packed red cells). Most often, the antibody demonstrated is anti-PI^{A1}, but rare instances in which antibodies to other antigens caused this syndrome have been documented. The ability to develop antibodies to the PI^{A1} antigen appears to be linked to HLA genes because only about 10% of exposed women make anti-PI^{A1}, and women who produce anti-PI^{A1} as a result of pregnancy have a high incidence of HLA-Drw52 (23).

The antibody response associated with posttransfusion purpura is uniformly IgG, and the platelet count usually correlates inversely with the antibody titer during the course of the illness, despite the fact that the antibody produced is an alloantibody. Thrombocytopenia appears to occur as an anamnestic response to a platelet alloantigen, although the mechanism whereby the patient's own antigen-negative cells are destroyed remains unclear.

The clinical course of posttransfusion purpura is that of rapid development of severe thrombocytopenia within 12 to 24 hours, accompanied by moderate to severe hemorrhage. An overall mortality of about 10% can be deduced from various studies. Bone marrow examination, if done, usually shows increased numbers of megakaryocytes, so that this syndrome resembles most drug-induced purpuras. The history of recent transfusion, however, along with either prior pregnancy or prior transfusion, should signal consideration of the diagnosis of posttransfusion purpura. If serology is available, demonstration of anti-PI^{A1} in the patient's serum is usually easily achievable, although platelet antigen phenotyping may be prevented by the extremely low platelet count. If left untreated, thrombocytopenia usually resolves over 1 week to 1 month. The most effective treatments are plasmapheresis (plasma exchange) and intravenous gamma globulin (94). Steroids alone do not appear to be of significant benefit, and transfusions of either antigen-positive or antigen-negative platelets neither improve nor worsen the thrombocytopenia.

Leukocytes and Febrile Reactions

Up to several percent of transfusions are associated with febrile reactions, which since the 1950s (8,33), have been attributed to the presence in the recipient of leukoagglutinating antibodies. Typically, the patient is more or less asymptomatic during the early phase of the transfusion (30–60 minutes). Then chills and fever intervene, usually along with increased blood pressure. Some patients also experience headache, nausea, or back pain. Occasionally, the fever is quite high, reaching 40°C. When a febrile reaction occurs, the transfusion should be stopped and the patient assessed for signs and symptoms of hemolysis and sepsis, the latter being a rare but serious complication of transfusion. Antipyretics may be used, and for patients who experience repeated febrile reactions, blood products processed through leukoreduction filters will abolish or cause less severe febrile reactions.

Not all febrile reactions are caused by recipient leukoagglutinins. Apparently, leukocytes in blood during storage may produce a variety of pyrogens and leukokines, including TNF-, IL-1, and IL-6. Platelet concentrates containing a relatively high level of TNF-a and IL-6 are associated with an increased risk of febrile transfusion reactions (68).

One of the most devastating sequelae of the transfusion of leukocytes, usually as unwanted companions of red cells or platelets, is transfusion-related graft-versus-host disease (GVHD). This syndrome ensues when transfused viable lymphocytes set up a graft-versus-host response in a blood recipient unable to mount a host-versus-graft response. Most often, this occurs because the blood recipient is immunocompromised due to an underlying medical condition or therapy. Transfusion-related GVHD has been convincingly documented in immunocompetent patients, however. For example, it has occurred when lymphocytes from a donor homozygous for a specific HLA-A/B allele are transfused to a recipient heterozygous for the same allele. Such a situation would allow a graft-versus-host response but not a host-versus-graft response on the basis of HLA-A/B antigens. Interestingly, no case of transfusion-related GVHD has been reported in HIV-infected patients. Patients most at risk for this syndrome appear to be those with congenital immunodeficiencies and those with hematologic malignancies.

Transfusion-related GVHD can be prevented by irradiation of cellular blood products prior to transfusion. Doses of 25 to 30 Gy are commonly used for this purpose.

Reactions to Plasma and Plasma Products

Plasma contains a complex mixture of proteins, including albumin, coagulation and complement components, and immunoglobulins. Many of these factors can engender a variety of reactions in patients who receive transfusions of either plasma or cellular blood products in which varying amounts of plasma remain.

Passive Transfer of Antibody

Plasma from healthy donors may contain both isoagglutinins (anti-A and anti-B) and alloantibodies to red cell, platelet, or leukocyte antigens. Transfusion of small

amounts of isoagglutinins, such as might be found in the plasma of group O packed red cells, is usually harmless. Transfusion of group O platelets usually involves a larger volume of plasma, however, and both positive DAT results and hemolysis have occasionally been observed following such transfusions (118). Transfusion of alloantibodies to red cell antigens is usually harmless because the amount of antibody transfused is usually insufficient to effect appreciable immune clearance of cells, even when the recipient's cells are antigen positive. At least one case in which anti-K from one unit destroyed antigen-positive cells from a second transfused unit in a K-negative recipient has been documented (1).

Transfusion of alloantibodies to platelet antigens has been documented to cause thrombocytopenia in several antigen-positive recipients (92). In at least some recipients, such antibodies may be of sufficiently high titer that only 10 mL of plasma is required to cause marked thrombocytopenia (95).

Transfusion of alloantibodies to leukocyte antigens may cause a unique and sometimes severe syndrome referred to as *transfusion-related acute lung injury* (TRALI). This syndrome is characterized by fever, chills, nonproductive cough, dyspnea, and hypotension. Radiographic examination of the chest most often demonstrates numerous nodular opacities in the lung fields, predominantly in the perihilar area and lower lung fields; vascular congestion and cardiac enlargement, typically found in the setting of cardiogenic pulmonary edema, are absent. The target antigens of the causative antibodies may be either HLA antigens or neutrophil-specific antigens. Most often the syndrome results from transfusion of leukoagglutinins, although the recipient's own leukoagglutinins also can cause a similar picture (31). Complement activation appears necessary for production of the syndrome in an animal model (93).

Immunoglobulin A-Specific Hypersensitivity Reactions

Approximately 1 in 700 individuals lack both serum and secretory IgA, and a substantial number of them develop anti-IgA class specific antibodies, sometimes without a history of a clear immunizing exposure. When the recipient has anti-IgA antibodies, only a few milliliters of plasma can elicit an immediate anaphylactic reaction, with initial sweating and nausea followed quickly by hypotension, cyanosis, seizures, and death. Thus, any patient with known IgA deficiency or with a history of a severe transfusion reaction of this nature should be given blood products that do not contain IgA. Red cells can be rendered plasma free by freezing and thawing (deglycerolizing) but not by routine washing. Other products must be obtained from IgA-deficient donors.

Urticaria

Hypersensitivity reactions marked by some degree of urticaria with or without other allergic symptoms (e.g., wheezing) are quite common, occurring in 1% to 3% of all transfusion episodes. Some may be due to anti-IgA of limited allotype specificity present in IgA-positive recipients, whereas others may be due to sensitivity to other donor plasma proteins.

NEONATAL ALLOIMMUNE CYTOPENIAS

All fetal blood cells, including erythrocytes, leukocytes, and platelets, carry antigens capable of stimulating maternal alloimmune responses when the mother's own cells lack the antigen. This was first recognized when maternal antibody was identified as the cause of HDN (52,93). Since then, cases of neonatal (and intrauterine) hemolytic anemia, thrombocytopenia, and neutropenia have been recognized as caused by maternal alloantibody.

HEMOLYTIC DISEASE OF THE NEWBORN

The immunologic basis of HDN is the combined existence of (a) paternal antigens expressed on fetal red cells, (b) incomplete separation of the fetal circulation from that of the mother, and (c) active transport of maternal IgG across the placenta. These three factors allow the mother to detect fetal antigens and to produce alloantibody in response and then allow the alloantibody to be transported into the fetal circulation. The vast majority of HDN is caused by maternal antibody to the Rh_o (D) antigen. This antigen is the most immunogenic of the Rh antigens. Because the mother is D negative but the fetus is D positive in about one in ten pregnancies in whites (63), a significant number of pregnancies are at risk for producing maternal alloimmunization to the D antigen. Because fetomaternal hemorrhage is often minimal until the time of delivery, when the average fetomaternal hemorrhage is 1.2 mL, fewer than 1% of at-risk women will have detectable anti-D at the time of delivery of a first D-positive child. Maternal anti-D will be detectable in 10% to 20% of pregnancies in which a D-negative woman bears a D-positive fetus for the second time. Before the era of immunoprophylaxis, about 1 in 100 second pregnancies was complicated by HDN, indicating that not all at-risk mothers produce anti-D, either because of insufficient exposure to fetal D-positive cells or because of maternal nonresponsiveness to this antigen. In addition, the ABO types of the fetus and mother also affect the likelihood of maternal alloimmunization. When small amounts of D-positive blood were transfused into ABO-compatible and ABO-incompatible recipients, more than 70% of ABO-compatible recipients made anti-D, whereas only about 15% of ABO-incompatible recipients responded similarly (99). When recipients of incompatible D-positive blood were then given ABO-compatible D-positive cells, their response rate was similar to that of the group that originally received the ABO-compatible D-positive cells. Maternal sensitization to D is similarly affected by fetomaternal ABO compatibility.

Since the inception of immunoprophylaxis and better treatment methods for HDN when it does occur, HDN occurs less frequently and causes even lower mortality. Pregnant women now undergo ABO and Rh typing early in pregnancy, along with screening for the presence of red cell alloantibodies. Rh-negative women often receive one or two doses of anti-D at between 28 and 34 weeks' gestation. Then, shortly after delivery, Rh-negative mothers receive anti-D if their infant's red cells are D positive. This combination of prenatal and postpartum immunoprophylaxis has reduced the incidence of anti-D HDN to well below 1 in 1,000.

Nevertheless, some cases of HDN do occur, as a result of either other (non-anti-D) alloantibodies or failure of the prophylactic approach. When an alloantibody is found in the serum of a pregnant woman, several factors contribute to decisions about management of the pregnancy. Antibodies with certain specificities tend to cause minimal or no HDN, whereas antibodies to other antigens typically cause moderate to severe disease. In addition to the factors discussed already for immune clearance, the strength of expression of particular antigens on fetal cells and the fact that IgM antibodies do not cross the placenta help determine whether an antibody causes significant HDN. Although ABO mismatch between mother and child is frequent, clinically significant ABO HDN is rare. The A and B antigens are poorly developed on fetal and neonatal red cells. Also, A and B antigens are expressed widely in the body, so that the IgG portion of maternal isoagglutinin that crosses the placenta to the fetus is adsorbed by numerous tissues other than red cells.

Following the titer of an alloantibody during pregnancy is usually not helpful in predicting the severity of HDN, although a rapidly rising titer is a relatively poor prognostic sign when the antibody is one generally associated with significant HDN. Amniotic fluid can be obtained by amniocentesis and examined spectrophotometrically for the presence of hemoglobin breakdown products (bilirubin), and this procedure provides quite a good indicator of the status of the fetus. As fetal maturity also can be assessed via amniocentesis, management then can include intrauterine transfusion of blood compatible with maternal antibody or premature delivery, followed by *ex utero* therapy, if these procedures are indicated. Once an infant is born, therapy may include phototherapy to break down bilirubin and exchange transfusion, with the aims of ameliorating anemia and prevention of hyperbilirubinemia (> 20 mg/dL), which can damage brain cells from bilirubin deposits (kernicterus).

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60 IMMUNOLOGIC ASPECTS OF FERTILITY AND INFERTILITY

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Genetic diversity in the population has numerous advantages, including increased resistance to disease and avoidance of inheritance of undesirable traits. Yet achieving diversity in humans, where the immune system is primed to prevent intermixing of host and foreign entities, is a formidable task. It requires special arrangements of tissues and cells in reproductive organs as well as profound local and systemic changes in normal immune responses.

Gametes and fertilized eggs are at risk and must be protected from both alloimmune and autoimmune processes. The gametes, sperm, and ova express novel surface and intracellular molecules that differ biochemically from somatic cell proteins and are therefore foreign to the host. Protection against autoimmmunity is required in both the ovary and testis. Sperm, which bear molecules that are entirely foreign to females, enter the female reproductive tract and must be protected against alloimmune reactions. Finally, mechanisms must be devised to prevent mothers from rejecting the semiallogeneic fertilized egg, which expresses both paternal and maternal genes during implantation and throughout the course of pregnancy.

Diverse approaches are used to ensure successful reproduction in humans and other mammals. Special tissue barriers are erected to avoid dissemination of gamete antigens into the blood and lymphatic circulations; unique cells called *trophoblasts* sequester the developing embryo from maternal immune cells; and multiple immunoevasive strategies are called into action on a local basis. These latter factors include recruitment of specialized subsets of leukocytes, production of immunosuppressive substances, as well as regulated expression of genes encoding complement regulatory proteins, major histocompatibility antigens, and display of apoptosis-inducing molecules. In short, the same devices used by tumors to escape surveillance and destruction are used during pregnancy to protect the developing embryo.

Not surprisingly, mechanisms erected to avoid autoimmunity and alloimmunity in gamete development and pregnancy may be overcome. Multiple disorders of reproduction are evident in the general population, and immune dysfunctions are likely to account for a certain proportion of cases of infertility and pregnancy failure.

This chapter first lays out the anatomic structures and components of the male and female reproductive systems and discusses the basic features that have evolved to prevent autoimmunity and alloimmunity during gamete development, fertilization, implantation, and pregnancy. Mechanisms that have developed to promote tolerance and avoid graft rejection during reproduction will be presented in detail. Features of autoimmunity and alloimmunity in reproductive organs are discussed, with emphasis on aspects of infertility that have emerged from studying natural disorders and conducting research in experimental systems.

TOLERANCE AND IMMUNE PRIVILEGE IN GAMETE DEVELOPMENT

Autoantigens of the testis and the ovary differ greatly in their ontogeny of expression and accessibility to the immune system. Thus, mechanisms of local immunoregulation and the extent of systemic tolerance to antigens of the two organs also differ.

Ovary

In mammals, the ovary is an almond-shaped structure characterized by an epithelial covering, an outer zone called the *cortex* where follicles develop, and an inner zone called the *medulla* where blood vessels emerge and depart. Stroma surrounding the follicles and vessels contains various populations of leukocytes, including macrophages. Developing follicles are composed of an outer layer of theca cells, an inner compartment of granulosa cells, and a central oocyte. The oocyte is surrounded by a thick glycoprotein coat known as the *zona pellucida* (ZP).

Ovarian Antigens

The ovary-specific antigens that might stimulate autoimmunity that have been investigated most extensively are located in the ZP and include the antigen ZP3 (1) and the oocyte cytoplasmic antigens, which include OP1 (2). In mice, these antigens can be recognized *in vivo* by circulating antibody and antigen-specific T cells in the first 3 days of life (3). Figure 60.1D shows an experimental situation where ovarian ZP is fully accessible to circulating IgG antibody and circulating immune complexes (4,5). This antigen also disseminates to the regional lymphoid nodes and organs to activate ZP-specific B cells in normal mice (6). ZP and oocyte antigens derived from apoptotic oocytes may serve as target T-cell antigens. Many oocytes in prepuberal ovaries and most developing oocytes in cycling adult women undergo apoptosis and are subsequently known as *atretic follicles*. In adult ovaries, the atretic follicles contain oocyte antigens as well as major histocompatibility (MHC) class II positive macrophages and dendritic cells. The oocyte antigens are the main target of $CD4^+$ pathogenic T cells (7,8).

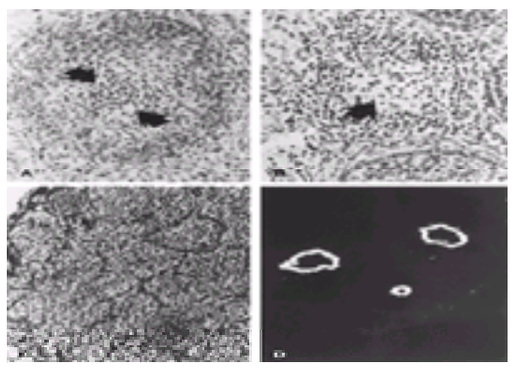

Figure 60.1. Immunopathology of murine autoimmune ovarian disease (AOD) induced by immunization with pZP3 in adjuvant.

Tolerance to Ovarian Antigens

Tolerance to the gender-specific ZP antigen has been demonstrated by comparing ZP3-specific peptide immune responses of normal female mice with male or ovariectomized female mice, which lack ZP (3). This study, which reported pathology in the ovarian graft and antigen specific T-cell cytokine production, suggests that antigen exposure during the neonatal period is not sufficient for tolerance to ZP; continuous ovarian antigen presence is required. Indeed, adult female mice depleted of ovarian antigens for only 7 days are no longer tolerant to the ZP antigen.

Tolerance to murine oocyte antigens includes a critical role for regulatory T cells (reviewed in 9). The thymus and peripheral lymphoid tissues of normal adult and neonatal female mice contain $CD4^+$ T cells with the capacity to induce autoimmune ovarian disease (AOD). These $CD4^+$ T cells do not express the interleukin-2 (IL-2) receptor a-chain (CD25) until activated (10,11). Regulatory T cells develop in the thymus as $CD25^+$ $CD4^+$ mature thymocytes (5%) and exist as a subset of $CD25^+$ $CD4^+$ T cells (5%–10%) in peripheral lymphoid organs (11,12). When $CD25^+$ thymocytes or peripheral T cells from normal mice are depleted, endogenous ovarian antigens rapidly stimulate pathogenic T cells, leading to severe AOD and oocyte antibodies spontaneously (13). Table 60.1 lists a number of murine experimental models for dissecting autoimmunity, of which several involve deletion of the thymus (11,14,15). In all these models, AOD is prevented on reconstitution with $CD25^+$ $CD4^+$ T cells from normal euthymic donors (reviewed in 9). These experiments led to the conclusion that pathogenic autoreactive T cells are present in normal female mice and that they are normally regulated by $CD25^+$ regulatory T cells. $CD25^+$ regulatory T cells operating to ensure self-tolerance also are found in tolerance to autoantigens of testis, thyroid, stomach, prostate, salivary and lacrimal glands, pancreatic islet, and the eye (Table 60.1).

Experimental autoimmune (allergic) orchitis (EAO) and experimental autoimmune oophoritis that result from immunization with tissue antigen:	
Classic EAO induced by immunization with testis antigen with adjuvant	(16–18)
EAO induced by immunization with testis antigen without adjuvant	(19,20)
Autoimmune oophoritis induced by immunization with a murine ZP3 peptide	(1)
Autoimmune diseases of ovary, testis (or other organs) that result from manipulations of the normal immune system:	
Thymectomize mice between d 1 to 4 after birth (DBX model)	(14,21–23)
Transfer adult murine $CD5^{low}$ or $CD25^-$ T cells to athymic mice	(10,24,25)
Treat normal mice with antibody to CD25	(15)
Treat neonatal mice with cyclosporine A	(26)
Engraft fetal rat thymus in athymic mice	(27)
Engraft neonatal mouse thymus in athymic mice	(28)
Transfer T cells from adult or neonatal thymus, neonatal spleen to athymic mice	(10)
Mice with a transgenic V_α protein of the T-cell receptor	(28)
Transfer RT6-depleted rat spleen T cells to athymic rats	(29)
Transfer $OX22^{high}$ (or $CD45RC^{high}$) rat spleen T cells to athymic rats	(30)
Other modes of autoimmune orchitis:	
Spontaneous autoimmune orchitis in dog (31), mink (32), rat (33), human (34)	
Postvasectomy autoimmune orchitis	(35,36)
Orchitis in rats with the transgenic HLA/B27 molecule	(37)

TABLE 60.1. Autoimmune Ovarian and Testicular Disease Models

Mechanisms of $CD25^+$ T-cell Regulation

$CD25^+$ T cells suppress the response of $CD25^-$ T cells *ex vivo,* and this approach has been exploited to investigate the mechanism of T-cell regulation (12,38). For effective suppression, the regulatory and the effector T cells may recognize the same or different antigenic peptides. Regulation requires antigen-presenting cells (APCs), T-cell receptor (TCR) engagement on both $CD25^+$ regulatory and $CD25^-$ effector T cell subsets, T-cell contact, and an anergic state in the $CD25^+$regulatory T cells. Some well-documented antiinflammatory cytokines, including IL-4, IL-10, and transforming growth factor-b (TGF-b), seem not to be involved.

Testis

The male reproductive tract has several compartments of which the testis is the major site of germ cell development. The testes comprise an outer epithelial cell covering and a series of folded seminiferous tubules embedded within stroma. The tubules are sequestered from the stroma by a thick basement membrane. Germ cell development occurs in waves emanating from the outermost border of the tubule, within which reside the Sertoli cells, toward the lumen, where mature sperm are released.

Testis Antigens and the Blood–Testis Barrier

Most testicular autoantigens are expressed in the haploid germ cells. Of these autoantigens, many are unique testicular isoforms of somatic antigens. Because these autoantigens first appear at puberty, they do not interact with lymphocytes early in life (reviewed in 39) and may not, unlike the situation in the ovary, induce early tolerance. Male germ cell antigens may be invisible, entirely ignored by the immune system because of being protected by a complete immunologic blood–testis barrier. In the seminiferous tubule, a formidable tissue barrier is formed by the peritubular myoid cells and the junctional complexes between the somatic Sertoli cells. This barrier effectively separates the haploid germ cells from circulating lymphocytes and antibodies, both of which reach the testis interstitium (40). Presumably, the barrier also limits the access of germ cell autoantigens to APCs outside the seminiferous tubules.

The idea that male germ cells are completely sequestered was challenged by data from experimental autoimmune orchitis (EAO) research. Studies in mice demonstrate that (a) antibody enters the rete testis to bind to spermatozoa (41); (b) autoantigens accessible to circulating autoantibodies exist on the diploid germ cells outside the blood–testis barrier (including the preleptotene spermatocytes that first appear in the second week of life) (Fig. 60.2) (42); (c) in the straight tubules, which link the seminiferous tubules to the rete testis, luminal germ cell antigenic peptides can reach MHC class II positive APCs outside the barrier to stimulate germ cell–specific $CD4^+$ T cells and elicit severe inflammation in that location (Fig. 60.3B) (9,43,44). These findings argue against the concept of a complete blood–testis barrier and emphasize the regional variations in the integrity of the blood–testis barrier within the normal testis.

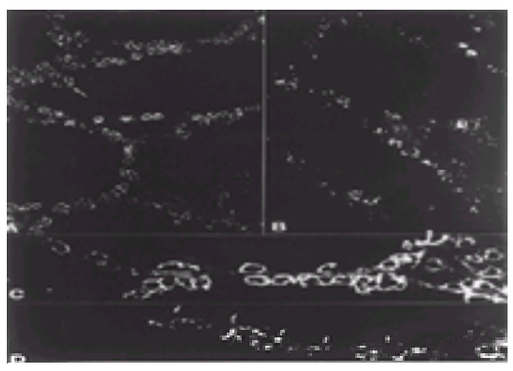

Figure 60.2. Autoantigenic germ cells are present outside the blood–testis Sertoli cell barrier. Mice were injected with testis homogenate in adjuvant and then tissue sections of testis were tested by direct immunofluorescence for bound immunoglobulin G (IgG) at day 7, 5 days before the onset of experimental autoimmune orchitis.

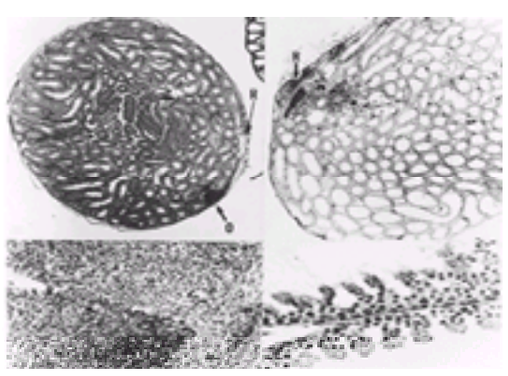

Figure 60.3. Patterns of inflammation in experimental autoimmune orchitis (EAO) are different when EAO is induced by active immunization and by adoptive transfer with pathogenic T cells.

Tolerance and Immune Privilege in the Testis

Immunoregulation against testis autoimmunity is dependent on mechanisms operative at three levels: (a) confinement of most of the germ cell autoantigens within a strong but regionally incomplete tissue barrier, (b) maintenance of tolerance by the same systemic regulatory T cells common to the ovarian autoantigens (22), and (c) local immunoregulation or immune privilege.

Immune privilege in the testis is demonstrated by the prolonged survival of tissue allografts, including parathyroid and pancreatic islets in the testis interstitium (45). Survival is extended despite the presence of circulating antibodies that readily enter the testis interstitium, which is drained by elaborate afferent lymphatics. The testis interstitium contains Leydig cells, resident macrophages, peritubular myoid cells, and Sertoli cells, which communicate via hormones, paracrine factors, and cell junctions (46). Factors produced by these cells may suppress T-cell response to mitogens or anti-CD3 antibody *in vitro*. Candidate suppression factors include IL-1, TGF-b, basic fibroblast growth factor, transglutaminase, prostaglandin E_2, and activin (47,48,49,50 and 51). Although Fas ligand is expressed in the testis, the contribution of Fas ligand to immune privilege by conferring T-cell apoptosis remains speculative (52,53).

ANATOMIC AND CELLULAR CONSTITUENTS OF THE UTERUS AND THE IMPLANTATION SITE

The preceding paragraphs document the special arrangements of tissues and developing gametes in the ovary and testis and discuss how these conditions may permit the development of autoimmune disease. Following release of the ovum from the follicle and fertilization, which occurs in the oviduct (also known as the fallopian tube), the fertilized egg descends into the uterus, which is hormonally and immunologically prepared for implantation.

Cycling Uterus

The uterus of women and experimental animals such as mice and rats is roughly separated into (a) the myometrium, which is composed of smooth muscle cells interspersed with connective tissue containing some leukocytes such as mast cells and macrophages; and (b) the endometrium. A transverse section of rat cycling uterus is illustrated in Fig. 60.4 (left panel). The major components of the endometrium, where implantation takes place, are the luminal epithelia, which continue as a lining for the endometrial glands, and the stromal compartment. In the endometrial stroma, leukocytes are scattered randomly and occasionally are gathered into lymphoid aggregates.

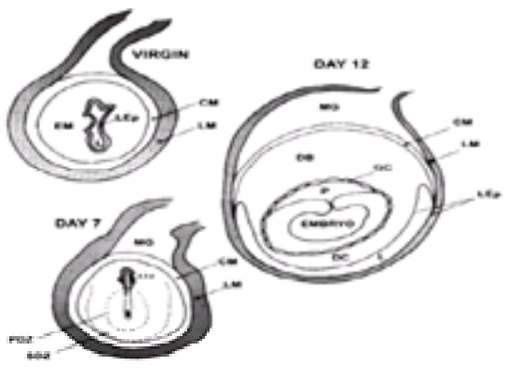

Figure 60.4. Schematic illustration of the cycling mouse uterus, postimplantation changes (gestation day 6) and arrangement of the uterus, placenta, and embryo at gestation day 12. A, amnion membrane; CM, circular muscle of the myometrium; DB, decidua basalis; DC, decidua capsularis; EM, endometrial stroma; GC, giant trophoblast cell layer of the placenta; L, uterine lumen; LEp, luminal epithelium; MG, metrial gland; P, placenta; PDZ, primary decidual zone; SDZ, secondary decidual zone; LM, longitudinal muscle. The position of the blastocyst within the lumen (day 6) and the embryo (day 12) are shown. (Adapted from Chen H-L, Kamath R, Pace JL, et al. Gestation-related expression of the interferon-g gene in mouse uterine and embryonic hematopoietic cells. *J Leuk Biol* 1994;55:617–625, with permission.)

Dramatic cyclic changes take place in the endometrium in response to ovarian steroid hormones circulating in the blood, that is, estrogens and progesterone. In general terms, estrogens drive cell proliferation during the first half of the cycle and progesterone production from the midpoint of the cycle onward supports implantation and pregnancy. Bulmer (55) systematically documented the specific types of leukocytes that populate the human endometrium during the menstrual cycle (Table 60.2). These leukocytes are likely to provide a measure of innate or natural protection against infectious disease and a limited degree of cell mediated immunity, but the uterus is not a major site of immune reactivity. Antibody in uterine secretions is derived mainly from blood, and the organ is refractory to stimulation of local immunity to sexually transmitted organisms (56,57).

Stage	Marker Expression[a]			
	CD45	CD56	CD3	CD14
Proliferative	160	85	75	52
Early secretory	190	95	80	52
Late secretory	325	190	75	80
Early pregnancy	320	250	60	120

[a] CD45 marks all leukocytes; CD56 marks natural killer lineage cells; CD3 marks T-lymphocytes; CD14 marks macrophages.
Adapted from Bulmer JN. Immune cells in decidua. In: Turpin M, Fernandez N, eds. *Immunology of human reproduction*. Oxford: BIOS Scientific Publishers, 1995:113–134.

TABLE 60.2. Leukocytes in the cycling and early pregnant uterus (Mean Cell Numbers per Four High-Power Fields)

Implantation Site

Implantation takes place during the late proliferative phase (human) or estrus phase (mouse, rat) (Fig. 60.4, center panel). During the time of transit of the fertilized egg from the fallopian tube (also referred to as the *oviduct*) to the uterus, the uterine epithelium is altered into a receptive profile, which includes reduction of cilia on luminal epithelial cells, changes in the integrins and other cell-to-cell and cell-to-substratum adhesion molecules, and alterations in the secretory profiles of endometrial cells (58,59). The changed state is referred to as the *implantation window* and is temporary in nature.

Implantation has been termed a mild inflammatory reaction. When the blastocyst penetrates the endometrial epithelial cell lining, extensive tissue damage occurs that is readily identified by blood pools and leukocytic accumulations (Fig. 60.5). Importantly, examination of tissues by histologic techniques reveals no evidence of graft rejection at the normal implantation site or during the course of uneventful pregnancy. Although events are not clear at the early human maternal–fetal interface, which is difficult to obtain and study, experiments in mice show that inflammatory cytokines such as IL-1 and interferon-g (IFN-g) are produced (60,61). These are not only present at the interface but also circulate in the mother's blood, where they could affect systemic immunity. Following implantation, the uterine epithelium seals over the implant, which then is sequestered within the modified endometrium, now called the *decidua*.

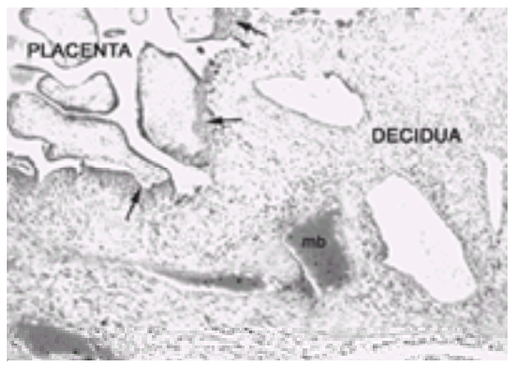

Figure 60.5. Histologic section of a gestation week 8 human placenta showing attachment and invasion of the decidua by extravillous trophoblast cells emerging from villi (*arrows*). Modified maternal arteries surrounded by fibrinoid maternal and containing pools of maternal blood (mb) mark the implantation site in the decidua. Stained with hematoxylin and eosin. Original magnification, ×100. (From the Boyd Collection, Department of Anatomy, Cambridge University, courtesy of G. Burton.) (See Color Figure 60.5.)

Growth and development of the embryo and extraembryonic membranes require many of the same factors as those used in the bone marrow to promote leukocyte maturation and development. These include both colony-stimulating factors and cytokines. One of the first examples of this overlap in function was colony-stimulating factor-1 (CSF-1), or macrophage CSF (M-CSF), which is produced at extraordinarily high levels in uterine epithelial cells during the periimplantation period in mice and in trophoblast cells in mice and humans (reviewed in 62). Both proinflammatory and antiinflammatory cytokines are present at early stages; tumor necrosis factor-a (TNF-a) is an example of the former (63), and TGF-b is an example of the latter (64). The specific functional roles of these powerful cytokines are as yet poorly documented but may well include programming placental cell growth and differentiation. TNF-a is, for example, known for its cell lineage-specific, differentiation-altering properties in the bone marrow (65,66).

TOLERANCE AND IMMUNE PRIVILEGE IN IMPLANTATION AND PREGNANCY

In the mid-twentieth century, immunologists were just beginning to understand the dimensions of transplantation and graft rejection. At this time, Medawar proposed the first set of potential explanations for the surprising willingness of mothers to tolerate genetically different tissues during pregnancy (67). His explanations included anatomic separation between the mother and fetus, antigenic immaturity of the fetus, and immunologic tolerance in the mother. Although the situation is considerably more complex than originally envisioned, it is now known that essentially all the explanations proposed by Medawar have an element of truth. Multiple mechanisms permit mothers to tolerate tissues expressing both self (maternal) and nonself (paternal) genes, and these include major accommodations and contributions from both the mother and the fetus (Fig. 60.6).

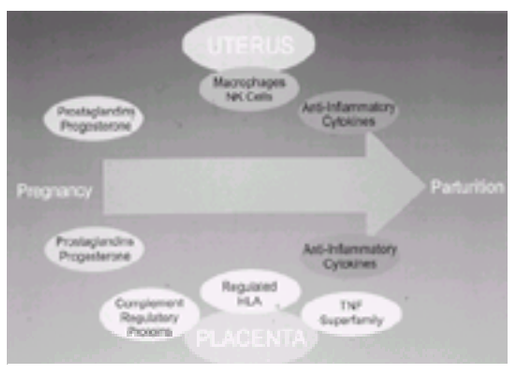

Figure 60.6. Schematic illustration of the progress of pregnancy toward parturition wherein maternal elements (uterus) and fetal elements (placenta) contribute to immunologic protection of the semiallogeneic fetus. NK, natural killer; HLA, human leukocyte antigens; TNF, tumor necrosis factor.

Maternal–Fetal Interface

Protection for the embryo against the maternal immune system components dedicated to ridding the host of foreign objects rests with the trophoblast layer. As illustrated schematically in Fig. 60.7, the inner cell mass from which the embryo proper develops is secluded beneath multiple layers of trophoblastic cells throughout pregnancy. These cells are derived from the external trophectoderm layer of the blastocyst. As illustrated in Fig. 60.8, one trophoblast subpopulation migrates into the decidua, where the cells are exposed to maternal leukocytes resident in this tissue. Later in pregnancy, these so-called extravillous cytotrophoblast cells form the chorion membrane. Another subpopulation syncytializes to form the outermost layer of the floating villi; this subpopulation is continually exposed to maternal blood leukocytes. New studies implicate the HERV-W endogenous retrovirus in syncytialization (68), and viruses also may play a role in the selection of certain human leukocyte antigen (HLA) class I genes for display by trophoblast (69).

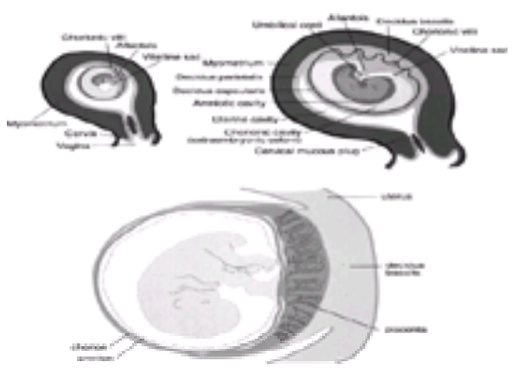

Figure 60.7. Schematic illustration of three stages of human pregnancy, from early implantation to late gestation. In the upper left-hand panel, the first trimester embryo resides in the uterus. In the upper right-hand panel, growth of the embryo and differentiation and development of both fetal and maternal structures have taken place in the second trimester. In the lower panel, the term fetus floats in amniotic fluid encased with the extraplacental membranes (chorion, amnion) and placenta. Note that at no time is the embryo/fetus directly exposed to maternal blood or tissues; the embryo/fetus is continuously secluded within a trophoblastic shell.

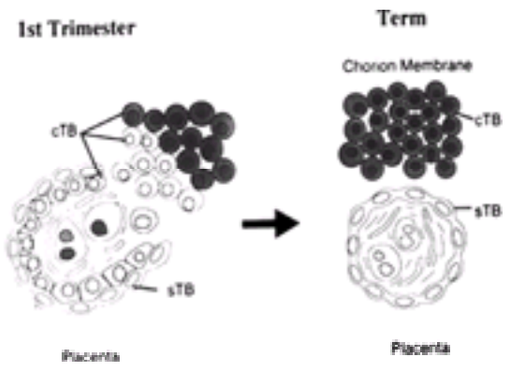

Figure 60.8. Schematic drawing of trophoblast subpopulations and their expression of membrane-bound human leukocyte antigen (HLA) class I molecules. Progenitor cytotrophoblast cells (cTB) merge to form syncytiotrophoblast (sTB) in the floating villi or proliferate and migrate in columns into the decidua to anchor the placenta and replace the endothelial cells of the maternal spiral arteries. Later in pregnancy, these migrating cells form the chorion membrane. Note that only these latter cells (*dark gray*) express membrane-bound HLA class I antigens.

The physical intimacy of fetal and maternal tissues during pregnancy raises the critical question of how protection against the maternal immune system might be provided. Several decades of study showed that multiple mechanisms have evolved that are contributed by both the mother and the extraembryonic tissues of the fetus (70,71). Some of the major systems are illustrated schematically in Fig. 60.6. Less emphasis has been placed on the reverse situation; the fetal immune system develops gradually and is unlikely to be capable of developing antimaternal immunity until mid to late pregnancy. For example, macrophages in the placenta do not express HLA class II antigens until the second trimester (72,73); so until that time, they are incapable of acting as fully effective APCs. The extent of the contributions of fetal immune mechanisms in relationship to maternal–fetal immunologic interactions remains to be explored in depth.

Uterine Privilege

Immune privilege in the pregnant uterus is provided by a number of mechanisms that are not operative in the cycling uterus.

Cellular Alterations in Pregnancy

A dramatic change in the relative proportions of uterine cells involved in innate and acquired immunity marks the first visible immunologic adjustment made in the mother to accommodate the semiallogeneic embryo. The invading fetal trophoblastic cells are met by vast numbers of natural killer (NK) cells and macrophages, which outnumber T- and B-lymphocytes (Table 60.2). The macrophages likely provide a measure of innate immunity to the decidua, as suggested in the early studies on mice (73). Functions of the uterine NK cells remain obscure; although they contain granzymes, TNF-a and perforin, knockout models fail to identify any major reproductive problems in the absence of these cytolytic molecules (74,75,76 and 77). Their major roles may be unique to pregnancy. For example, murine uterine NK cells appear to participate in both decidual and placental growth (77,78) as well as in development of blood lacunae around the placenta through production of nitric oxide (NO) (79). Some g/d T cells appear to be present, and there is evidence for a population of double-negative (DN, $CD4^-/CD8^-$) T-lymphocytes (80,81). The roles of these latter cells remain unclear; even though small in terms of absolute numbers, the cells could have critical functions.

Soluble Immunosuppressive Agents Progesterone, prostaglandins, and T-helper cell 2 (Th-2)-type cytokines.

Uterine immune privilege is provided in part by immunosuppressive molecules. Progesterone is the dominant hormone of pregnancy and has the ability, in high concentrations, to simulate corticosteroids, a well-known suppressor of immune responses. For example, progesterone inhibits the production of TNF-a mRNA and protein in macrophages (82,83). Other critical soluble modulators include prostaglandin E_2 from resident macrophages and decidual cells as well as antiinflammatory cytokines, such as IL-10. Wegmann and co-workers first proposed that pregnancy is a Th2 phenomenon (84), and studies in mice confirm that high levels of Th2-type cytokines are typical of pregnancy (61). Because Th2-type cytokines drive antibody-mediated responses in preference to cell-mediated immune responses, the cytokines probably account for the systemic bias in mothers favoring antibody production. This condition of pregnancy has a major impact on women with preexisting autoimmune disease, exacerbating some, such as systemic lupus erythematosus, and alleviating others, such as rheumatoid arthritis (85).

Placental Privilege

The placenta and its attached membranes, which interface directly with maternal blood and tissues, have a remarkable ability to protect themselves from maternal immune cell attack. The mechanisms utilized by trophoblast cells vary with their state of differentiation and anatomic location. As mentioned, it is this unique cell type that protects the embryo and components of the extraembryonic membranes derived from the inner cell mass. As illustrated in Fig. 60.6, multiple devices are used by these tissues to avoid maternal immune cells and antibody cytotoxicity.

Protection Against Cell-Mediated Immune Attack: Membrane-Bound Placental Molecules [HLA, indoleamine 2,3-dioxygenase (IDO), TNF superfamily]

Regarding prevention of immune cell attack, perhaps the best studied of the mechanisms is control of expression of the major histocompatibility antigens. In human placentas, extravillous trophoblast cells migrating into the decidua express a unique pattern of class I HLA, with HLA-G, HLA-E, and HLA-C predominating (86,87). Figure 60.9 shows HLA-G protein and mRNA in these cells. The HLA-G and -E antigens encoded by these genes, which have few alleles in comparison with HLA-A, HLA-B, appear to interact with uterine NK cell and possibly also uterine macrophage inhibitory receptors, which include CD94/NGK2A, ILT2, and immunoglobulin-like transcript 4 (ILT4) (88). Class I antigens on trophoblast also interact with TCRs on $CD8^+$ cells (89). Based on interactions in other contexts, the consequences probably include activation of killer inhibitory pathways in NK cells and macrophages and possibly death of $CD8^+$ T cells because of activation of the Fas/Fas ligand programmed cell death pathway by soluble HLA class I antigens (90,91).

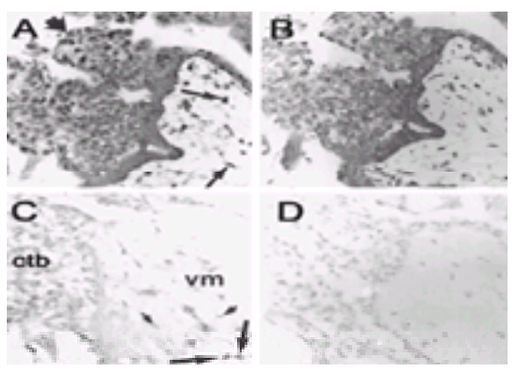

Figure 60.9. *In situ* hybridization and immunohistochemical staining demonstrate that human leukocyte antigen-G (HLA-G) mRNA and protein are present in discrete populations of cells in first-trimester placentas. **A**: HLA-G mRNA is evident in migrating trophoblast cells (*large arrow*) as well as macrophage-like villous stromal cells (*smaller arrows*). **B**: A sense version of the HLA-G probe does not hybridize to placental tissues. **C**: HLA-G protein is identified using the mouse monoclonal antibody, 87G, specific for HLA-G1. Signal is present in migrating cytotrophoblast cells (ctb) and in some villous mesenchymal cells (vm). **D**: The immunostaining control is negative. Paraformaldehyde-fixed tissue was used for (A,B), and flash frozen, acetone-fixed tissue was used for (C,D). Original magnifications: ×250. (See Color Figure 60.9.)

In contrast to the migrating extravillous cells, syncytiotrophoblast forming the outermost layer of the placental floating villi, which is exposed to maternal blood, seems to express no membrane-bound HLA class I antigens, although there is evidence for class I message and for the presence of soluble class I antigens in this layer early in pregnancy (92,93). Syncytiotrophoblast in term placentas lacks HLA class I mRNA (94), and this is reflected in a lack of membrane-bound HLA class I protein (86,87). None of the subpopulations of trophoblast cells expresses HLA class II antigens *in vivo*, which may be due to the trophoblast-specific repressor of gene expression

identified by Murphy and Tomasi (95). Thus, the overall pattern in trophoblast is of strict control over the expression of genes that could encode potentially dangerous, paternally derived foreign MHC (HLA-A, -B, -D). This is accompanied by expression of antigens that effectively prevent killing by cells of the innate immune system (HLA-G, -E).

Recent experiments in mice suggest that a second mechanism preventing attack on trophoblast by maternal T-lymphocytes is expression of an inhibitor of tryptophan metabolism, IDO in trophoblast cells. T-lymphocytes require tryptophan and in its absence are inactivated. Studies by Munn et al. point to a role for this system in protection of early mouse trophoblast and suggest that there is an MHC-related aspect (96).

Apoptosis-inducing members of the TNF supergene family also may have an important role in protecting the placenta and its membranes from killing by maternal immune cells. The ligands identified in or on human trophoblast cells include TNFa, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL) (97). TRAIL localization to the syncytiotrophoblast microvillous membrane in early human pregnancy is shown in Fig. 60.10. These powerful molecules, which are expressed in both membrane and soluble forms, could kill activated immune cells that bind to trophoblast by transducing an apoptotic signal through specific receptors on activated leukocytes, as is the case in other immune privileged organs, such as the eye (98). Studies in mice suggest that FasL may be of particular importance (99), with trophoblast cell FasL preventing maternal immune cell attack on the placenta by interacting with leukocyte receptors (Fig. 60.11). These include TNF-R1, Fas, and the TRAIL apoptosis-inducing receptors, DR4 and DR5. Evidence for this active, aggressive pathway in placentas is not strong at present, but the placenta is clearly protected from the reciprocal pathway. Killing of placenta cells is abrogated by synthesis of soluble receptors (TNF), exhibition of nonfunctional receptors (Fas), and expression of decoy receptors (TRAIL DcR1) (100,101 and 102).

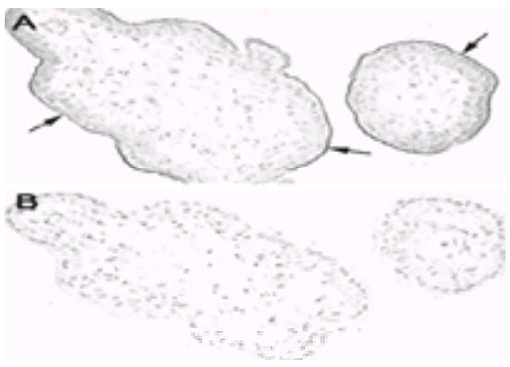

Figure 60.10. Immunohistochemical stain shows that TNF-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) supergene family, is localized to the microvillous membrane of early human early syncytiotrophoblast (*arrows*).

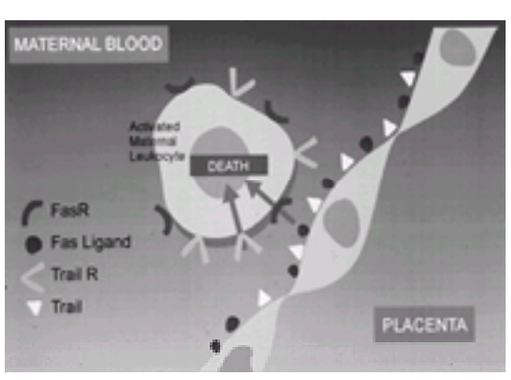

Figure 60.11. Schematic drawing of a potential pathway by which two tumor necrosis factor (TNF) superfamily molecules, FasL and TRAIL, could protect placentas from leukocyte toxicity. The two ligands, when expressed on trophoblast, could interact with specific receptors on leukocytes and transduce apoptosis-inducing signals, resulting in death of the leukocyte.

Protection Against Cell-Mediated Immune Attack: Soluble Immunomodulators

In placentas, as in pregnant uteri, immune privilege is also conferred by immunosuppressive molecules. For example, production of progesterone, prostaglandin E_2, and antiinflammatory cytokines, such as IL-10 and IL-4, characterize the human placenta (103,104,105,106,107 and 108). Production of antiinflammatory cytokines by placental cells may be driven by progesterone, as is the case with lymphocytes (109). IL-10 may have an additional role, that is, stimulation of production of HLA-G (110). As in the uterus, Th1-type cytokines also are produced; these appear to have major roles in (a) the initial event of implantation, (b) immune privilege throughout pregnancy, (c) differentiation and development of the placenta, and (d) parturition.

Protection against Cytotoxic Antibodies

Trophoblast is noted for its extremely high expression of the complement regulatory proteins, that is, CD46 (membrane cofactor protein, MCP), decay-accelerating factor (DAF), and CD59 (111). This system is believed to be of critical importance; mothers routinely produce high titers of antibodies to paternally derived HLA and unique trophoblast antigens such as placental alkaline phosphatase. Figure 60.12 shows that two of these molecules, CD46 and DAF, are differentially distributed in trophoblast subpopulations in first-trimester placentas, and are well positioned to prevent complement-mediated lysis. A study in gene-deleted mice showed that, in the absence of the complement regulatory protein encoded by the *Crry* gene, complement is deposited in the placenta and a major inflammatory reaction results in placental disruption and fetal demise (112). This constitutes the first direct evidence supporting a role for this family of proteins in placental immune privilege.

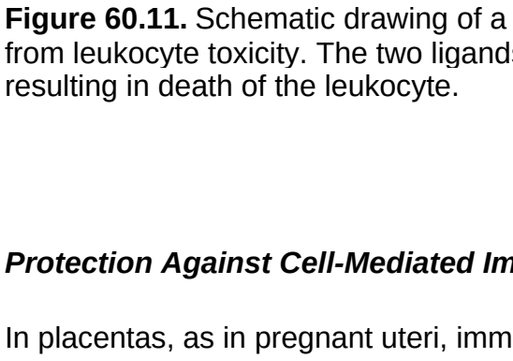

Figure 60.12. Immunohistochemical staining of human first-trimester placentas for membrane cofactor protein (MCP, CD46) and decay accelerating factor (DAF, CD55). Note that both these complement regulatory proteins are expressed but are differentially located, with MCP being prominent on villous cytotrophoblast (left panel, *arrows*) and DAF being prominent on migrating cytotrophoblast cells (right panel, *arrows*). Original magnifications: ×250. (See Color Figure 60.12.)

Type of Placentation and Immune Responses

The systems described here seem to have developed slowly during the course of evolution. It is generally held that the more extensive the invasion of the uterus by placental cells, which is great in humans, less vigorous in rodents, and not a feature of the horse implantation site, the more sophisticated, restrictive, and diverse are

the mechanisms developed to provide protection to the embryo from elements of the host (maternal) immune response.

Antibody Transport into the Fetus

Many maternal antibodies reach the fetal blood circulation, where they constitute the major immunologic protection of the infant up to the age of 6 months. IgG antibodies are routinely transported across the syncytiotrophoblast via a specific class I–like molecule, the neonatal FcR (113,114), and into the stromal of the placenta, which is contiguous with the cord and embryo. Stromal cells and ultimately the embryo itself are protected from potentially harmful maternal antipaternal IgG antibodies by the placental macrophages known as *Hofbauer cells*, which ingest immune complexes (115). When the antibody load is excessive, as is the case in hemolytic disease of the newborn due to anti-Rh and other erythrocyte antibodies, the placenta is overburdened. Harmful antibodies reach the fetal circulation, and damage to fetal cells ensues. These destructive antibodies are likely to employ the same pathway from mother to fetus via syncytiotrophoblast transport, diffusion through the placental villous stroma, and transport across the fetal capillary endothelium (116) that is used by the beneficial antibodies.

Maternal Systemic Immune Responses

The question of whether mothers are immune compromised in a systemic sense has been difficult to resolve; however, the consensus of considerable experimentation is that there is little or no generalized depression of immune responses in pregnancy. Instead, selective suppression may occur. As discussed, Th2 cytokines circulate in mothers and maternal production of antibodies remains intact; multiparous women are excellent sources of antibodies to paternal HLA. Yet there is evidence that maternal B-lymphocytes specific for paternal HLA are partially deleted during pregnancy (117). Similarly, T-lymphocytes specific for paternal HLA are difficult to demonstrate (118). Experiments in transgenic mice suggest that pregnancy selectively depresses maternal T cells recognizing paternal H-2 class I (119). This suppression in pregnancy extends protection to tumors of the same haplotype as the embryo but not to tumors of other haplotypes.

Mothers harbor circulating trophoblast cells for as long as 27 years (120,121). It has been shown that some of these cells localize to lesions in scleroderma, suggesting that certain “autoimmune” diseases may have an alloimmune basis (122).

IMMUNOLOGIC BASIS OF INFERTILITY: GONADS AND GAMETES

Basic research on autoimmunity has generally outpaced our understanding of clinical autoimmune disease. Human ovarian and testicular autoimmune diseases remain incompletely defined with respect to prevalence, mechanism, diagnosis, and treatment. On the other hand, the clinical relevance of experimental models for most autoimmune diseases has not been firmly established. Nevertheless, research based on models of experimental autoimmune disease of the gonads has contributed significantly to the overall concepts of autoimmune disease pathogenesis and prevention.

Autoimmune Disorders in the Ovary: Clinical and Experimental Conditions

Autoimmune disorder in the ovary (AOD) is a known cause of human premature ovarian failure and is also a component in the different forms of autoimmune polyendocrinopathy (123,124). The diagnosis of human AOD depends on the detection of serum autoantibodies to ovarian antigens and, in rare ovarian biopsies, the histologic finding of noninfectious inflammation (reviewed in 125). The most commonly detected autoantibodies react with steroid-producing cells common to ovaries, placenta, adrenal, and testis, with the P_{450} side-chain cleavage enzyme and the 17a-hydroxyl enzyme as candidate antigenic molecules (126,127). Indirect immunofluorescence studies also detect antibodies to oocyte cytoplasm and the ZP (128). The clinical diagnosis of AOD is often elusive, however, because of the paucity of ovarian immunopathologic data and the uncertainties associated with the method and target antigens for measurement of autoantibody and autoreactive T-cell responses. As a result, the true prevalence of immunologic human premature ovarian failure has not been defined.

Pathogenesis

The pathogenesis of AOD has been investigated in several murine models (Table 60.1). Two of these are particularly informative: (a) AOD induced by immunization with a murine ZP3 peptide (pZP3) that contains both T-cell epitopes and a native B-cell epitope (1), and (b) AOD that develops spontaneously in mice thymectomized between days 1 and 4 after birth, usually on day 3 (14). This model is referred to as D3TX. Figure 60.13 summarizes the recent findings and presents a working hypothesis for mechanisms underlying the pathogenesis of murine AOD.

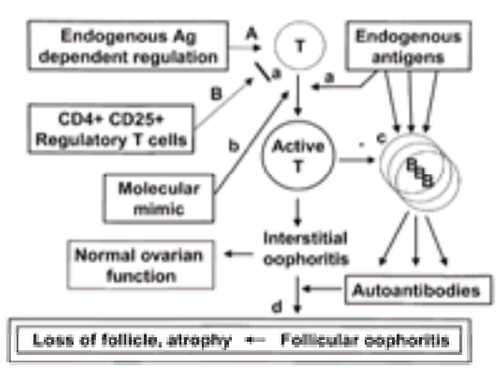

Figure 60.13. Proposed mechanisms for autoimmune disease prevention and induction based on studies of murine autoimmune ovarian disease (AOD). In the pathway marked (**A**), persistent endogenous antigen is required for maintenance of tolerance to oocyte antigen. The pathway marked (**B**) illustrates one known tolerogenic mechanism where $CD25^+$ regulatory T cells play a central role. Disease may occur when (a) the $CD25^+$ T cells are depleted, which permits endogenous antigens to stimulate a pathogenic T cell response; (b) T cells are stimulated by nonovarian peptides that mimic ovarian peptides; (c) autoreactive B cells are rapidly stimulated by endogenous antigen to produce autoantibodies to the ZP3 antigen; (d) antibody binds to the zona pellucida and T cells are retargeted, leading to ovarian atrophy. In the absence of autoantibodies, T-cell reactivity does not interfere with normal ovarian function.

Autoimmune disorder of the ovaries is induced when regulatory $CD25^+$ T cells are depleted by D3TX. In these mice, endogenous ovarian antigens spontaneously stimulate pathogenic T-cell responses within 2 to 3 weeks, and this leads to ovarian inflammation, followed rapidly by ovarian atrophy and development of oocyte antibodies. AOD also can be induced by stimulation by exogenous antigens through the mechanism of T-cell epitope molecular mimicry. Thus, immunization with foreign or unrelated self-peptides with partial sharing of amino acid residues appears critical for T-cell induction, which can occur commonly; of 16 randomly selected nonovarian peptides, 44% induced AOD (129,130). At least one infectious agent has been implicated in the pathogenesis of AOD. The enteric nematode (the rodent pinworm) operates as a cofactor and, together with a nonimmunogenic form of ovarian pZP3, elicits AOD in neonatal mice. Infected mice are imprinted with long-term memory for the autoantigen and mount a rapid and intense autoimmune response when challenged at a later date (131). Although most pathogenic T cells that elicit AOD belong to the Th1 $CD4^+$ subset, there is evidence that Th2 $CD4^+$ T cells also elicit AOD and result in eosinophilic oophoritis. In pinworm-infected mice, autoreactive T cells produce IL-4 and IL-5 but not IFN-g, and the ovaries are infiltrated by numerous eosinophils (131). Human ovarian inflammation with dominant eosinophilic inflammation also has been reported (132).

An unexpected mechanism of autoantibody induction and an unexpected action of autoantibody in tissue inflammation were uncovered during the study of murine AOD induced by pZP3 (Fig. 60.13). Ovarian antibodies are stimulated spontaneously and rapidly by endogenous ovarian antigens in mice where T cells for ovarian antigen have been activated (133,134). Thus, mice immunized with a pZP3, which lacks native B-cell epitopes, develop IgG ZP3 antibodies that recognize distant B-cell epitopes of ZP3, and the antibodies do not cross-react with the pZP3 immunogen. The antibody response is abrogated in mice with ovarian ablation. Thus, autoreactive B cells to ovarian antigens are not tolerized but are primed by endogenous antigens. This discovery, made in parallel with studies on diversification of autoantibody responses to lupus autoantigens, provides the critical evidence that endogenous antigens can drive a diversified antibody response. Although ZP antibodies do not cause AOD, they strongly influence the distribution of AOD induced by $CD4^+$ T cells. As mentioned earlier, atretic follicles in the ovarian interstitial space are the prime targets for pathogenic T cells. Although T cell-mediated interstitial oophoritis may be severe, its occurrence is compatible with normal ovarian function (Fig. 60.1B) (7). When ZP antibody is also present, the T cell-mediated inflammation is retargeted or shifted to the ovarian follicles, leading to ovarian atrophy (Fig. 60.1A,C) (8). Finally, the critical costimulatory molecules required for pZP3 T-cell activation and AOD induction have been identified; both CD28 and CD40 ligand pathways are required and sufficient for pZP3 pathogenic T- and B-cell activation (135).

In yet another approach to studying the pathogenesis of AOD, disease-associated genetic loci were mapped and their mechanisms elucidated. Studies on genetic loci that influence AOD induced by D3TX have uncovered six non-H2 chromosomal loci associated with ovarian inflammation, autoantibody induction, and ovarian atrophy

(Table 60.3) (136,137, Roper et al., in preparation).

Locus	Phenotype	Chromosome (cM)[a]	Colocalization[b]
Orch1	EAO/orchitis	17	H2
Orch2	EAO/orchitis (BALB/c substrains)	Unmapped	eae
Orch3	EAO/orchitis	11 (48)	*eae7, Idd4*
Orch4	EAO/orchitis	1 (110)	*Ste1c*
Orch5	EAO/orchitis	1 (20)	
Orch6	EAO/orchitis	8 (34)	*eae14*
Bphr	*B. pertussis*-induced histamine sensitivity	6 (37)	*eae*
Epd1	EAO/epididymitis	16 (4)	
Vbt1	EAO/vasitis	1 (33)	
Aod1	D3TX AOD oophoritis	16 (24)	*eae11*
Aod2	D3TX AOD atrophy	3 (30)	*eae8, Idd3*
Aod3	D3TX AOD ovarian autoantibody	11 (17)	
Aod4	D3TX AOD ovarian autoantibody	7 (9)	
Aod5	D3TX AOD antinuclear antibody	6 (67)	

[a] Centromeric assignment based on the MGD map (www.informatics.jax.org/).
[b] Colocalization with other autoimmune disease susceptibility loci.
Adapted from Tung KSK, Fuji E, Teuscher C. Autoimmune diseases of the spermatozoa, ovary, and testis. In: Theofilopoulos A, ed. The molecular pathology of autoimmune diseases. Gordon and Breach Science Publishers/Harwood Academic Publishers (in press).

TABLE 60.3. Loci Controlling Susceptibility and Resistance to Murine Experimental Allergic Orchitis (EAO) and Embryonic Day 3 Thymectomy (D3TX)-Induced Autoimmune Ovarian Dysgenesis (D3TX-AOD)

Autoimmune Disorders in the Testis: Clinical and Experimental Conditions

Immunopathology

Male infertility can result from autoimmune disease that affects the testis or its excurrent ducts. Spontaneous autoimmune orchitis has been reported in spontaneously infertile animals, including mink, dogs, and rats (31,139). Some human testicular diseases have immunopathologic features that resemble the testicular changes of autoimmune orchitis of the infertile mink. They include (a) granulomatous orchitis of noninfectious basis that presents as enlarged testis, a differential diagnosis of testicular tumor (140); and (b) aspermatogenesis without orchitis in testis with local immune complexes (34). Epididymal granulomas of noninfectious origin also may have an autoimmune basis (reviewed in 125).

Model System and EAO Development

As in AOD, there are many murine models of EAO (Table 60.1). EAO can be induced by immunization with testis antigen, including the testis-specific isoform of hyaluronidase (PH20), or it can occur spontaneously following D3TX (Table 60.1) (22,141). In addition, vasectomy leads to autoimmune responses to spermatozoa in human and animals, and EAO is a known complication of vasectomy in the guinea pig and rabbit (35,36). Cloned T cells that transfer EAO are predominantly of the Th1 type, and passive transfer of EAO by Th1 cell clones is largely ameliorated by neutralizing antibody to TNF-a (44). T cell transfer results in testicular inflammation that is initiated in a unique region, the straight tubules, blocking the outflow of sperm and fluids from the seminiferous tubules (Fig. 60.3) (9). In contrast, EAO induced by active immunization of testis antigen in adjuvant results in diffuse EAO that affects the seminiferous tubules and the straight tubules equally. Thus the differential permeability of the blood–testis barrier dictates the distribution of EAO within the target organ.

A major question is how inflammation, which initiates in the testis interstitium, overcomes immune privilege and bypasses the blood–testis barrier. EAO is induced by immunization with testis antigen in adjuvant (23). Following injection of testis antigen and adjuvant (or adjuvant alone), and before the onset of EAO, there is a dramatic accumulation of MHC class II–positive, F4/80-positive cells in the interstitium. Thus, recruitment or activation of local APC to the testis, including macrophages and dendritic cells, occurs in response to adjuvants. The relevance of this finding to EAO is supported by an *in vitro* study on Sertoli cell responses to cytokines (142). When puberal Sertoli cells are stimulated by TNF-a, they express intercellular adhesion molecule-1 (ICAM-1), become more adhesive to T cells, and secrete the proinflammatory cytokine IL-6. It is therefore possible that, in response to cytokines produced in the testis microenvironment, activated macrophages that are induced or recruited into the testis interstitium may provide the proinflammatory cytokines that can alter integrity of the blood–testis barrier and direct the antigen specific T cells to the boundary of the tubules. This leaves unanswered the question of the location and nature of the APCs and the source of the germ cell peptide. In addition to macrophages, other testicular cells also produce TNF-a and IFN-g (143,144). Thus, once tissue injury has occurred, endogenous proinflammatory cytokines may propagate the inflammatory destruction in EAO. The intrinsic testis cytokines may be perhaps of greater relevance in murine EAO induced by testis cell injection without adjuvant (20) (Table 60.1).

Genetic Components

Genetic analysis of inbred mouse strains indicates that the susceptibility or resistance to EAO is strongly influenced by both H2 and non-H2 genetic loci. As shown in Table 60.3, nine chromosomal regions have been linked to EAO. As in the case of the *aod* gene loci, many of the *eao* loci are found in chromosomal regions that govern the susceptibility to other autoimmune diseases, such as experimental autoimmune encephalomyelitis and insulin-dependent diabetes of the nonobese diabetic (NOD) mice.

AUTOIMMUNE AND ALLOIMMUNE RESPONSES TO SPERM

Male and female infertility may result from antibody to sperm-specific antigens, and the response may originate in either the male or the female reproductive tract. Sperm antibodies have been reported in patients with cystic fibrosis, and development of antibodies follows vasectomy, testicular trauma, torsion, biopsy, tumor, and infection (145,146,147,148,149 and 150). Most sperm antibodies, however, are detected without associated abnormalities in the reproductive tract. In these idiopathic cases, the trigger of the immune response to sperm is not known. It has been postulated that defective local immunoregulatory mechanisms in the male or female reproductive tract may be important.

Despite many years of research, a direct link between antisperm antibody and male or female infertility has not been conclusively established. Most research effort has been devoted to develop reliable methods for sperm autoantibody detection. Popular methods include the mixed agglutination reaction (151) and immunobead assays (152). It has been reported that altered semen quality is more frequent when both IgM and IgG antibodies are detected and less frequent when IgG alone is present (153). Retrospective analysis suggests that a low fertilization rate may be associated with sperm decorated with combined IgA and IgG head-bound antibodies (154,155). Antisperm antibodies may impede sperm penetration of the cervical mucus in the postcoital test, where the number and motility of sperm within the cervical mucus are evaluated 8 to 12 hours after sexual intercourse (156). Antibodies also may alter and affect various stages of sperm–egg fusion (157), including the acrosome reaction (139,158,159), ZP binding (160,161 and 162), oocyte fusion (32,163,164,165,166 and 167), and early cleavage of fertilized oocytes (168). Although medical treatment of infertility associated with sperm antibodies is not satisfactory at present, some successes have been reported with the use of assisted reproduction techniques (169).

Research on contraceptive vaccines has driven new efforts to identify functional sperm and oocyte antigens involved in fertilization events. Complete infertility has been documented in female guinea pigs immunized with the testis isoform of hyaluronidase, PH20 (170). Significant reduction of fertility has been observed in female mice injected with a chimeric peptide that contains a B-cell epitope of mouse ZP3 (171,172). Additional sperm antigens that conferred significant infertility in female mice also included the testis-specific LDH-C4 (173) and SP17 (174). In addition, many potentially important human and animal sperm and oocyte molecules have been identified by means of serum antibody from infertile patients or with monoclonal antibodies that affect fertilization events (175,176).Therefore, research on a contraceptive vaccine based on sperm and oocyte antigens will help to determine their relevance in fertilization events and therefore their role in human infertility.

IMMUNE FACTORS IN EARLY PREGNANCY TERMINATION

The preimplantation human blastocyst produces chorionic gonadotropin (hCG), which facilitates the identification of potential pregnancy. Using this biochemical marker and sensitive assays, it has become clear that perhaps as many as 50% of potential pregnancies are lost prior to or during implantation (177). Whether any of these losses can be accurately referred to as “immunologic,” that is, based on maternal recognition and rejection of normal embryos expressing foreign (paternal) antigens, is problematic, and cause and effect relationships are unclear.

Implantation and Maternal Immunorejection

In mothers who spontaneously abort their fetuses, Th1 rather than the expected Th2-type cytokines are produced when their blood mononuclear cells are incubated

with preparations made from tumor-derived trophoblast cell lines (178). In inbred mice, macrophages producing high levels of NO flood into resorption sites (179). These observations support the idea that some mothers perceive embryos as foreign and mount immune rejection responses. If the blastocyst is fully adequate and intact, however, the embryo should be entirely protected from trophoblasts using the mechanisms described previously. If the blastocyst is genetically deformed and not fully intact, the mother might perceive exposed paternally derived antigens and mount a graft rejection response. In cases of recurrent abortion, a secondary immune response would be expected to cause early rejection. Alternatively, some mothers may lack essential components of the networks that provide immunologic protection to the embryos, such as appropriate expression of complement regulatory proteins, apoptosis-inducing TNF superfamily members, and HLA-G or -E. These and other aspects of infertility remain poorly explored.

Infection

The periimplantation period is the time at which interruption of pregnancy is most common. Yet pregnancy still may be disrupted by various physiological conditions. Infection of the decidua and membranes appears to be one of the most disastrous situations, although vaginosis is also common (180,181). The pathways leading from infection to preterm labor are not fully understood, but clearly involve immune mechanisms and cells. Chemotactic substances such as IL-8 are produced, and both neutrophils and macrophages swarm into infected membranes. In many cases, lipopolysaccharides (LPS) from Gram negative bacteria activate the leukocytes, leading to further inflammation, production of prostaglandins and other potent modulatory substances such as TNF-a. The sum of these interactions is stimulation of a cascade that culminates in myometrial contractions and the induction of labor. A major therapeutic tool in these situations is the administration of glucocorticoids to the mother.

Recurrent Pregnancy Loss and Leukocyte Immunization

Based on early clinical results (182), some fertility specialists are committed to immunizing potential mothers with paternal or third-party leukocytes as a means for improving reproductive success. This strategy developed because homozygosity within couples, particularly at some HLA loci, is associated with reduced fertility (183,184), and some researchers thought that leukocyte immunization would introduce a needed measure of immune recognition and stimulation. A multicenter study showed conclusively that leukocyte immunization is not effective and strongly suggested that reproductive outcomes might be worsened rather than improved (185).

SUMMARY AND FUTURE PERSPECTIVES

The complexity of the reproductive process results in multiple points at which immunologic factors might impinge on reproductive success (Table 60.4). These range from potential recognition of secluded gamete antigens to overwhelming cytokine responses in infections. Information emerging from studies on specific aspects of immunity have permitted recognition and dissection of many of these factors and can be expected to yield further insights in the future. Conversely, the situation of pregnancy, where the immune system is diverted for the benefit of diversity, has permitted a better understanding of several aspects of transplantation and tumor immunology.

Disorder	Potential Causes
In situ loss of gametes	Autoimmune disease (recognition of unique gamete antigens)
Destruction of sperm	Alloimmune recognition in the uterus by maternal immune system
Implantation failure	Inappropriate expression of HLA on trophoblast
	Lack of production of IDO (tryptophan inhibitor) in trophoblast
	Inappropriate expression of complement regulatory proteins
	Incorrect balance of Th1- and Th2-type cytokines
	Inadequate progesterone to drive Th2 response
Preeclampsia	Lack of production of IL-10 and/or HLA-G
	TNF-2 allele producing excessive TNF-a
IUGR	Lack of nitric oxide
Immunorejection	Defective or deficient complement regulatory proteins
	Aberrant expression of TNF superfamily ligands/receptors
Premature labor	Infection and excessive Th1-type cytokines, prostaglandins
Scleroderma	Residual trophoblast in mothers

HLA, human leukocyte antigen; IDO, indoleamine 2,3-dioxygenase; IL-10, interleukin-10; IUGR, intrauterine growth retardation; Th-1, T-helper cell type 1; Th-2, T-helper cell type 2; TNF, tumor necrosis factor.

TABLE 60.4. Disorders of Fertility Associated with Immunologic Factors

Fertility remains suboptimal in the general population. Although many pregnancy losses are attributable to genetically incorrect fetuses, others result from inappropriate or inadequate measures to protect the fetus in either the embryo/fetus or the mother. As subtle features of the immune system are elucidated, it is reasonable to expect application to this problem and improvement in fertility rates and the overall success in pregnancy.

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61 ALLERGENS

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In 1873 Charles Blackley (26) published *Researches on Cattarhus Aestivus*, which established that grass pollen was the cause of hay fever in England. At the same time, Wyman (286) demonstrated the association between ragweed pollen and hay fever that occurs during late summer in the United States. Blackley introduced skin tests, pollen counts, and nasal and bronchial challenges; but perhaps most important, he demonstrated that patients with positive wheal-and-flare skin tests to pollen extracts developed symptoms at that time of the year when grass pollen grains were airborne. Thirty years later Noon (156), convinced erroneously that pollen caused symptoms because it was toxic, started immunizing patients with pollen extracts and reported the first successful trial of desensitization. Over the next 30-year period, a series of enthusiasts applied skin testing and desensitization injections to many allergens and diseases. Studies on these extracts established that most allergens were proteins, although the activity in grass pollen extracts was so heat stable that some authors initially thought the allergens could not be proteins.

During the early twentieth century, antibodies were generally demonstrated by precipitin reactions, and sera from patients with hay fever did not give rise to precipitin reactions with extracts of the relevant pollen. This led to the erroneous description “supersensitivity without immunity” (265). That the serum of allergic patients contained a specific active substance was first demonstrated by Prausnitz and Küstner in 1921 (190). They showed that local injection of serum from a person who was allergic to fish (Küstner) could passively transfer wheal-and-flare skin sensitivity to a nonallergic individual (Prausnitz). This P-K test was used extensively to establish the specificity of the response to allergens. However, the nature of the active substance (or reagin) remained unclear. In 1935 Cooke et al. (49) demonstrated that patients treated with desensitization injections develop neutralizing antibodies in their serum in addition to the P-K activity.

Further progress in understanding allergens and the immune response to them did not come until the 1960s when modern immunochemistry made possible both the purification of allergens and the purification of immunoglobulin E (IgE). First, King (119) purified ragweed antigen E (now Amb a 1), and then Marsh (137) purified rye grass allergen I, rye I (now Lol p 1). The terminology for purified allergens was established by an International Union of Immunologic Societies (IUIS) subcommittee report: each allergen is assigned the first three letters of the genus, followed by the first letter of the species (not italicized) and an arabic number for the order of purification (121,138). Thus Fel d 1 was the first allergen purified from the domestic cat, *Felis domesticus*. In 1966 Ishizaka et al. (105) proved that the active factor in serum was a novel class of immunoglobulin, IgE, and went on to establish that IgE binds to a specific high-affinity receptor on mast cells and basophils (104). These studies proved that the “toxicity” of allergens was directly attributable to their interaction with specific antibody, which in turn triggers the release of histamine and other mediators from mast cells and basophils. Crude extracts of allergens contain many different proteins as well as low-molecular-weight substances that may irritate the skin or cause symptoms when inhaled. Furthermore, many proteins may have biologic activities that influence the immune response. However, most purified allergens have no effect on the skin, nose, or lungs of nonallergic individuals (119,137,172). The purification of IgE led to the production of anti-IgE and the development of the radioallergosorbent test (RAST) by Wide et al. (275). It also became possible to radiolabel allergens and, with double-antibody assays, to study further the immune response in allergic individuals (171,181). As the assays improved, it became clear that patients with immediate hypersensitivity have serum IgG antibodies, nasal IgG and IgA antibodies, and serum IgE antibodies. Some nonallergic individuals have IgG antibodies, but most have no antibody response to allergens (172). This conclusion led to a general view of allergy as a state of enhanced immune responsiveness such that repeated low-dose exposure (usually by the inhaled route) produces an immune response that includes local IgA antibodies as well as serum IgG and IgE antibodies. Thus an allergen is an otherwise benign protein that commonly gives rise to IgE responses in individuals who are genetically predisposed. In practice, most allergens are soluble proteins that are repeatedly inhaled because they become airborne either outside or indoors.

OUTDOOR ALLERGENS

Pollen Grains

Most flowering plants are fertilized by pollen grains landing on the stigma and triggering the production of a pollen tube. This process is rapid, is absolutely specific, and depends on the fact that all pollen grains rapidly release species-specific proteins. The plants can be broadly divided into those that are wind pollinated and those that are insect pollinated. The wind-pollinated plants release large numbers of pollen grains, which can float for miles (Table 61.1). In contrast, the insect-pollinated plants (e.g., roses, tulips, magnolia trees) produce relatively few grains, and these grains are generally not found in the air. Some pollens are released but are so large (larger than 40 µm) that they remain airborne for only a short time (e.g., the pollens of the Virginia pine and most cereal crops). Most important wind-borne pollens are 20 to 30 µm in diameter and have a surface that is specifically designed to help them remain airborne (Fig. 61.1). Thus the number of pollen grains airborne is influenced by the number of plants, the number of grains released, and their aerodynamic properties. In most areas where these factors have been studied, there is a good correlation between the number of pollen grains that become airborne and their importance as causes of sensitization and seasonal hay fever. Grass pollen is the most common outdoor pollen and the dominant cause of hay fever in northern California, New Zealand, England, and many other countries. In Sweden the highest pollen count by far is from birch trees, which is the primary cause of hay fever there (19,264).

TABLE 61.1. Major Sources of Pollen Associated with Hay Fever

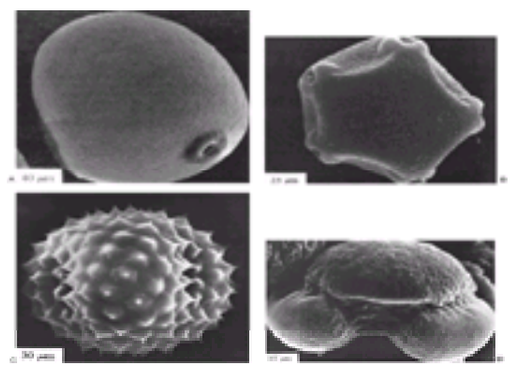

Figure 61.1. Scanning electron micrograph of pollen grains showing pores and overall size.

In the United States, there are dramatic regional and seasonal variations. In much of the Midwest and East Coast, short ragweed is the most common airborne pollen during the fall season and the most common cause of hay fever (159). In the Rocky Mountains and the Southwest, mountain cedar and the chenopod amaranth group are the major sources of pollen and the most important causes of symptoms. Release of pollen from a wind-pollinated plant varies from day to day and during the day. The main factor influencing release appears to be decreasing relative humidity (for ragweed, 70% or less). Therefore most plants release the largest quantity of pollen during the early morning on sunny days, but this release is delayed by mist, clouds, or rain. The pollen seasons for various plants vary dramatically in different parts of the country (223,279). The trees and grasses generally pollinate in the spring or early summer; and the farther south they are geographically, the earlier pollination occurs. By contrast, many of the summer-flowering weeds, including ragweed, pollinate slightly later in the South.

The seasons for most plants are based more on changes in the length of the day than on temperature (223,279). The release of allergens from pollen grains has been studied in detail for ragweed, grass, and birch (19,137). In each case, release is rapid, although the rate may be slower for some proteins. Estimates of the quantity of major allergen in grass, ragweed, or tree pollens are generally close to 0.5 ng per grain (137). Thus a pollen grain can be seen as ideally designed to deliver proteins to the nasal mucosa, and the most important plants are those that release a large number of pollen grains that remain airborne.

Fungal Spores

Fungal spores are released by many varieties of fungi and are often present in the air in large numbers (i.e., more than 1,000/m^3) (Table 61.2). Fungal spores vary in size from about 2 µm in diameter (e.g., *Penicillium*or *Aspergillus*spp.) to about 20 µm in length (e.g., *Alternaria*spp.) (30,220). Spores of 2-µm diameter have a volume about 1/1,000 that of a pollen grain, which is 20 µm in diameter. The biologic role of a spore is as a seed, designed to germinate when the conditions are optimal (Fig. 61.2). Spores have no need to release proteins rapidly and indeed are commonly protected by a waterproof shell. Spores of *Aspergillus fumigatus*do not contain measurable quantities of the allergen Asp f 1 but start to produce it within 6 hours of germination (10,225). These results suggest that *A. fumigatus*spores become a source of this allergen only after germination. In contrast, easily measurable quantities of the *Alternaria*allergen, Alt a 1, are found in the outside air during the late summer (162). It is not clear whether this allergen is associated with spores, hyphae, or some other fragment. Fungal spores germinate in conditions of high humidity (i.e., 80% or greater relative humidity); in addition, many fungi, particularly mushrooms, release spores at times of high humidity. Spore dispersal is triggered by absorption of humidity (i.e., the opposite of the conditions for pollen release). The Basidiomycetes produce large numbers of airborne spores. In New Orleans, it has been reported that epidemics of asthma occur during the peak appearance of Basidiomycete spores (202). In addition, surveys of patients with asthma have confirmed that 15% to 20% of them have positive skin tests to extracts of Basidiomycete spores (128). Although it is clear that large numbers of fungal spores are airborne in the outside air, their role in allergic disease is difficult to define. Many fungal spores are much smaller than pollen grains, but these small spores should not be regarded as quantitatively or qualitatively equivalent to pollen grains as a cause of allergic symptoms. The thermophilic Actinomycetes produce large numbers of spores, which are an important cause of hypersensitivity pneumonitis. They grow in moldy hay, garbage heaps, and bagasse, but they have not been associated with immediate hypersensitivity or asthma.

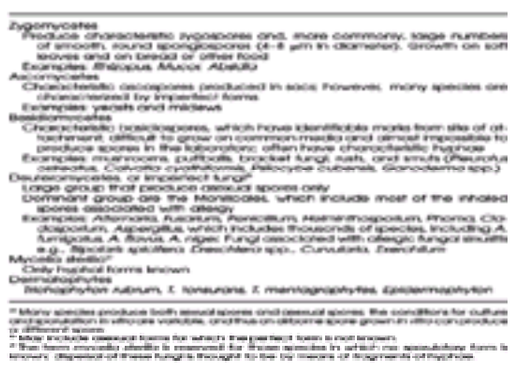

TABLE 61.2. Fungi in Allergic Disease[a]

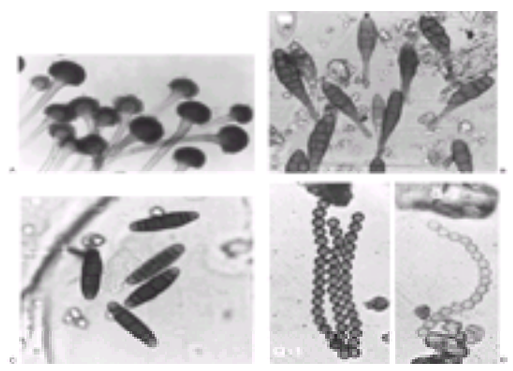

Figure 61.2. Fungal spores and spore heads.

Other Outdoor Allergens

The outside world contains hundreds of animals and plants that could produce inhaled allergens. In addition to the large numbers of insect species, there are nematodes, many species of mites, algae, protozoa (including *Naegleria*and *Acanthamoeba*species), and ferns that can release antigenic material. Thus the allergic symptoms that many patients report when mowing lawns, moving leaf mold, or simply on a windy day could be due to a wide range of antigens in addition to pollen grains or fungal spores. For many of these sources, skin test extracts are not available or are poorly characterized. Indeed, for most of these allergens, in the absence of a seasonal increase in the quantity of the antigen airborne or good extracts for skin testing, it is difficult to evaluate their importance as a cause of symptoms. The origin of allergens produced by insects is variable. The scales on the wings of some moths are an important source of allergen particles. For the nimitti fly, allergens are

components of hemoglobin present in the single particle of meconium that these flies produce after they hatch (113,140). The allergen in Japanese river flies also is a hemoglobin that some species produce in larger quantities when they are growing in polluted (i.e., oxygen-poor) streams (106).

The relevance of outdoor insects has been clearly established in several parts of the world. In the Sudan, the nimitti fly breeds in pools of stagnant water along the Nile. It hatches in vast numbers and can give rise to both IgE antibodies and allergic symptoms (113). Similarly, hatching of lake flies in Wisconsin and river flies in Japan has been clearly associated with local epidemics of allergic disease including severe asthma attacks (106). Positive skin tests have been reported to a variety of other outdoor species, including moths, crickets, ants, beetles, and cockroaches. It is safe to assume that symptoms develop in some patients because of exposure to these insect species. However, in the absence of epidemics occurring at the time of increased exposure, it is difficult to assess their importance relative to other outdoor sources.

INDOOR ALLERGENS

Estimates suggest that North Americans spend at least 23 hours per day indoors, much of this time in their own houses, and the time spent outdoors is likely to decrease further (189). Positive wheal-and-flare tests with extracts made from house dust were first reported in 1921 (115). During the 1920s and 1930s, various antigens that contribute to house dust were defined, such as cat dander, fungi, horsehair, and kapok (Table 61.3). However, several investigators believed that there was antigenic activity in house dust that was not explained by the then-known allergen sources. Indeed, some postulated the existence of a specific house dust "atopen," suggesting that the antigen is derived from human dander. Many species of mites were known during the nineteenth century, but they were not recognized as a major feature in houses. The first suggestion that mites were an important source of house-dust allergens came from Dekker (56) during the 1920s, who recognized mites in bedding dust. His work was largely ignored, though, perhaps because it was not possible to grow mites or produce extracts at that time. In 1967 Voorhorst et al. (267) demonstrated a strong correlation between the allergenicity of house dust and the presence of mites of the genus *Dermatophagoides*. More important, they developed techniques for growing mites so that it became possible to study mites and to produce skin-test extracts (266). In many parts of the world, a strong association between asthma and dust-mite sensitivity was rapidly recognized (44,142,217). This association was not so evident in the United States, and reports from Chicago, Kansas City, and Boston suggested that cockroach sensitivity might be as important in relation to inner-city individuals with asthma (23,98,250). Similarly, studies in the dry climate of northern Sweden showed that among individuals with asthma, animal-dander sensitization was more common than mite sensitization (62). For dust-mite allergens, there is consistent evidence that higher domestic exposure correlates with a higher percentage of children who become sensitized. However, the evidence is not clear for exposure to animals; indeed, several studies have suggested that children living in a house with a cat or a dog have a *decreased* risk of asthma and sensitization (87,200,228,235). There are reports of separate areas with a strong association between chronic asthma and sensitivity to dust mites, cockroaches, and animal-dander allergens (227,229). However, these findings do not diminish the conclusion that the allergens most commonly associated with chronic asthma are primarily indoors and that they show much less seasonal variation than do the outdoor allergens (174,176).


Arachnids
Dust mites
Dermatophagoides pteronyssinus
Dermatophagoides farinae
Euroglyphus maynei
Blomia tropicalis
Storage mites
Predator mites: Cheyletidae
Spiders
Insects
Cockroaches
Blattella germanica (German)
Periplaneta americana (American)
Blatta orientalis (Asian)
Others: crickets, flies, beetles, fleas, moths, midges, wood roaches
Mammals
Cats, dogs, ferrets, horses, rabbits, guinea pigs, Vietnamese pigs
Fungi
Growing indoors: multiple species, including *Penicillium*, *Aspergillus*, *Rhizopus*, and *Mucor* (growing on surfaces or rotting wood)
Growing outside: enter with incoming air (e.g., *Alternaria*, *Cladosporium*)
Pollens
Derived from or entering the house*
Miscellaneous
Horse hair in furniture, tepees
Food dropped by inhabitants
Rodents
Pets: Mice, gerbils, guinea pigs, hamsters
Wild mice, rats

* In domestic houses, the only source of incoming air is leakages (i.e., doors, windows, unused chimneys, ventilation systems). Humidifiers and systems for air entry. Air exchanges in domestic houses varies from 0.3 to 0.5 air changes per hour.

TABLE 61.3. Sources of Indoor Allergens

Dust Mites

Mites are eight-legged members of the order Acari (Fig. 61.3). They are photophobic, sightless, and absorb water from the environment through a hygroscopic material exuded from their leg joints. The pyroglyphid mites, which live on human skin scales and dominate the fauna of house dust, include *Dermatophagoides pteronyssinus, Dermatophagoides farinae,* and *Euroglyphus maynei* (7,24,256,274). The only other mites that are found with any regularity in bedding dust (in the United States, United Kingdom, and Australia) are predator mites of the genus *Cheletidae* and the tropical *Blomia tropicalis* (7,24). Many species of storage mites contaminate stored grain and other foods; they are common in barn dust and may be found in house dust. Farmers often develop immediate sensitivity to them (257). Exposure of these allergic individuals to dust containing storage mites can cause immediate symptoms of "barn asthma," distinct from farmer's lung disease, which is a delayed response not associated with immediate hypersensitivity (50). Mites found in houses are referred to as domestic mites, but the term house-dust mites is reserved for pyroglyphid mites. Mites excrete fecal pellets with a chitinous peritrophic membrane that maintains their size but does not prevent the proteins in them from escaping (Fig. 61.3B). The digestive enzymes produced by mites are important allergens, and fecal pellets are a major form in which these mite allergens accumulate and become airborne (37,246,247). Mites reproduce by laying eggs, which hatch in approximately 10 days; the larvae then go through several stages before becoming adults. The adult male and female mites of the major species are relatively easy to distinguish by microscopy, but the larval stages are difficult to distinguish. In the United States *D. farinae* was reported to be the most common mite in some areas (256). In contrast, in southern England, Brazil, and coastal Australia, *D. pteronyssinus* is the most prominent mite (11,166,226). The primary reason for these differences appears to be that one of the *D. farinae* larval stages can survive long periods of low humidity. Thus *D. farinae* is the most common mite in areas where there is a dry winter but a hot, humid summer [e.g., Cincinnati (8)], but *D. pteronyssinus* is generally more common in areas of the United States that have continuous humidity (i.e., the Southeast, Pacific Northwest, and Gulf Coast).

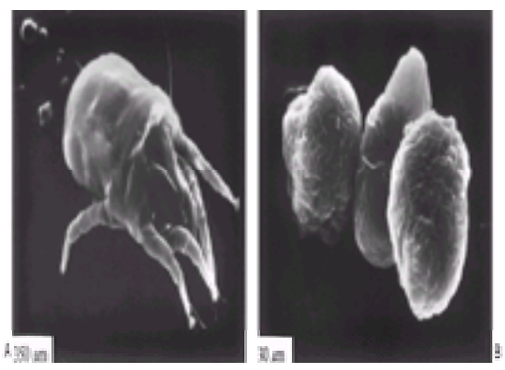

Figure 61.3. Adult dust mite *Dermatophagoides pteronyssinus*. The mite has eight legs, and the pads are clearly visible on the front legs. Several fecal pellets also are visible to illustrate the difference in size.

Because of their photophobia and susceptibility to dehydration, mites require a nest in which to live and breed. Mattresses, sofas, carpets, bedding, pillows, and clothing are suitable sites for growth if humidity and temperature conditions are adequate (24,274). In most houses, the highest levels (up to 1,000 mites/g of dust) are found in bedding or sofas, although high levels can be found in any fabric or soft material (177). Mites have "sticky" pads on their feet. Consequently, when dust is collected from carpets or sofas, the mites recovered are mostly dead; even repeated vacuum cleaning does not reduce the number of live mites in a carpet. Effective measures to reduce exposure to mites include the following: physical barriers that are impermeable or have pores small enough to exclude fecal particles [i.e., 6 µm or less in diameter] (261); washing bedding weekly at temperatures that kill mites (130°F) (135,165); removing carpets, upholstered furniture, and curtains that can act as nests for mite growth; controlling humidity with air conditioning or dehumidifiers (relative humidity less than 55% or absolute humidity less than 7 g/kg) (125,177); and regular vacuum cleaning with a cleaner that has a good filtration system (262,282). In areas where the humidity and temperature are high during the summer, houses should either have air conditioning or be kept simple, without carpets, curtains, or upholstered furniture.

Cats and Other Domestic Animals

The importance of cats (*Felis domesticus*) as a cause of sensitization has been clear since the introduction of skin tests. Indeed, a history of increased symptoms on exposure to a cat or on entering a house with a cat is one of the most reliable features of an allergic history. After purification of Fel d 1 by Ohman et al. (161), it became possible to assay for cat allergen. Initially, it appeared that saliva was the major source of cat allergen. Although it was known that allergen came off the cat when air passed over it, it was assumed that the allergen reached the fur when the cat groomed itself (Fig. 61.4) (239). A series of experiments has now cast doubt on the importance of saliva. First, when a cat was shaved and physically restrained from licking itself, allergen accumulated on the skin (39). Second, when cats were repeatedly washed, the allergen on the fur or the allergen that was becoming airborne was dramatically reduced 1 week later, although cats lick themselves for hours after being washed (51,76). Finally, when the gene for Fel d 1 was cloned, it was possible to develop RNA probes and demonstrate that the gene for Fel d 1 is expressed strongly in the glands of the skin (146). It now seems likely that the skin is the main source of cat allergen, and that small flakes of dander are the likely form in which the allergen becomes airborne. Although cat urine contains some allergen, it is not an important source of Fel d 1 in house dust.

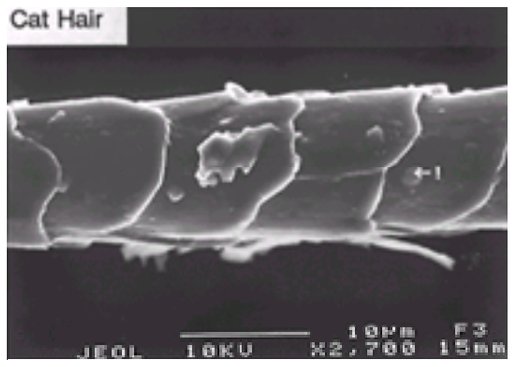

Figure 61.4. Scanning electron micrograph of a cat hair showing typical structure and multiple small “flakes” that may be typical of the dander that becomes airborne. The actual particles that become airborne have not been identified (×2700).

The allergen released by cats appears to be “sticky”; it adheres to walls and accumulates in large quantities in carpets and sofas (281). Between 90% and 98% of the cat allergen in a house has been estimated to be in the furnishings; therefore, it is not surprising that cat allergen persists in a house for many weeks after the cat has been removed (280). Rapid cleaning of a house after the cat has been removed requires removing the carpet and furniture and washing all surfaces (281). Although it is clear that cat allergen is present in the furnishings and that the allergen remains airborne after the cat is removed, the relative contribution of the cat and of the reservoirs (carpets, sofas, walls) to airborne allergen is not clear (51,71,258,281). Individual cats show tremendous variation in the total quantity of Fel d 1 that they carry (15). Based on airborne allergen measurements and estimates of the total quantity of Fel d 1 on cats, the amount of Fel d 1 shed has been calculated to be ~0.002% of the total allergen present on the cat (15). Thus other factors that influence how particles carrying cat allergen become airborne may be as important as the amount of allergen on the animal. There is currently much debate regarding the role of cat washing in reducing airborne allergen levels and the quantity of allergen in the reservoirs. In a blinded, comparative controlled study, weekly washing of cats was reported to have no effect on airborne Fel d 1 levels (123); however, this study did not measure quantities of allergen removed by washing. There is no doubt that washing cats, and even the simple measure of wiping cats with a damp cloth, can remove large quantities of cat allergen and cause a reduction in airborne Fel d 1 levels immediately after washing (15,51,168). However, it appears that, for most cats, reductions in airborne Fel d 1 are not maintained 1 week later (15).

Dogs also are an important source of airborne allergens, and the major allergen Can f 1 can be measured. However, in most countries, dogs seem to be less important than cats in this regard, in large part because they spend more time outdoors. Studies in Virginia and Delaware strongly suggest that animals kept outside are less likely to provoke sensitization. In addition, washing the dog is often a weekly routine for dog owners. Nevertheless, it has been reported that, to control airborne levels of Can f 1, it would be necessary to wash the dog at least twice a week (93).

Cockroaches and Other Insects

Cockroaches were first recognized as a significant allergen among inner-city patients during the 1960s (23,98,250). These results have been confirmed and linked to results on exposure levels in houses (32,73,185). Although direct comparison of cockroach allergen levels with other indoor allergen levels is not yet possible, the relative levels of cockroach allergen in Chicago, Wilmington (Delaware), and central Atlanta are high relative to mite or cat allergens (32,73). Many species of cockroaches live in houses, but the German (*Blattella germanica*) and American (*Periplaneta americana*) varieties are the most common. More is known about the allergens of *B.germanica*. When roaches are grown in the laboratory, large quantities of debris (or frass) accumulate at the bottom of the bottle. Frass, which consists of feces, shed skin, and saliva, is rich in allergens. The feces contain allergen, but allergen also accumulates on the outside of the insects and can be recovered by washing (187,252). This finding suggests that cockroach allergens are secreted or excreted. Indeed, the aspartic proteinase, Bla g 2, is probably a digestive enzyme secreted in the feces (12).

Various insects are used for biology research and pharmaceutical testing protocols. There are many reports of laboratory personnel who become allergic to the locusts, moths, crickets, and cockroaches with which they work. There is little doubt that a wide range of insects is capable of sensitizing humans, but the relevance of other insects in homes is not clear. Although homes can be heavily infested with crickets, beetles, flies, or spiders, the situation is usually transient and is less common than infestation with cockroaches. Occasionally, individuals become allergic to a wide range of indoor arthropods, but these cases are not common compared with those related to allergens from mites, cats, dogs, or cockroaches.

Rodents

IN ANIMAL HOUSES

Rodents are by far the most common mammals used for medical research, and allergic reactions to them are a major problem among professional animal handlers and research workers (2,45,117,191). The main source of allergen is protein in the rodents' urine (153,154,178). This finding is not surprising because rodents, especially male rats, have heavy proteinuria (154). Assays have been developed for measuring mouse (Mus m 1) and rat (Rat n 1) allergens, but only limited data are available for allergen levels in domestic houses (170). The urinary allergen accumulates in the bedding of the rodents and probably becomes airborne as a dried layer on small fragments of the bedding material. Several factors increase the exposure and symptoms of laboratory workers, including the use of fine sawdust rather than large wood chips or an absorbent paper material, drying the bedding, and the direction of the airflow in the animal house (178,238,240). Especially designed biodegradable foams and other synthetic bedding materials have been recommended as a method of decreasing exposure. Because some animals, particularly rats, develop pneumonia without high levels of airflow, federal law requires that animal houses have high air-exchange rates (at least 10 air changes per hour). High quantities of rat, mouse, guinea-pig, and rabbit urinary allergens accumulate in cages. Therefore IgG antibodies develop in a large proportion (i.e., ~50%) of animal handlers, regardless of whether they become allergic. As many as 20% of exposed individuals develop positive wheal-and-flare skin tests and serum IgE antibodies. In general, symptoms of rhinitis and asthma on exposure to experimental animals correlate well with the presence of IgE antibodies (or positive skin tests), whereas those individuals with IgG antibody alone generally report no symptoms (179). The high prevalence of IgG antibodies without allergy to rat allergen among animal handlers may, in part, reflect “selection bias” by the workers. However, exposure is very high, and it has been reported that these IgG antibodies are predominantly of the IgG4 isotype. Thus the response of the animal handlers to rat urinary proteins may reflect a modified T-helper type 2 (Th2) response.

IN HOMES

Rodents are found in houses as pets (usually in cages) or as pests. The many case reports of children and adults who become allergic to pet rodents and improve after the animal is removed leave no doubt that a rodent in a cage can give rise to sensitization and symptoms. Although there are no studies on the distribution of pet rodent allergens in houses, it is assumed that the cage is the dominant source. Rodent cages should not be allowed in the bedrooms of children with symptoms or a strong family history. That wild rodents in domestic houses can be a problem is established by positive skin tests to rodent urine among individuals with asthma, particularly in New York City, and by measurements of airborne rat and mouse allergen in their houses (237). The lack of positive skin tests among individuals with asthma living in the suburbs suggests that the levels of allergen in houses with an occasional wild mouse are not sufficient to give rise to sensitization or symptoms.

NONINHALED ALLERGENS

Commensal Fungi

The human body carries many commensals, including bacteria and both opportunistic (e.g., *Aspergillus* spp.) and nonopportunistic (e.g., dermatophytes or yeast on the skin) fungi. Although these organisms do not usually provoke symptoms, they can, ranging from trivial irritation such as tinea versicolor to major problems such as trench foot or bacterial sinusitis. During the 1930s, it was thought by some that bacteria and fungi could become allergens (48). However, the lack of antibiotics and systemic antifungal agents made it difficult to determine if these “intrinsic” allergens contribute to allergic disease. Nonetheless, there were repeated reports of positive immediate hypersensitivity skin tests to a wide range of commensal organisms.

The first noninvasive fungus to be recognized as an allergen was *Aspergillus fumigatus.* The association between positive skin tests to *A. fumigatus* and allergic bronchopulmonary aspergillosis (ABPA) was established before the discovery of IgE (18). It is now understood that this fungus can give rise to several diseases associated with different immune responses, including allergic asthma, ABPA, allergic sinusitis, aspergilloma, and locally or systemically invasive aspergillosis (18). The genus *Aspergillus* includes many species that are widely distributed, and it is not clear why the single species, *A. fumigatus*, is so common in the lung. In part it may reflect the thermotolerance of this species, which unlike most other fungi can flourish at temperatures from 10°C to 38°C. Evidence suggests that *A. fumigatus* produces specific toxins that may contribute to both its colonization of the lung and its toxicity (10). *Aspergillus* is a colonizing organism in the lungs of children with cystic fibrosis (65,291). As judged by the total IgE level, and specific IgE and IgG antibodies, this colonization may start as early as age 5 years. Given the evidence that the *A. fumigatus* allergen Asp f 1 is produced only after germination of the spores, the presence of an antibody response indicates that the patients are (or have been) colonized. The important issue is whether *A. fumigatus* colonization of the lungs of patients with cystic fibrosis contributes to their lung damage. Approximately 20% of children with cystic fibrosis have IgE antibodies to *A. fumigatus*, although most have IgG antibodies alone. There is no reason to assume that colonization is more damaging in individuals with an allergic response than in those with IgG antibody alone, because the enzymes and toxins produced by *A. fumigatus* may be directly damaging to the lung. Indeed, *A. fumigatus* serine proteinase has recently been shown to degrade human lung elastin and collagen and to produce lower respiratory tract destruction when injected intratracheally in a mouse model (100). To date, the main treatment for ABPA has been systemic glucocorticoids; however, a recent trial of the systemic antifungal, itraconazole (231), reported improvement in patients with ABPA. This antimicrobial therapy provides promise for the treatment of this chronic debilitating condition.

Although *A. fumigatus* dominates colonization of the lungs in those with mycetomas or ABPA, other fungi have been implicated. In particular, allergic bronchopulmonary curvulariosis and candidiasis have been documented. There also is a rare but important condition called allergic fungal sinusitis (77), which is characterized by production in the sinuses of an “allergic” mucus with the consistency of peanut butter and containing Charcot-Leyden crystals. Although this allergic mucus can erode bone, this erosion results from pressure, and there is no penetration of the periosteal or meningeal layers. It is possible to have extension into the orbit and even the frontal lobe without infection of the bone, orbit, or meninges. Cases have involved several fungi but most commonly those of a group including *Bipolaris spicifera, Drechslera*, or *Excerohilum* spp. (Fig. 61.2). In all cases, the patients are atopic and have positive skin tests to the relevant fungus. Other investigators have suggested that fungal infection contributes to a large proportion of cases of chronic sinusitis (188,293). However, general experience is that fungal colonization is present in only about 6% to 7% of cases (46). Fungi also can colonize the lungs of patients with asthma and those with chronic obstructive pulmonary disease with or without an IgE antibody response. Perhaps the most common organism involved is *Candida albicans*. Currently it is difficult to assess whether *C. albicans* is a harmless saprophyte or whether it has a pathogenic role in allergic disease. Serum IgE antibodies to *C. albicans* have been reported in patients with allergic bronchopulmonary candidiasis, hyper-IgE syndrome, and allergic asthma (21,79). The yeast *Malassezia furfur* (previously known as *Pityrosporium ovale*) also has been recognized as an allergen in patients with atopic dermatitis (108). *M. furfur* is a “cause” of both seborrheic dermatitis and tinea versicolor, but the nature of the immune response is not established. Recently, moderate-to-severe atopic dermatitis has been shown to be strongly associated with sensitization to *M. furfur* (205); these findings suggest that fungal allergens contribute to the severity of allergic disease.

Perhaps the single most common fungal colonization of humans is with dermatophytes of the genus *Trichophyton*. Worldwide, *T. mentagrophytes* and *T. rubrum* are the most common cause of athlete's foot and infection of the toenail beds (onychomycosis). *Trichophyton* is unusual in that it is associated with distinct immune responses in different individuals. Positive immediate hypersensitivity skin tests have been associated with chronic infections characterized by low-grade inflammatory lesions that tend to persist and the presence of high titers of serum IgG and IgE antibodies to *Trichophyton* antigens (85,110,224,270). In contrast, delayed type hypersensitivity, a form of cell-mediated immunity, has been reported in individuals with acute, highly inflamed lesions; sera from subjects with delayed-type hypersensitivity contain only low-titer IgG antibodies (81,111,112,283). The association between the severity of the clinical dermatophytosis and different immune reactions has led researchers to speculate that delayed-type hypersensitivity responses are required to eradicate *Trichophyton* infection, and immediate hypersensitivity responses are not protective. Chronic dermatophytosis appears to be associated with atopy; the prevalence of allergic history in patients with chronic dermatophytosis (36% to 49%) is higher than in the general population (85,109,111). Furthermore, as early as the 1930s, Wise and Sulzberger (278) suggested a relation between dermatophyte colonization of the feet and allergic symptoms. Subsequent studies proposed that a variety of allergic diseases including asthma, chronic sinusitis, and urticaria were related to dermatophytosis (173,270,273). This theory was supported by anecdotal reports of improvement of allergic symptoms after the use of antifungal drugs. Although allergic patients may exhibit positive immediate or delayed-type hypersensitivity skin tests to *Trichophyton*, in a few patients with positive immediate skin tests and chronic fungal colonization, *Trichophyton* appears to play a role in their allergic disease. The availability of highly efficacious systemic antifungal therapies has made it possible to examine the role of *Trichophyton* in allergy. A significant improvement of allergic symptoms in a group of patients with late-onset asthma treated with fluconazole has recently been reported (271); these findings provide strong evidence of a role for *Trichophyton* antigens in allergic disease. Such cases are intriguing, given that *Trichophyton* has never been isolated from the respiratory tract. It is assumed that sensitization may occur by absorption of antigen from the skin or nail beds. Several *Trichophyton* antigens have now been characterized; two allergens designated Tri t 1 (30 kd) and Tri t 4 (~80 kd), derived from *T. tonsurans*, were purified with standard immunochemical techniques (59,283). Subsequent studies used molecular cloning techniques to identify allergens derived from *T. rubrum* that exhibit a high degree of amino acid sequence identity with distinct families of serine proteinases: the prolyl oligopeptidase Tri r 4 (~80 kd) and the class D subtilase, Tri r 2 (29 kd) (285). The availability of purified natural and recombinant *Trichophyton* allergens made it possible to study humoral and cellular responses to these antigens. Immediate hypersensitivity responses were found to be associated with high titers of antigen specific IgG and IgG4 antibodies and with the presence of IgE in the serum, whereas delayed-type hypersensitivity responses were associated with only low titers of IgG (283,285). Furthermore, T cells derived from individuals with different immune responses had distinct cytokine profiles (214). These recent advances open the door to further studies aimed at understanding the relation between dermatophyte colonization, the immune response to dermatophyte antigens, and allergic disease.

Bacterial Allergens

Early investigations of bacterial allergy were marred by inadequate evidence about the nature of the antigens and a lack of controlled trials (48). This situation led to the consensus opinion that immunotherapy with bacterial antigens is not efficacious. This view should not be interpreted as evidence that patients do not make IgE antibody responses to bacteria. Although positive wheal-and-flare skin responses to a wide range of bacteria have been reported, there is less evidence about the nature of the allergens or about serum IgE antibodies. Good evidence for IgE antibodies to *Staphylococcus aureus* has been reported for patients with hyper-IgE syndrome (21). Patients with atopic dermatitis may also have IgE antibodies to enterotoxins of *S. aureus* (130,203). The staphylococcal enterotoxins have many of the properties of a superantigen, which may influence the immune response (89,130). Recently, an IgE antibody–binding protein derived from *S. aureus* was identified by screening a genomic DNA library with sera from patients with atopic dermatitis (101). This finding suggests that previously unidentified bacterial antigens may play a role in allergic disease. Thus there seems to be a strong case for reevaluating the immune response to common bacterial commensals in atopic patients, particularly those with atopic dermatitis or chronic sinusitis (152).

Venom Antigens

The venom produced by members of the order Hymenoptera—honeybees, yellow jackets, wasps, hornets, and fire ants—consists primarly of low-molecular-weight peptides (MW, 1,000 to 3,000), which are potent causes of pain and local swelling (119,120,244). The venom also includes proteins such as phospholipase A_2 and hyaluronidase; and in a few patients who are stung, IgE antibodies develop to these proteins. Such patients are at risk of large local reactions or anaphylaxis on subsequent stings. Detailed study of these patients has revealed various degrees of cross-reactivity among venoms (197). Patients who are allergic to honeybees, yellow jackets, wasps, or hornets can be skin-tested and treated with “pure” venom, a form of immunotherapy that is highly successful (99,289). It has long been known that beekeepers can become tolerant to stings, and that they have IgG antibodies to phospholipase A_2. In addition, Aalberse et al. (1) reported that this antibody was predominantly of the IgG4 isotype. Several honeybee venom proteins have been cloned and the recombinant proteins expressed; these proteins have been used for diagnosis and treatment (68,122,145,149,219). Recently peptides of bee venom phospholipase A_2 that contain T-cell epitopes have been shown to induce T-cell anergy, which is associated with interleukin (IL)-10 production (3,4,148). In contrast to bee venom, fire ant venom is difficult to obtain, and patients are generally treated with whole-body extracts, although the venom can be used for diagnosis (230). Fire ant stings also produce pustules, which may reflect an added toxicity of one of the peptides. There is considerable interest in cloning fire ant venom proteins for use in diagnosis and treatment (207).

Biting Insects

Although many biting insects (e.g., mosquitoes, deer flies, black flies, horse flies, and fleas) can produce local reactions resembling immediate hypersensitivity, systemic reactions are rare. In most cases, the quantity of protein delivered with the bite is probably inadequate to produce a systemic reaction, even if the patient has developed IgE antibodies (94). Two salivary allergens from mosquitoes (Aed a 1 and Aed a 2) have been cloned and expressed, and monoclonal antibodies that recognize other salivary proteins have been produced (167,287). Occasional cases of anaphylaxis and IgE antibodies have been reported after bites by deer flies or

reduvid bugs of the genus *Triatoma. Triatoma*is a large insect that feeds on mammals including humans; it has a painless bite, leading to its designation, the “kissing bug.” The allergen of *Triatoma protracta*is an 18-kd salivary protein (36), and successful immunotherapy has been reported with salivary gland extract (198).

THE IMMUNE RESPONSE TO ALLERGENS

A major objective of purifying and defining allergens has been to understand why these antigens selectively induce one type of immune response. Immediate (type I) hypersensitivity is mediated by IgE antibodies, and this form of immunity is the classic response to allergens. Although antibodies are the primary effector molecules of type I immunity, T lymphocytes of the Th2 phenotype play a major role in induction of the allergic response. This subset of T cells produces an array of cytokines including IL-4, IL-5, IL-6, and IL-13, which exert their effects on T lymphocytes themselves but also on other cell types including B cells and antigen-presenting cells such as monocytes and dendritic cells. IL-4 serves as a growth factor for B cells and induces antibody isotype switching to IgE and IgG4 production. On initial exposure to allergen, T lymphocytes are primed, and IgE antibody production is induced; these antibodies bind to receptors on the surface of mast cells. On subsequent exposures to allergen, cross-linking of these IgE antibodies by allergen triggers degranulation of the mast cells and release of histamine, which causes allergic symptoms. Thus, to experience allergic symptoms, an allergic individual must be repeatedly exposed to allergen. Although the events that initiate a Th2 response are not yet clear, the detailed understanding of allergens has not changed the view that low-dose, crepeated exposure to a soluble protein is the best way to induce IgE antibody responses. Knowledge of allergens is currently being used to develop strategies to abrogate the allergic response in susceptible individuals. Such approaches include altering B-cell epitopes so that they are no longer capable of binding IgE and identifying T-cell epitopes (allergenic peptides) that may be used to induce preferentially a cellular response, while failing to stimulate the humoral arm of the response (see later). One theory that has gained favor in the last decade is that by stimulating a distinct subset of T lymphocytes (Th1 type) it may be possible either to modulate an ongoing allergic response or to induce protective immunity to allergens. Cell-mediated (delayed-type hypersensitivity) responses are mediated by Th1 cells; these cells are characterized by a cytokine profile that is distinct from that of Th2 cells (i.e., IFN-g and IL-2), and their development is dependent on IL-12. Although allergen-specific cells with a Th2 phenotype have been isolated from allergic patients (27,251,276), there is limited evidence that in humans, immunotherapeutic strategies have provoked a Th1 type of response (199) (discussed at the end of the chapter). Our current knowledge of allergen structure, including the definition of T-cell and B-cell epitopes, provides a basis for investigating the immune response and for designing new approaches to immunotherapy.

IMMUNOCHEMISTRY OF ALLERGENS

Properties of Purified Allergens

A major objective of the early studies on allergens was identification of the special properties of “atopic allergens” that induce immediate hypersensitivity. The wide range of allergen sources (see Table 61.1,Table 61.2 and Table 61.3), however, made it unlikely that these proteins would have common features. The allergens that have now been purified are soluble proteins or glycoproteins with molecular masses between 5 and ~80 kd (Table 61.4). For inhaled allergens, the primary immune response occurs in the lymph nodes draining the nose, tonsils, or peritonsillar lymphoid tissue (141,171,241). Because particles landing on the nasal epithelium are propelled posteriorly and swallowed within about 15 minutes, proteins must be released rapidly in mucus to be able to penetrate the epithelium and basement membrane. The respiratory tract basement membrane has an approximate molecular-mass cutoff of 60 kd (208). Respiratory epithelium includes a dense network of Ia-positive dendritic cells that collect foreign antigens (96,155,209). These cells then pass via the lymphatics to the local lymph nodes (96). Any foreign protein that can penetrate the mucous membrane is likely to be highly immunogenic. The primary characteristics of an allergen are that it is a soluble foreign protein that is delivered repeatedly to the nasal mucosa in sufficient dose to cause an immune response.

TABLE 61.4. Important Allergens

Allergens can be identified in several ways. Most of the allergens listed in Table 61.4 were initially purified by classic immunochemical techniques based on salt concentrations (e.g., ammonium sulfate), size (Sephacryl), and charge (electrophoresis or anion-exchange resins). The purified proteins were studied by RAST, skin testing, or crossed radioimmunoelectrophoresis (CRIE) to establish the importance of the allergen. The term *major allergen*has been used loosely and is usually taken to mean that at least 60% of the individuals with IgE antibody to the source material (e.g., rye grass pollen or dust mites) have IgE antibody directed against this particular protein (e.g., Lol p 1 or Der p 1) (119,137,172). However, when CRIE is used to determine if IgE antibody is present against a given protein band, the technique is so sensitive that many proteins are considered to be major allergens. Similarly, estimates that an extract contains 15 or more allergens based on CRIE or Western blot after sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) are difficult to evaluate. In general, allergens should be purified to establish their allergenicity and importance relative to other proteins in the same extracts. For many allergens (e.g., those derived from dust mites, grass pollen, cat, and alder pollen), the major allergens represent a large proportion of the protein in the extract (28,37,119,137). This finding would be expected if the quantity of allergen reaching antigen-presenting cells in the nasal epithelium were the limiting factor in the immune response. However, other allergens, which represent only a small proportion of the total protein, are “highly immunogenic” for some individuals (143). Another criterion for the importance of a protein allergen is an estimation of the percentage of serum IgE antibody against the source material that is specific for the purified allergen (37,74,206). Reliable estimates of this kind have been reported for the major allergens of ragweed, rye grass, *D. pteronyssinus, D. farinae*, and cat (37,55,74,181,206). In each case, patients have been identified in whose sera more than 50% of the IgE antibody against the source is directed against the purified *major*allergen.

Sequencing of Allergens: Biochemical Properties

The first sequence of an allergen was reported by Elsayed and Apold (66) for codfish muscle allergen M in 1973 and was followed by the sequence of Amb a 5 by Mole et al. in 1975 (143). At that time, it was still considered possible that the amino acid sequence of allergens would reveal a characteristic feature or that allergens would have repeated epitopes that allowed them to cross-link IgE antibodies. It is now considered unlikely that any proteins of this size would include repeated B-cell epitopes (20). Furthermore, because essentially the whole surface of a protein can be immunogenic, it is unlikely that the sequence data alone would give any information regarding allergenicity (20). The partial amino acid sequences of many purified natural proteins have been obtained by Edman degradation (e.g., Der p 1, Der p 2, Lol p 1, Fel d 1; Table 61.4). More recently, molecular cloning techniques have resulted in determination of the full amino acid sequence of numerous allergens. The amino acid sequence of Der p 1, reported in 1988 (43), showed a high degree of sequence identity with actinidin and papain (43,233). Subsequently, other mite allergens were identified as enzymatic homologs and their function characterized *in vitro*; for example, the trypsinlike serine protease Der p 3 and a-amylase Der p 4 both showed enzymatic activity (182,233). Many major allergens show no sequence identity with known enzymes (e.g., Fel d 1 and Der p 2) (34,42,90,146); furthermore, enzymatic homologs may lack enzymatic activity (285). Although it seems unlikely that the allergenicity of a protein can be attributed solely to its enzymatic properties, several groups have speculated that the biochemical activity of dust-mite allergens may enhance their immunogenicity or allergenicity. The cysteine proteinase, Der p 1, was shown to cleave the low-affinity IgE antibody receptor (FceRII) from human B-cell lines; this results in the release of soluble CD23, which is thought to be involved in stimulating the growth and differentiation of a variety of cell types, including T and B lymphocytes, and in the autocrine regulation of IgE antibody production (88). More recently, Der p 1 has been shown to cleave the a subunit of the IL-2 receptor (CD25) from the surface of human peripheral blood T cells (211). Thus it has been proposed that Der p 1 may suppress propagation of Th1 cells, thereby promoting a Th2 bias. Other studies have shown that Der p 1 (and Der p 9) stimulates IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) production by bronchial epithelial cells (118) and that several group 3 allergens can generate anaphylatoxins by proteolysis of the complement components C3 and C5 (139); each of these effects was inhibited by protease inhibitors. A more recent study showed that Der p 1 disrupts intercellular tight junctions *in vitro*by cleaving the adhesion protein occludin, thereby facilitating transepithelial allergen delivery (269). Pollen enzymes also have been reported to detach airway epithelial cells from their substratum *in vitro*(83), suggesting that epithelial injury can be mediated by different allergens. Thus by a variety of different mechanisms, the proteolytic activity of some allergens may contribute to the pathogenesis of allergic disease. However, there is still no evidence for the relevance of enzymatic properties *in vivo*in humans. In addition, it remains to be determined whether enzymatic activity plays a role in the induction of the allergic response. Given the diverse nature of allergens, this seems unlikely.

Allergenic Cross-Reactivity

Classically, cross-reactivity between allergens has been determined by testing the ability of one allergen to inhibit antibody binding to another. Such experiments are typically carried out with human sera containing allergen-specific IgE or monoclonal antibodies (mAbs). mAbs may give a different impression of cross-reactivity from that of natural human IgE antibodies. For example, IgE specific for Der p 1 can be largely absorbed from serum on a solid phase of Der f 1 (91). In contrast, almost all mAbs raised against either Der p 1 or Der f 1 do not bind to other group 1 proteins (35,131). Thus group 1 mite allergens have common (or group-specific) determinants (recognized by human IgE and mAb 4C1-B8), as well as species-specific determinants (recognized by different mAbs).

Knowledge of the amino acid sequence of proteins has provided insight into cross-reactivity between allergens. For example, the major grass pollen allergens such as the group 1 allergens of rye grass, timothy, and June grass show sequence homology and extensive cross-reactivity (6,137). Many proteins have highly significant sequence identity (30% to 40%) but do not cross-react immunologically (e.g., the group 5 allergens of short and giant ragweed, or Lol p 1 and Lol p 2 allergens of grass pollen). As a rule, proteins with more than 70% identity cross-react to some degree (e.g., group 1 mite allergens), whereas those with 85% or more homology show extensive cross-reactivity (e.g., group 2 mite allergens). Amino acid sequencing of allergens has revealed potential cross-reactivity between allergens from diverse sources. For example, tropomyosin was recently identified as a major cockroach allergen (204). Shrimp and house dust mite tropomyosin were recently identified as allergenic, and there is now strong evidence for cross-reactivity of tropomyosin allergens derived from diverse organisms (14,196,204). An association between sensitization to pollen and symptoms from specific foods has long been recognized (22,61). The ubiquitous actin monomer–binding protein, profilin, derived from birch pollen, was first identified as an allergen by Valenta et al. (254). Subsequent studies described profilins as allergens in grasses, weeds, fruits, and vegetables, and thus it was postulated that profilins may be responsible for the cross-reactivity between pollen and foods in allergic patients. Many profilins have now been cloned from different plants, yielding clues regarding cross-reactivity of allergens from diverse sources (13). Perhaps the best-defined cross-reactivity is between birch pollen and a group of foods that includes apples, plums, and hazelnuts (61). Approximately 30% of patients with hay fever related to birch or the closely related alder pollen are aware of tingling of their lips and itching at the back of the throat with or without visible swelling on exposure to these foods ("oral allergy syndrome"). Molecular cloning has identified homologs of the birch pollen allergen, Bet v 1, in different food sources, which may explain this cross-reactivity (see footnote to Table 61.4). The sensitizing allergen source (pollen or plant food) remains controversial; because apple sensitization is far more common in countries where birch pollen is an important allergen, it seems likely that the primary sensitization in most cases is induced by exposure to birch pollen. This theory is supported by recent findings that most IgE epitopes in plant food recognized by patients with oral allergy syndrome are present in pollen allergens (114).

Recombinant Allergens

Once an allergen has been cloned, it can often be produced *in vitro* with bacterial (e.g., *Escherichia coli*), yeast (e.g., *Pichia pastoris*), or mammalian (Baculovirus) expression systems. Pure allergens expressed in this way have major advantages for diagnosing and treating allergic disease and for standardization. With molecular cloning techniques, it is now possible to identify IgE reactive clones from cDNA or genomic DNA libraries and to sequence these putative proteins. However, the possibility still remains that not all B-cell epitopes will be present in the recombinant protein. Full definition of an allergen generally requires (a) purification of the natural allergen and demonstration of its importance, (b) development of mAbs that react with the natural protein, and (c) amino acid sequencing of the full-length protein. However, molecular cloning techniques are increasingly being used to identify novel allergens, particularly those of bacterial and fungal origin, for which the natural form has not been characterized. Many recombinant allergens have now been produced and their immunoreactivity tested (255). Initially, although production of recombinant protein was achieved, some allergens exhibited reduced immunoreactivity, as judged by IgE antibody binding compared with the natural form. However, in general, such problems can been overcome by using different expression systems for production of the recombinant protein. For example, the immunoreactivity of recombinant Fel d 1 (r-Fel d 1) expressed in bacteria was not comparable with that of the natural protein, but r-Fel d 1 produced in baculovirus showed essentially the same IgG- and IgE-binding properties as natural Fel d 1 (102). Reasons for variations in immunoreactivity of recombinant proteins produced in different expression systems are not clear but may include incomplete or incorrect folding of the peptide chain and lack of (or altered) disulfide bond formation or posttranslational modification. Several allergens, including Fel d 1, the *Trichophyton* allergen Tri r 2, and the major cockroach allergen Bla g 2, are glycoproteins; recent advances in eukaryotic expression systems have allowed the production of recombinant allergens that are glycosylated to a degree comparable with the natural form. In a few cases, for reasons often unknown, it has not been possible to express certain allergens in their recombinant form. However, problems in protein expression have occasionally been overcome by altering the DNA sequence encoding the allergen, so that codon use is more appropriate for the host expression system. The availability of recombinant allergens is a valuable advance in the field of allergy. It provides unlimited source material for immunologic studies of allergens and for the development of novel treatments. For those allergen sources in which the immune response is directed against a limited number of proteins, it is possible that recombinant proteins will replace native extracts for diagnosis and immunotherapy.

Allergen Structure: B-Cell Epitopes

The development of mAbs made it possible to "map" B-cell epitopes on the surface of allergens and investigate the relation between epitopes that bind IgE and those that bind murine mAbs. Studies of this kind have been reported for many allergens, including those derived from mite, cat, and pollen (34,38,67). These studies were useful for studying cross-reactivity (see earlier) and, more recently, for evaluating techniques used to modify the antibody-binding properties of allergens with a view to developing molecules with reduced allergenicity. Various techniques used to alter the reaction of allergens with IgE antibodies have incorporated different conditions of heat and pH, and chemical treatment with glutaraldehyde, urea, and tannic acid (104,137,160). Often a slight modification of the molecule could prevent interaction with IgE antibodies (22,119,132). Some proteins are highly sensitive to denaturation, whereas others are resistant. For instance, Der p 1 is labile, and Der p 2 is relatively stable to heat and chemical denaturation (132).

Epitope mapping of conformational determinants does not yield information about the structure of the epitope but merely about the relation of one epitope to another (i.e., distinct or overlapping). Elucidating the tertiary structure of allergens is critical for defining B-cell epitopes and for producing recombinant allergens with reduced allergenicity. Knowledge of the amino acid sequence of several allergens has allowed computer-assisted molecular modeling based on sequence identity with proteins of known structure. More recently, nuclear magnetic resonance techniques have been used to determine the structure of recombinant Phl p 2 (58), Der p 2 (147), and Der f 2 (103). Furthermore, the structure of several allergens has been determined by x-ray crystallography, including profilins derived from birch pollen (69) and *Arabidopsis thaliana* (245); the major horse allergen, Equ c 1, and the bovine lipocalin, Bos d 2 (78,126,201); and the major grass allergen Phl p 2 (Fig 61.5) (70). Such studies have allowed identification of major epitopic regions involved in IgE binding (69,70,194) and have provided valuable insight into allergenic cross-reactivity.

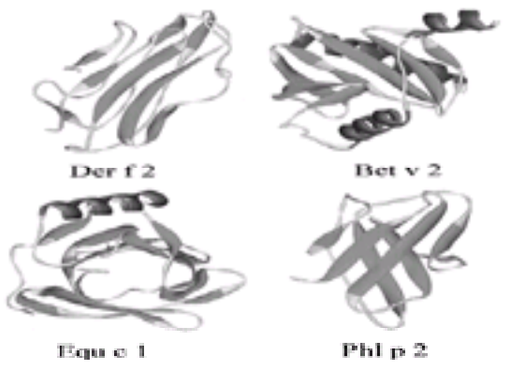

Figure 61.5. Three-dimensional structures of the mite allergen, Der f 2 [Protein Data Bank file 1AHK.pdb (103)], the birch pollen profilin, Bet v 2 [1CQA.pdb (69)], the horse allergen, Equ c 1 (126), and the major timothy grass pollen allergen, Phl p 2 [1WHO.pdb, (70)]. Figure kindly prepared by Dr. Anna Pomés. (See Color Figure 61.5.)

After the amino acid sequences of mite and other allergens were determined, several groups synthesized peptides and tested their interaction with IgE antibodies (107,259). These experiments assumed that some allergens contained linear B-cell antigenic determinants. This concept remains controversial because B-cell antigenic determinants are typically conformational. In 1991, Van't Hof et al. (259) defined a peptide of 14 amino acids (residues 65 to 78) derived from Der p 2 that bound IgE antibodies (259) and determined that the IgE response was polyclonal (260). In the case of Der p 1, four peptides were selected for study using modeling of the molecule based on its homology with papain and actinidin (107). In that study, peptides of 14 to 17 amino acids were shown to cause histamine release *in vitro* that correlated with IgE antibody levels to Der p 1. The authors speculated that allergenic peptides could bind to albumin to create divalent molecules capable of triggering histamine release. Recently, epitope-mapping studies of group 2 mite allergens have been carried out with site-directed mutagenesis to disrupt disulfide bonds (80,215,216,242). The findings of these studies support a role for conformational, *not* linear, B-cell antigenic determinants in IgE antibody binding. In contrast to allergens derived from dust mites, there is considerable evidence that food-derived allergens contain linear B-cell epitopes. For example, epitope analysis of the peanut allergen Ara h 3 identified four peptides (10 to 15 amino acids in length) that bound serum IgE (193). In this study, antibody binding was reduced or abrogated by single amino acid substitutions. Furthermore, heat-induced conformational changes of Ara h 1 have no effect on IgE antibody binding (124). These findings support a role for

linear IgE epitopes in peanut allergy. Despite this evidence, it seems most likely that conformational B-cell epitopes dominate the allergic response to most allergens.

T-Cell Responses to Allergens: T-Cell Epitopes

Although allergic responses are mediated by IgE antibodies, T lymphocytes of the Th2 subset play a major role in these responses (see earlier). For T cells to carry out their effector functions, they must first be activated to proliferate and differentiate into armed effector T cells capable of producing the relevant cytokines and rapidly responding to antigen. T cells are activated upon recognition of foreign antigen presented on the surface of an antigen-presenting cell in the form of peptide bound to a major histocompatibility complex (MHC) molecule. Antigenic peptide is generated within the antigen-presenting cell by enzymatic cleavage. Th2 lymphocytes are $CD4^+$ T lymphocytes that recognize antigenic peptide in the context of MHC class II molecules. Antigenic peptides that bind to MHC class II molecules are heterogeneous in length, ranging from 15 to more than 20 residues. These peptides or T-cell epitopes are *linear*epitopes. Most allergic patients have circulating T cells that proliferate and produce interleukins *in vitro*in response to allergen extracts, purified allergens, and specific peptides (25,169,195). Knowledge of the amino acid sequence of allergens has made it possible to identify T-cell epitopes using synthetic peptides derived from allergens to stimulate T-cell proliferation *in vitro*. Immunodominant antigenic determinants for a wide variety of allergens have been identified by this method: these include group 1 and group 2 mite allergens (97); the major cat allergen, Fel d 1 (136); the major birch pollen allergen, Bet v 1 (63); and many other pollen-derived allergens (210). Other studies have mapped T-cell epitopes of the major olive allergen, Ole e 1 (33), the group 5 grass pollen allergen, Phl p 5 (150), and the major cow dander allergen, Bos d 2 (292). T-cell epitopes of fungal allergens derived from *Aspergillus*(Asp f 1) and *Trichophyton*(Tri r 2) have also recently been mapped (41,284). The initial aim of such studies was to identify epitopes associated with allergic disease that may serve as targets for novel therapeutic strategies. Although in principle this theory was attractive, studies revealed that most individuals exhibit a unique pattern of T-cell epitope recognition; thus no specifically “allergenic” epitopes derived from any major allergen have been identified. An alternative approach was to compare atopic and nonatopic individuals to identify significant differences between these two groups. However, many of these studies were confounded by several factors. First, insufficient numbers of patients were selected for study. Second, the assay design was not optimized; this is critical, given that many allergens are relatively weak inducers of T-cell proliferation *in vitro*. In addition, the chances of a single antigenic peptide stimulating T-cell proliferation *in vitro*are restricted by the low precursor frequency of antigen-specific T cells in the peripheral blood. Thus it is imperative that T-cell proliferation assays using antigenic peptides incorporate a high number of replicate wells (at least 12 per peptide). A recent study used rigorous assay design and statistical techniques to identify highly significant differences in the T-cell repertoires associated with immediate and delayed hypersensitivity to the *Trichophyton*allergen, Tri r 2 (284). This study identified an amino-terminal immunodominant T-cell epitope specifically associated with delayed hypersensitivity. A peptide containing this epitope failed to stimulate proliferative responses in T cells derived from individuals with immediate skin tests. The mechanism of T-cell hyporesponsiveness in individuals sensitized to *Trichophyton*is not yet clear.

Peptide immunotherapy has been proposed as a method for modulating T-cell function, thereby abrogating the allergic response. In the early 1990s, subcutaneous injection of Fel d 1 peptides was reported to induce T-cell “tolerance” to whole antigen in a mouse model (29). Similar models were developed for other allergens; however, initial studies in humans failed to demonstrate this effect (213). Nevertheless, recent studies have demonstrated that allergen-derived peptides injected intradermally can induce a late asthmatic reaction in individuals allergic to cat (82). These findings suggest that antigenic peptides may indeed have the capacity to modulate the immune response. It is possible that the use of better techniques for defining T-cell epitopes and the testing of different immunotherapy regimens will allow the rational design of peptide therapies. Testing the clinical and immunologic responses to injections of “T-cell epitopes” is one of the most interesting experiments in immunotherapy since Noon (156) first introduced desensitization in 1911.

STANDARDIZATION OF ALLERGENS

Before the discovery of IgE and the purification of major allergens, extracts were standardized by measuring protein [1 protein nitrogen unit (PNU) = 0.01 µg protein N] or by the weight of pollen used for extraction (1 Noon unit = the allergen extracted from 1 µg of pollen). When good-quality pollen is extracted in saline at 4°C for 24 hours, these units or a simple weight/volume ratio provides a reasonable guide to the potency of the extract. However, when the source material is more complex (e.g., fungi, cat, mites, or insects) or the extraction conditions are altered, these assays are not reliable. For example, extraction in pyridine at pH 13.0, which increases protein, destroys many allergens, including Amb a 1 and Der p 1 (16,17). Therefore standardization of allergen extracts should include either a direct assessment of potency on the skin (or basophil count) of allergic individuals or, preferably, an *in vitro*measurement of the allergen content.

The first generally applicable *in vitro*measurement of allergen was RAST inhibition, which is still widely used for standardization. It is therefore important to understand the arguments against continuing to base standardization on RAST inhibition. The technique uses a solid-phase, cellulose disc or beads covalently linked to a known allergen extract. This step is inherently difficult because the choice of extract used for the solid phase influences the results and implies some preexisting decision about the correct form of the extract. (A typical example is the difference between mite extracts made from whole culture and those made from “purified” mites.) The second problem with RAST inhibition is that it requires a serum pool derived from individuals who have high levels of IgE antibodies to the relevant allergen. In theory, a large serum pool can be established and maintained as a reference. However, RAST inhibition uses “large” quantities of serum, and even the serum pools established at the U.S. Food and Drug Administration (FDA) have lasted only a few years. Thus all results based on RAST inhibition must be qualified and are reproducible only if the solid phase and the serum pool are the same (75). In conclusion, RAST inhibition is a reasonable technique for comparing the potency of a series of extracts or monitoring the consistency of batches of extract manufactured with an exact protocol. However, RAST inhibition cannot provide a system of units that remains constant over time or that can be compared between one allergen extract and another.

Skin testing allergic patients with a series of extracts as a method of standardization has been rejected in the past because of the difficulty in establishing that the patients tested on different occasions were equally allergic. Indeed, the World Health Organization (WHO) specifically rejects the idea of using any *in vivo*test of a standard to establish the potency (157). Nonetheless, skin testing has the advantage that the extract is tested on allergic patients, and it has been recommended by several groups as a technique of standardization.

More recently, the Center for Biologics Evaluation and Research (CBER) at the FDA in the United States adopted the allergy unit (AU), which also is based on a system of skin testing (249). The primary unitage for AU is established by skin testing 15 patients who are “typically allergic” with an intradermal end-point dilution system (13 to 15 = 100,000 AU; 11 to 13 = 10,000 AU; nine to 11 = 1,000 AU). The primary problem is the method of choosing “typical” allergic individuals; the current protocol suggests that patients are chosen on the basis of their history and a positive prick test. Because the extract to be tested is used to identify allergic individuals, this method is not ideal. Furthermore, the choice of patients based on allergic history may not be applicable to many fungi or to the main perennial indoor allergens. For each new supplier, the allergy unitage of the extract must be established and then each batch standardized by RAST inhibition. Thus the AU system, owing to reasons described earlier and its expense in time and reagents, cannot be regarded as a stable unitage system. In 1993 a modified AU, or bioequivalent unit (BAU), was introduced, in which the mean skin test dilution is converted to BAU. This system was initially adopted for cat allergen extracts; and although it avoids problems of discontinuity of the AU, it still is not ideal.

The alternative method of standardization is the use of measurements of defined allergens in absolute units. This method is possible only for those allergen extracts from which the major allergen (or allergens) have been purified and for which there are suitable methods for measurement (Table 61.4). For the indoor allergens and for many pollens, major allergens can be measured with mAbs and results derived by reference to international standards. Each international standard has been allotted international units (IU) by WHO, but these standards can be used to standardize assays using absolute units. Because the mAbs represent a constant resource and the two-site immunometric assays use little extract to establish a control curve, it is possible to maintain units based on an international standard over many years. The WHO international standard for *D. pteronyssinus*was established in 1980; it is maintained as glass-sealed, freeze-dried ampules, is stable, and has already provided fixed unitage (micrograms of Der p 1 per gram of dust) in a prospective study over a 10-year period (72,182,226).

Recombinant allergens have clear potential advantages for standardizing extracts. Although in theory it is possible to create a defined mixture with known concentrations of all the important allergens, there are still significant problems. All recombinant proteins must be expressed in bacteria, yeast, or mammalian cells, and thus they may be contaminated with other cellular proteins. In addition, some recombinant proteins are expressed as fusion proteins (typically glutathione *S*-transferase), which allow affinity purification but then must be cleaved from the allergen. Some purification techniques also can contaminate recombinant proteins; for example, purification over an mAb column may introduce a trace of mouse immunoglobulin, which is a potential allergen. Hence, there are potential risks involved when attempting to produce pure recombinant proteins. Nevertheless, development of new expression systems (e.g., *Pichia pastoris,*which secretes expressed proteins extracellularly) and refinement of existing ones has made it possible to produce large quantities of pure recombinant proteins. However, before these proteins can be used for clinical diagnosis or treatment, the reactivity of each recombinant protein must be compared with purified natural allergen.

In conclusion, the current situation with allergen standardization is unsettled and includes skin testing, RAST inhibition, protein nitrogen units, and the measurement of major allergens. There are arguments for using recombinant allergens as a source of standards, but such use depends on establishing that the recombinant proteins truly match the native proteins. The area in which the most progress has been made and that offers the simplest approach is measurement of representative major allergens. With mAb technology, technically simple assays for many allergens are now available or possible and can provide standardization of allergen content in absolute units.

QUANTITATION OF ALLERGEN EXPOSURE

Identification and Measurement of Outdoor Allergens

During the nineteenth century, the pollen count was introduced with the use of a sticky slide and microscopic identification of adherent pollen grains (26). Hundreds of airborne (wind-pollinated) pollens have been identified, and excellent atlases give seasonal variations in specific pollens in different geographic areas (223,279). Pollen can be collected in many ways. Techniques that depend on sedimentation and wind velocity, such as gravity slide (or Durham) samplers, give only semiquantitative information. Because sedimentation is affected by particle size and the volume of air sampled is unknown, it is not possible to derive data on the number of pollen grains in the air from these methods. Currently, the standard for analyzing pollen levels is a rotorod sampler, in which the sticky rods are rotated for 20 to 60 seconds every 10 minutes (222). The volume of air sampled is calculated from the size and velocity of the rods, and the results are expressed as pollen grains per cubic meter of air. The rotorod has been estimated to be about 90% efficient at collecting pollen grains of about 20 µm diameter, and the results have been validated by comparison with techniques in which a known volume of air is sampled through an impactor. All pollen-counting techniques depend on microscopic examination of the pollen grains. Interpretation of pollen counts depends to a large degree on local experience. In some areas, the pollen count is an excellent guide to symptoms, and it is possible to predict from a given count that many or most of the patients who are at risk will develop symptoms, although some patients require several days of exposure before they reach maximal sensitivity. This phenomenon, originally described as “priming” by Connell (47), probably reflects in part the recruitment of basophils and mast cells to the nasal epithelium (84). In addition, some patients who have strong positive skin tests and IgE antibodies do not develop symptoms on exposure. Thus there cannot be a specific number for the pollen count that predicts that all skin test–positive individuals will develop symptoms. Nonetheless, at a pollen count of 200 grains/m^3, all patients who are going to get hay fever do experience symptoms; and when the pollen count is less than 5 grains/m^3, it is unusual for patients to experience symptoms.

In general, immunochemical techniques for assaying airborne pollen allergens are not yet simple enough to replace the pollen count. A luminescence immunoassay has recently been used to quantitate airborne birch (Bet v 1) and grass (Phl p 5) allergens (95). Interestingly, in these experiments, allergen levels correlated with pollen grains during dry weather; but after rainy days, large amounts of grass allergen were present in the absence of pollen grains. These results suggest that the development of allergen-specific quantitative assays will provide a more accurate method for measurement of outdoor allergens. The airborne ragweed allergen Amb a 1 also has been measured (221,290). The results showed that some allergen is airborne on particles less than 5 µm in diameter, which is far smaller than the size of a ragweed pollen grain. The quantities of allergen on small particles relative to large particles are not clear (31,221). There has been considerable speculation about the source of small particles carrying grass and weed pollen allergens; fragments of grains and other parts of the plant have been proposed (221,232). Grass pollen can fragment in a wet environment and release multiple starch granules, which then become airborne in a form that could enter the peripheral lung (234). At present, it remains true that the primary correlate of seasonal hay fever symptoms is the number of whole pollen grains airborne on a given day. For some patients allergic to pollen, there is a clear correlation between the pollen count and increased asthma symptoms (186). In many areas, particularly on the East Coast of the United States, the correlation between ragweed pollen counts and asthma is not close, which could be explained if asthma symptoms relate to inhalation of a different particle. It has not been shown, however, that “pollen asthma” correlates with airborne levels of a different ragweed particle. It is more likely that inhalation of small numbers of pollen grains causes progressive increases in bronchial hyperreactivity over a period of weeks. Thus the poor temporal correlation between asthma and pollen counts reflects the cumulative and delayed inflammatory effects of pollen exposure.

Outdoor fungal spores also can be collected on a rotorod and identified microscopically. However, the smaller fungal spores are collected inefficiently on the rod, with perhaps as few as 10% of 1- to 2-µm particles being collected (222). Identification of fungal spores is more difficult than identification of pollen grains. The large spores of *Alternaria* and *Helminthosporium* are highly characteristic and can be identified rapidly; in contrast, the spores of *Aspergillus, Penicillium* spp., and *Basidiomycetes* are small, round, and difficult to distinguish. Many samples include fragments of hyphae that cannot be identified; whether these fragments carry significant allergen has not been determined. Immunochemical assays for airborne fungi have been developed for *Alternaria* antigen Alt a 1, and they appear to correlate with *Alternaria* spore counts (162). *Alternaria* and *Cladosporium* spores can release preformed allergen rapidly in saline. However, *Basidiomycetes* and *Aspergillus* spores release protein only when they are ruptured (10,128,225). One of the *Aspergillus* allergens, Asp f 1, is expressed only after the spore germinates (9). Alternative techniques for collecting fungal spores include the Burkhard trap and various modifications of an Anderson multistage sampler. With the Burkhard (or Hirst) trap, air is drawn through an orifice and hits a slowly moving sticky surface. The surface is then examined for fungal spores. With the Anderson sampler, each stage has a sticky surface or a culture medium, so that individual colonies of viable particles can be identified. The problem is confounded because the various fungi grow better on different media and may require special media to produce spores. There is no satisfactory approach for all fungi; for many species, the small, round spores (1- to 2-µm diameter) are indistinguishable microscopically, whereas others, notably the *Basidiomycetes*, do not germinate on normal culture media.

Measurement of Indoor Allergens

Measurement of indoor allergens is necessary for understanding their role in allergic disease, for evaluating the relevance of indoor exposure to an individual patient, and for developing improved avoidance measures. Many of the sources of indoor allergens (e.g., cats, cockroaches, pet rodents) are easily identifiable. In addition, mites can be identified microscopically and counted in dust samples. Apart from a few fungal spores, none of the particles carrying indoor allergens (cat dander, mite fecal particles, *Penicillium* spores, rodent urinary residues, or cockroach debris) can be identified microscopically in dust or air. There is no indoor equivalent of the pollen count, and we must depend on immunochemical techniques.

Before the discovery of dust mites and the purification of indoor allergens, there was no method for evaluating the allergen content of house dust extracts. Thus, the house dust extracts used in allergy practice had no standardization other than protein nitrogen units (PNUs) or the weight/volume ratio (wt/vol), both of which were almost meaningless. With RAST inhibition, it is possible to evaluate the mite allergen content of house dust or mite extracts, using mite extract on the solid phase (248). However, RAST inhibition cannot be used to evaluate the overall potency of a house dust extract because the dust extract is too heterogeneous.

After its purification, Fel d 1 was measured in samples from houses or in allergen extracts by radial immunodiffusion (161). This work was followed by radioimmunoassay of Der p 1 and other mite allergens (37,247). Since the early 1980s, exposure to indoor allergens has been measured by RAST inhibition or assay of representative major allergens (e.g., Fel d 1, Der p 1, Der f 1, Bla g 2, Can f 1). The use of a single major allergen as an index of exposure implies only that concentrations of this allergen correlate with the levels of other allergens derived from the same source, not that this antigen is the only or even the most important allergen. The assays for major allergens have progressively incorporated mAbs and are standardized relative to national or international standards (34,35,72,131,133). The assays used most widely today are two-site mAb enzyme-linked immunosorbent assays (ELISAs); they are sensitive down to about 1 ng of antigen, are highly specific, and are technically simple (133).

There are two approaches to obtaining samples from a house (collecting reservoir samples and airborne samples) and it is important to understand the basis for each. Because these allergens are inhaled, it would be logical to measure the quantities that are airborne. Such measurements can provide important information about the quantities inhaled and the size(s) of the particles carrying allergens (71,175,178,237,239,258,288). However, the quantities that are airborne are often 5 ng/m^3 or less, and the airborne concentration is dramatically influenced by domestic disturbance. Therefore, collecting airborne samples is technically difficult and requires sampling large volumes of air (a disturbance in itself) or using sensitive assays (175). In modern houses, the air-exchange rate is often low (£ 0.2 air changes per hour), so particles the size of a pollen grain fall rapidly (Table 61.5). In contrast, outdoor air is almost never still, and pollen grains can fly for hundreds of miles. For analyzing the reservoirs of allergen in a house, dust samples can be obtained from carpets, sofas, mattresses, bedding, and kitchen floors with a hand-held vacuum cleaner. These samples are then sieved, weighed, and extracted. Typically, 100 mg of fine dust is extracted in 2 mL of buffered saline. Inasmuch as the relevant allergens are freely soluble, extraction for 4 hours is adequate; and the extracts can be stored frozen. Results are generally expressed as micrograms (or units) of antigen per gram of dust. The reasons for expressing results as a concentration of allergen rather than as the total quantity recovered were considered in detail by two international workshops (176,182). The main argument for using the allergen concentration is that it is difficult to standardize recovery of dust; the current conclusion regarding dust mites is that measurement of the *concentration* of group 1 mite allergen in a reservoir sample is the most reliable index of exposure.

Allergen	Diameter (µm)	Estimated quantity of major allergen/particle (ng)
Outdoor		
Pollen		
Virginia pine	~90	—
Rye grass	30	0.5
Short ragweed	20	0.5
Birch	25	—
Fungal spores		
Aspergillus spp.	2	<0.01
Penicillium spp.	2	—
Alternaria	20 × 10[a]	?
Cladosporium	10 × 5[a]	—
Indoor		
Dust-mite fecal pellets	15–30	0.2
Rat urinary allergen	~7	—
Cat allergen	2–20	—
Bronchial provocation		
Droplets produced by nebulizer	~2	~10^{-5}

[a] Dimensions of nonspherical spores given as length × width.
NOTE: Particle size dictates the maximum quantity of allergen that can be carried, the floating velocity, and to some extent, the distribution in the respiratory tract.

TABLE 61.5. Apparent Aerodynamic Size of Particles Carrying Airborne Allergens

When assessing a house, it is normal practice to collect four or five samples of dust from the bedding, bedroom floor, living room floor and sofa, and kitchen (Table 61.6) (32,73,182). In most houses, the highest level of mite allergen is present in bedding but may be present in dust from a downstairs sofa or from a carpet on an unventilated (typically a concrete slab) floor. In general, there is good correlation between the number of mites and the concentration of mite allergen (100 mites/g is almost equivalent to 2 µg of group 1 mite allergen/g of dust). For cat allergen, the highest level is generally found on the living room floor or sofa. In a house with a cat, these dust samples almost always contain at least 8 µg Fel d 1 per gram; however, the concentration is variable and can be as high as 4,000 µg/g. Comparable highest levels of mite allergen in bedding or carpets are about 100 µg/g. Cat allergen may also be present in houses where a cat is not present, and Fel d 1 levels as high as 80 µg/g have been reported. It is assumed that this cat allergen is transported from other houses on the clothing of the occupant or visitors. Cat allergen is present at high concentration in dust collected from the clothing of individuals who live in a house with a cat. Cockroach allergen is generally found in the highest levels in dust samples obtained from kitchen cupboards or floor. Although there is a good correlation between the presence of cockroach allergen and the identification of cockroaches, high levels of Bla g 2 have been found in houses without signs of roaches (32,73). In most published studies, the highest level of allergen in the house has been taken as the index of exposure for each allergen. In situations in which it has been analyzed, this approach appears to be more reliable than use of the mean level of allergen in the house. However, high concentrations of allergen in the bedding or pillow of a child are probably more important than a comparable level in the furniture or carpets in a room that the child does not use. Thus although the highest concentration of allergen found in the house is most often used as the index of exposure, there may well be situations in which an alternative approach is more appropriate.

Area	Site	Mite		Cat	Cockroach	Gras
		Der p 1 (µg/g)	Der f 1 (µg/g)	Fel d 1 (µg/g)	Bla g 2 (units/g)	pollen Lol p 1 (µg/g)
Central Virginia	Floor	<0.4	<0.4	124.0	<0.5	n.d.
Central Virginia	Floor	42.3	3.1	<0.2	<0.5	n.d.
Central Virginia	Bedding	<0.4	<0.4	<0.2	<0.5	<0.2
Los Alamos, New Mexico	Floor	<0.4	<0.4	1,130.0	<0.5	n.d.
Atlanta	Floor	16.0	4.6	<0.5	148.0	n.d.
Atlanta	Kitchen	<0.4	<0.4	<0.5	180.0	n.d.
Atlanta	Bedroom	62.5	1.2	<0.5	<0.5	n.d.
Northern California	Carpet	1.9	0.2	2.2	<1.0	148.0

n.d., not done.

TABLE 61.6. Allergen Levels in House-Dust Samples from Various Areas in the United States

AIRBORNE INDOOR ALLERGENS

The objective of measuring indoor allergens is to provide an estimate of the allergen entering the lungs. Ideally, this would be done with personal monitors. These types of monitors have been used to assess the exposure of children to cat allergens in school (5). In addition, nasal filters have been developed that can identify particles carrying inhaled allergens (163,164). However, measurements of personal exposure are made difficult by major differences in domestic exposure during domestic activity; changes in exposure when the face is in close proximity to allergen reservoirs (e.g., sofas and pillows); and air disturbance caused by air cleaners. Airborne cat allergen was first measured by collecting air samples and testing them for their ability to release histamine *in vitro*(71). Subsequently, airborne cat allergen was measured with RAST inhibition or immunoassays for Fel d 1 (134,237,239,258). These experiments have used filters, cascade impactors, and cascade impingers to separate the various particle sizes (Fig. 61.6). Although the details of the studies were different, the overall conclusions were similar: (a) detectable cat allergen is airborne in most houses with a cat; (b) some of the airborne Fel d 1 is associated with particles 5 µm or smaller in diameter; and (c) during disturbance (e.g., vacuum cleaning), the quantities of airborne allergen in houses associated with small particles were comparable to those necessary to produce acute bronchial provocation (134,239,258). Detailed study of houses with a cat suggests that both the cat and the reservoirs contribute to airborne allergen. The cat itself releases large quantities of cat allergen in a range of particle sizes. If the ventilation rate is low (i.e., 0.2 to 0.5 air changes per hour, which is typical of a *tight*domestic house), small particles accumulate. As the ventilation rate increases, the proportion of large particle allergen increases; so in an animal-house room with 12 cats in cages and a ventilation rate of ten air changes per hour, almost all the airborne cat allergen was associated with large particles (51,134). In a house with a cat, allergen can be controlled only by reducing the reservoirs, performing regular vacuum cleaning, and arguably by regularly washing the cat (see earlier) (15,51).

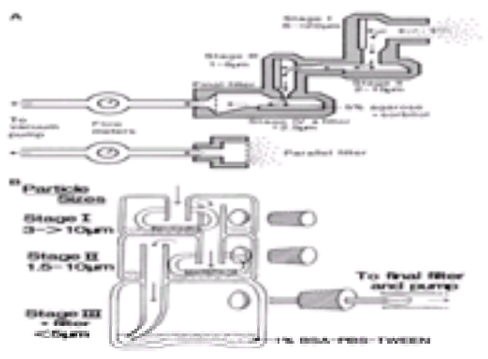

Figure 61.6. Evaluation of particle size of airborne allergens indoors with techniques that depend on terminal velocity; that is, they measure the size of the particles as an equivalent sphere of unit density.

Airborne mite allergens were not measured until a radioimmunoassay for Der p 1 was developed in 1980. Those studies demonstrated that mite allergen became airborne almost exclusively on particles 10 micrometers or larger in diameter (247). Furthermore, the major route by which mite allergen became airborne was as fecal particles. In keeping with this size particle, mite allergen is not airborne under undisturbed conditions, and its concentration decreases rapidly after disturbance (178,239,247,288). The techniques for determining particle size measure terminal velocity and depend on the impaction of particles as they pass through progressively smaller openings. Thus the particle sizes given are those of an equivalent sphere (Fig. 61.6). For the dust mite, it seems likely that the airborne particle is approximately spherical; however, for cat allergen, it is equally possible that the airborne particles are flakes, which behave aerodynamically, similar to a sphere of 2 to 5 µm in diameter. Group 2 mite allergen also is airborne on large particles, although they may not be fecal particles, as the quantity of airborne group 2 allergen is too high to be explained by fecal particles (52). The observation that mite allergen is airborne only during disturbance has been confirmed (288). It is not possible to standardize household disturbance among the various studies, so it is difficult to evaluate the level of exposure of patients in houses based on airborne levels of mite allergen (175,182,192).

The quantities of airborne mite allergen during disturbance have been reported as 5 to 200 ng/m^3. Given that fecal particles contain about 0.2-ng group 1 mite allergen, this figure represents 25 to 1,000 fecal particles per cubic meter. Because of the strong association between mite allergy and asthma, it would be useful to know how much inhaled antigen enters the lung. Some early studies suggested that few inhaled ragweed pollen grains entered the lungs, but those studies assumed that particles this size would induce acute changes in the forced expiratory volume at 1 minute (FEV_1); they also used forced inspiration, which would tend to decrease the number of particles entering the lung (31,277). In two studies on the entry of different-sized particles into the lung (236,243), it was found that under conditions of quiet breathing, particles of 20-µm diameter land predominantly on the pharynx, and only 5% to 10% enter the lungs. Even for optimally sized particles of about 2-µm diameter, only 30% to 40% enter the lungs. Thus for 1 hour of disturbance or equivalent activity, it is possible to estimate that between 5 and 100 mite fecal particles per day enter the lungs. This exposure is dramatically different from that used for bronchial provocation, where 10^7 to 10^8 droplets of about 2-µm diameter are inhaled within 2 minutes. For airborne cat allergen, there is a wide range of particle sizes; and if the particles are not spherical, the actual mass could be greater than the equivalent sphere. The clinical response when a patient who is highly allergic to cats enters a house with a cat suggests that there can be a sufficient *number*of particles airborne to produce acute bronchospasm. In contrast, it is unusual for a patient who is allergic to mites to report rapid onset of symptoms on entering a house, even if the furniture and carpets are highly infested with mites. These observations suggest that exposure to mite allergens occurs at the time of disturbance and that exposure consists of a few particles per day. As with pollen grains, it appears that the primary role of fecal particles in asthma is to contribute to a chronic increase in bronchial hyperreactivity rather than to cause acute symptoms at the time of exposure (180).

The data on airborne levels of other indoor allergens are less complete. Cockroach and rodent urinary allergens have been measured in houses (237). Airborne cockroach allergen is associated with particles 10 µm or larger in diameter, is detectable with disturbance, and falls rapidly (53,54,57,144). Rat and guinea-pig urinary

allergens have been measured in animal quarters (178,238). The results for urinary allergens are similar to those for cat allergen, because allergen is airborne most of the time, and the particle sizes are smaller than those of mite or pollen allergens (178). In keeping with this fact and the high overall airborne levels, some allergic patients have acute attacks of asthma on entering an animal house. Until recently, measurement of indoor fungal allergens was not practical because of the paucity of sensitive immunoassays and the large differences in the expression of allergens on different spores (30,220). Recently an enzyme immunoassay for extracellular polysaccharides of *Aspergillus* and *Penicillium* was developed as a marker of fungal exposure (60). In that study, levels of extracellular polysaccharide were found to be correlated with total culturable fungi and with house dust mite allergens. In addition, an ELISA for measurement of Alt a 1 has allowed quantitation of this allergen in samples of house dust (253). Such assays are critical for determining the relationship of exposure to fungal allergens in houses to development of allergic disease.

RELATIONSHIP BETWEEN EXPOSURE TO ALLERGENS AND DISEASE: ESTABLISHING THRESHOLD LEVELS FOR EXPOSURE

For many pollens, the relationship between the pollen count and symptoms of hay fever provides strong evidence for a causal nature and demonstrates that airborne allergen levels are relevant to whether symptoms will be present in allergic individuals (158,159,186,223,279). For the role of indoor allergens in asthma, where there is no clear seasonal variation, the evidence for a causal relationship is less direct. In 1965 Hill (92) listed the evidence that was needed to conclude that exposure to an environmental dust was causally related to a respiratory disease (Table 61.7). The association between dust mite allergens and asthma is complicated because there are two distinct phases to the relationship: sensitization and the subsequent development of bronchial hyperreactivity and symptomatic asthma (Fig. 61.7). For some of the criteria listed in Table 61.7, the evidence relates to the relationship between sensitization and asthma, whereas for others (e.g., the dose/response relationship), the evidence is about the relationship of allergen exposure to sensitization. Perhaps the most convincing evidence comes from what Hill referred to as experimental (i.e., provocation and avoidance studies). Given the increasing evidence that asthma is an inflammatory disease, it is striking that the only challenge to the lung that consistently induces this kind of eosinophil-rich inflammation is inhalation of allergen by an allergic patient. Challenges with sulfur dioxide, passive smoke, ozone, and viruses can produce changes in lung function but do not on their own produce an eosinophilic infiltrate (129), although there may be important interactions between the effects of these substances and the effects of allergen exposure. Thus exposures to rhinovirus and to ozone have been reported to enhance the subsequent response to allergen provocation. Studies now show that reducing the exposure to allergen of patients with asthma who are allergic to mites can reduce symptoms and decrease bronchial hyperreactivity. The studies are categorized as (a) those in which the patients are moved to a sanatorium (116,183,212,263,272) or a hospital room, and (b) those in which avoidance measures are carried out in the patient's house (64,151,268). These studies have indirectly provided evidence about the levels of mite allergen in a house at which symptoms and bronchial hyperreactivity are maintained. To be effective in a controlled trial, allergen must be decreased by about 90%, and the levels should be decreased below 2 µg group 1 mite allergen per gram of dust if possible. With these criteria for effective reduction, good evidence for efficacy is available for covering mattresses, washing bedding at 130°F, removing carpets, and treating carpets chemically with either benzyl benzoate or 3% tannic acid (86,135,165).

Criterion	Evidence
Strength of association	Odds ratios ≥ 7 (and ≤ 30) have been reported in many studies
Consistency of association	Dust-mite sensitization has been demonstrated among individuals with asthma in the Netherlands, United Kingdom, Denmark, Japan, Australia, Brazil, and United States
Specificity	Asthma is the only lung disease associated with exposure to mite (or cat) allergens
Dose response relation	At least four studies have shown a dose-response relation between mite allergen levels and the prevalence of sensitization
Temporality	In a prospective study, exposure to high levels of mite allergen preceded evidence of sensitization and asthma
Experimental evidence	
Challenge studies	Bronchial provocation of allergic individuals can produce bronchospasm, increased bronchial hyperreactivity, and eosinophil infiltrate
Avoidance studies	Sanatoria and hospitals; patients' houses

SOURCE: Hill AB. The environment and disease: association or causation. *Proc R Soc Med* 1965;58:295, with permission.

TABLE 61.7. Evidence for a Causal Relation between Dust-Mite Allergen Exposure and Sensitization and Asthma: Criteria of Hill

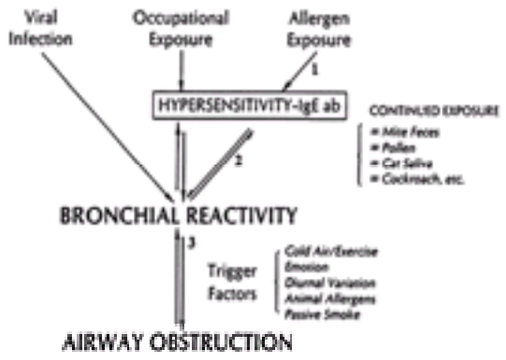

Figure 61.7. Role played by allergens in chronic asthma. The initial role of allergen exposure is in sensitization, including the production of immunoglobulin E (IgE) antibodies. For this phase (*1*), there is extensive evidence for a dose/response relationship. Patients who are sensitized are at increased risk for the development of such symptoms as rhinitis, asthma, and atopic dermatitis. However, the level of exposure necessary to induce symptoms or bronchial reactivity (*2*) varies widely among patients. Some individuals who have IgE antibodies are not aware of symptoms. Most of the triggers of acute asthma (*3*) are nonspecific, but some allergens can cause acute bronchospasm. The latter appears to be more common with allergens that are airborne on small particles (e.g., cat dander or rat allergens).

Threshold values have been proposed for several allergens. It is important to recognize the difference between these “thresholds” and those used for toxic chemicals. For chemical exposure, a threshold value is the level below which there is considered to be no risk. For allergens, the threshold value is a level below which 10% or fewer of the at-risk population develop sensitization (176,177,182,189). The other distinction is that with toxic gases (e.g., radon and formaldehyde), it is assumed that the whole population is at risk and that the risk of disease increases progressively with a dose above threshold. In contrast, with allergen exposure, “nonatopic” individuals are at low risk of sensitization or disease, even with high levels. Moreover, thresholds for sensitization may be different from thresholds for causing disease among sensitized individuals. It was initially proposed that 2 µg group 1 allergen per gram of dust was the threshold for sensitization to mite allergens (note: sensitization is not necessarily restricted to group 1 allergens), and that 10 µg group 1 allergen per gram of dust was a level above which individuals allergic to mites were more likely to have acute attacks (176,177,182). The proposed threshold for sensitization to mite allergens has been supported by numerous studies (40,127,218,226). In contrast, evidence for a simple relationship to symptoms is less clear. The fact that many allergic individuals do not develop asthma implies that for these individuals, the dose necessary to induce symptoms in the lungs is higher than that necessary to sensitize them. For other patients, including those with severe symptoms, the level of exposure necessary to induce symptoms is *lower* than that necessary to induce sensitization. Thus a simple threshold for symptoms may be less appropriate than one for sensitization. Indeed, the 10 µg/g level of mite exposure may be regarded more appropriately as a level above which those patients allergic to mites who are going to have symptoms do have symptoms.

THE MODIFIED Th2 RESPONSE TO ALLERGENS AS A MODEL OF TOLERANCE AND TARGET FOR IMMUNOTHERAPY

Allergic disease has increased dramatically in the last few decades and for reasons that remain largely unclear. Despite extensive speculation about a shift from Th1 responses early in life to those of a Th2 type, the evidence for Th1 responses to common allergens is limited to *in vitro* T-cell data. Thus there is very little evidence for high-titer IgG antibodies, delayed hypersensitivity skin tests, or lung diseases other than asthma. By contrast, there are now several clinical situations in which “tolerance” to an allergen has been demonstrated in the presence of an IgG4 antibody response. These include bee venom, rat urinary allergen, the response to immunotherapy, and most recently, the response to Fel d 1 among children living in a house with a cat. Among a cohort of children living in the United States, those children whose houses had more than 20 µg Fel d 1 per gram of dust were *less* likely to be sensitized to cat allergen (228). In this study, the prevalence of IgG antibodies correlated closely with exposure, and was predominantly of the IgG4 isotype; in contrast, the prevalence of IgE antibodies was decreased at higher allergen levels. These findings are in keeping with a “modified Th2” response (184). As mentioned earlier, previous evidence from Aalberse et al. (1) showed that the immune response to immunotherapy and the response of beekeepers to phospholipase A_2 is characterized by a predominance of IgG4 antibodies. Inducing a modified Th2 response with increased IgG4 and decreased IgE antibodies may be a more attractive immunotherapeutic strategy than stimulation of a cell-mediated Th1 response that has the potential to induce tissue damage.

CONCLUSIONS

Allergens are those antigens that commonly give rise to an IgE antibody response in humans. In keeping with animal models, this form of immune response is most often induced by repeated “low-dose” exposure to allergens such as pollens, fungal spores, cat dander, and mite fecal pellets. The word “allergen” is commonly used to describe either the source (e.g., ragweed pollen) or the purified protein (e.g., Amb a 1). Studies with purified allergens have shown that the immune response to allergens includes IgG and IgA antibodies, a T-cell response, and IgE antibody. With the development of protein and DNA sequencing, it has become possible to study the nature of allergens and the epitopes that react with T cells and IgE antibodies. In keeping with their sources, allergens are a very diverse group of proteins. Although many allergens are enzymes and several groups of related proteins have been defined, no characteristic features that relate to allergenicity have appeared. An allergen is best defined as a soluble foreign protein of 5 to 80 kd that is inhaled (or ingested) repeatedly, so that it commonly gives rise to IgE antibody responses in individuals who are genetically predisposed.

The purification of allergens has made it possible not only to study the properties of the allergen but also to measure the concentrations in extracts and in the environment. For the indoor allergens, in which none of the airborne particles can be identified microscopically, measurement of major allergens has proved important for quantitating exposure and analyzing the particle sizes of airborne allergens. This technique in turn has provided better understanding of the ways in which allergens contribute to asthma and of the appropriate measures to control exposure. Measurement of mite, cat, and cockroach allergens in houses has provided quantitative data about the relationship between exposure and sensitization. For dust mite allergens, these measurements have suggested a threshold for exposure above which the risk of sensitization is increased. These results provide important evidence about the role of allergens in asthma. By contrast, for cat allergen exposure, the dose/response relationship is not simple, and high exposure can induce a form of tolerance. Thus threshold levels cannot be simply applied to cat exposure. Understanding the role of allergens in allergic disease is directly relevant to several aspects of treatment; evidence about exposure has provided a scientific basis for evaluating methods of allergen avoidance in the treatment of asthma. In addition, the measurement of major allergens provides an objective method for standardizing allergen extracts, using micrograms of allergen per milliliter. Methods of this kind are increasingly being adopted in Europe. Finally, with molecular biology techniques, it is now possible to define epitopes on allergens that interact with T cells and B cells, thereby allowing the design of peptides or altered molecules as treatments that act selectively on T cells. Thus advances in understanding the proteins that give rise to immediate hypersensitivity have provided a cornerstone for investigating allergic disease.

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62 GENETICS OF ALLERGIC DISEASE

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It is now generally accepted that there exists a genetic component to many common chronic disorders, including both allergy (atopy) and asthma. Studies are in progress to determine which genes, and which polymorphisms within these genes, are responsible for increasing an individual's susceptibility to allergic diseases. Most likely, multiple genes interact with each other and with environmental factors to produce atopic disease (Fig. 62.1). Additionally, individuals with symptomatic allergies may have specific gene variants that influence the progression (or severity) of the disease and even the response to treatment (pharmacogenetics).

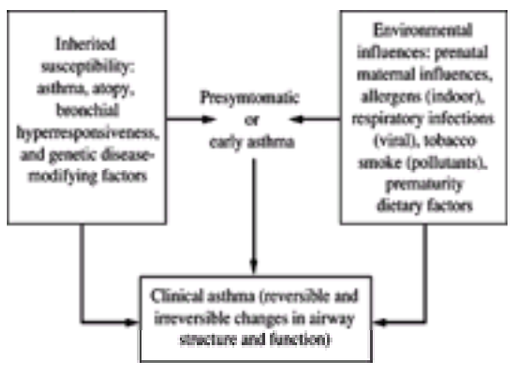

Figure 62.1. Gene/environment interactions in the development of childhood asthma.

Delineation of the role of genetics in allergy and asthma will further our understanding of the pathogenesis of these disorders (Table 62.1). Genetic tests in conjunction with phenotypic information in a specific family may be useful in early disease diagnosis, possibly even before symptoms occur, when preventive strategies may be appropriate. Better understanding of the disease process and the nature of the genes in the development and expression of atopic disorders involved also may lead to new therapeutic approaches. Thus these studies have widespread public health implications, given the high frequency of allergies in the general population.

• To understand the heritable component of the disease or trait and gene-environment interactions
• Elucidate important disease mechanisms
• Develop new therapeutic interventions
• Develop methods for early diagnosis and disease prevention

TABLE 62.1. Benefits of Genetic Studies in Allergy and Asthma

PHENOTYPE DEFINITION

In performing genetic studies, it is first necessary to clearly define the phenotype(s) (trait or disease) that will be studied; such definition as this is an important part of the overall experimental design (Table 62.2). It is reasonable to ascertain populations (e.g., families) through one or multiple index cases, or probands, who are affected by recognized standards, which should be clearly defined by standardized methods. Issues often arise regarding the affection status of relatives who have only some of the disease characteristics. For example, the “allergic” phenotype can be defined in several ways, ranging from a definition based solely on symptoms or objective measures such as total serum or specific immunoglobulin E (IgE) levels and/or response to allergen skin tests, to the combination of both subjective (symptoms) and objective traits. Multiple accepted definitions may exist, but a clear definition must be provided so that the results and potential differences between studies can be distinguished.

• Identification of a population
• Clinical assessment
• Linkage analysis: genome screen
• Fine mapping and gene localization
• Identification of the gene and the relevant sequence variant
• Gene-association studies
• Gene function and expression

TABLE 62.2. Steps Toward Finding a Susceptibility Gene for Allergy and Asthma

In studies of common disorders such as allergy and asthma, where interactions between genes and environmental factors are involved, associated phenotypes, which may be easier to measure objectively, are often used. For example, high total serum IgE levels have been shown to correlate with the clinical expression of allergy and bronchial hyperresponsiveness, which is an important component of allergic and asthmatic phenotypes (1,2,3 and 4). Therefore the results from positional cloning studies of total serum IgE levels may provide important information on susceptibility genes for both allergy and asthma. Identifying gene(s) and sequence variants for the regulation of total serum IgE levels represents one component (possibly a very important component) in the overall complicated processes involved in the development of allergic diseases.

In addition to total serum IgE levels, other measures can be evaluated as associated phenotypes in genetic studies of allergy and asthma (Table 62.3). Often laboratory measurements such as total serum IgE, specific IgE levels, or skin-test responsiveness to common allergens are used in studies of allergy, and bronchial hyperresponsiveness is used in studies of asthma. The advantages of these measures are the ease in which they may be obtained in all family members or individuals in a specific population and the possibility of analyzing these traits as quantitative measures without having to specify a specific clinical cut point. In addition, it may be possible to define the overall allergic phenotype based on the various clinical and laboratory measures with statistical techniques such as principal component analysis. This analysis simultaneously incorporates data from multiple measures such as clinical history, skin-test responses, and specific and total serum IgE levels into one outcome measure (5).

Atopic parameters
Total serum IgE
Specific IgE
Allergen skin tests
Bronchial hyperresponsiveness
Direct (methacholine, histamine)
Indirect (adenosine, exercise, etc.)
Asthma
Physician diagnosis
Symptoms and laboratory results
Severity classification
Airflow obstruction and reversibility
Combinations of "atopic" and "asthmatic" parameters

IgE, immunoglobulin E.

TABLE 62.3. Possible Phenotypes

GENETIC EPIDEMIOLOGY

Twin Studies

For both allergy and asthma, twin studies have been performed to estimate the extent of the genetic contribution. Comparison of frequencies of different allergic and asthmatic phenotypes in monozygotic (MZ) versus dizygotic (DZ) twins suggests the presence of a strong heritable component because MZ twins have the same phenotype more often than do DZ twins (6,7). However, twin studies are not useful for gene mapping or positional cloning studies because MZ twins share identical genotypes; DZ twin families may be used, but these twins are equivalent to full siblings, who are usually easier to ascertain.

The observed difference in the level of a quantitative trait in members of MZ versus DZ twin pairs provides information on the degree of heritability, whereas the difference observed between members of a MZ twin pair is more reflective of environmental influences. For example, in a study by Hopp et al. (8), the intrapair correlation coefficient for total serum IgE levels was 82% in 61 MZ twin pairs and 52% in 46 DZ twin pairs, giving a heritability estimate (the ratio of genetic variance to the observed total variance) of 0.61. This result provides strong evidence for a genetic component; however, the nongenetic component caused by environmental factors is still substantial. These estimates should be interpreted with caution, because the extent of the environmental components may vary in different individuals and populations. For example, in families or in a population that is exposed to a homogeneous environment, the estimate of heritability will be higher (smaller environmental component) than that in those exposed to a less homogeneous environment, even if the genetic component is the same.

Risk to Relatives

Significant evidence of familial aggregation also provides evidence for a genetic component to the phenotype, although aggregation also is influenced by common environmental factors or by an interaction of environmental and genetic factors. The risk ratio (l), which is the relative's risk compared with the prevalence of the disease or trait within the general population, is often used to estimate the degree of familial aggregation. These risk ratios can also be used to estimate the number of chromosomal loci involved in the disorder (9). However, the accuracy of these odds ratios is sensitive to the accuracy of the overall population prevalence (which may differ significantly between populations and is dependent on phenotype definition). In common disorders, such as allergies, risk ratios may show only a two- to threefold increased risk to a relative of an affected individual, supporting the presence of multiple genes.

Another method for evaluating evidence for a genetic component is to determine the correlation between family members for a quantitative trait. For example, a significant correlation between parents and offspring and between offspring was observed for total serum IgE levels in a study of more than 1,000 individuals from 200 Dutch families (10). An even stronger correlation was observed among siblings (p .001), supporting the presence of a strange genetic component for atopic phenotypes. A portion of this correlation was probably caused by environmental factors, although a significant correlation between parents (unrelated individuals) was not found. An interactive effect between genes and the environment also may be important.

Segregation Analysis

Segregation analysis may be performed to determine whether a specific genetic or environmental model explains the observed familial aggregation and may provide specific information on the mode of inheritance. The observed number of family members with the trait is compared with the expected number by using various models of inheritance, such as recessive, dominant, polygenic, codominant, and an environmental model. For example, the observation that children of two parents with elevated IgE levels also have high IgE levels suggests recessive inheritance of this trait. The frequency of children in such families with low levels would reflect the degree of incomplete penetrance of the susceptibility gene. To perform segregation analysis, families have to be ascertained in a standard manner that may need to be adjusted for in the segregation analysis. For example, all families in a specific study may be ascertained through one affected parent, but if they were selected though an affected parent and an affected child, the results will be biased in favor of a dominant model of inheritance. For genetic linkage studies, families are usually selected because of multiple affected members and are, usually, not appropriate for segregation analysis.

Another significant problem with segregation analysis is that usually only one- or two-locus models are fitted to the data, which are poor approximations to the true situation in common disorders that involve multiple genes (11). Therefore most recent studies concentrate on linkage analysis instead of segregation analysis.

When segregation analysis can be performed, it yields useful information pertinent to linkage analysis and gene identification. In segregation analysis, the best-fitting or most likely model of inheritance is determined by comparing the likelihoods from the different models tested (dominant, recessive, polygenic, or environmental) with the likelihood of the general model. Models that differ significantly from the general model can be rejected. Estimates of gene frequency and the frequency of each genotype as well as the degree of penetrance are obtained. The best-fitting model and parameter estimates are useful in subsequent linkage analysis. In numerous family studies, total serum IgE levels have been examined as a quantitative trait with segregation analysis (10,12,13,14,15,16,17,19 and 20), but there have been few segregation studies for other allergic or asthmatic phenotypes.

An analysis of data from 200 Dutch families is described as an example of segregation analysis for total serum IgE levels (10). These 200 families (1,171 family members) were ascertained through a parent (proband) who was initially evaluated between 1962 and 1975 at Beatrixoord Hospital, Haren, the Netherlands (a regional referral center for patients with asthma and other obstructive airways diseases). Patients with symptomatic asthma without a current asthma exacerbation were referred to this hospital and admitted for a standardized allergy and pulmonary evaluation. At the time of initial testing, all probands had asthma symptoms, were hyperresponsive to histamine (PC_{20} FEV_1 histamine £32 mg/mL, 30-second method), and were younger than 45 years. Among the 200 families, 166 families consisted of two generations, 33 families of three generations, and one family of four generations. Total serum IgE (IU) was measured by solid-phase immunoassay (Pharmacia IgE EIA: Pharmacia Diagnostics). Log (total serum IgE levels) were used because they are approximately normally distributed. Because log(IgE) values were higher in male subjects and in younger individuals, the effect of sex and age was included in the segregation analyses.

In the one-locus segregation analysis, both the sporadic model and mixed environmental model were rejected. Although single-gene models were rejected, several mixed mendelian models (major gene plus a residual effect) were not rejected. The mixed recessive model (i.e., a major recessive gene and with significant residual genetic effects) was the best-fitting (p = .75) and the most parsimonious model, although only slightly better than the mixed additive model. The estimate of the gene frequency q_a (corresponding to a high IgE level) under the mixed recessive model was 0.57, which results in genotype frequencies of 0.68 for *AA/Aa*, and 0.32 for genotype *aa* (assuming Hardy-Weinberg equilibrium). This recessive gene had a large effect on IgE levels and was responsible for 32.4% of the adjusted $\log_{10}$(IgE) levels in these families. The mean IgE level for genotype *aa* (μ_{aa}) was estimated to be 209 IU/mL, significantly higher than the mean (29 IU/mL) for the other genotypes (μ_{AA}, μ_{Aa}) and a clinically relevant value.

Because the control of IgE is probably regulated by more than one gene, a two-locus segregation analysis was performed; in this analysis, the mixed two major gene models (i.e., two major genes and residual genetic effects) fit the data significantly better than the one major gene model. Among the several mixed two major gene models that were tested, a model with a major recessive gene and a dominant modifier gene was the most parsimonious model. The estimate of the gene frequency for the first recessive gene (q_a) was 0.55, similar to the gene frequency of the mixed recessive model under one-locus segregation analysis. The estimate of gene frequency for the second dominant gene (q_b) was 0.8. The second gene modifies the effect of the first recessive gene so that a portion of the individuals who were homozygous for the high-risk allele at the first gene did not have high IgE levels. Individuals with genotype *aaBB* (homozygous for the high-risk allele at the first gene and homozygous for the low-risk allele at the second locus) had normal IgE levels (mean = 23 IU/mL), whereas individuals with genotypes *aaBb* and *aabb* (29.6% of the total sample) had high IgE levels (mean = 282 IU/mL). The two major genes were responsible for 51.3% of the adjusted $\log_{10}$(IgE) variance. The first gene was responsible for 40.6%, and the second gene was responsible for 9.0% of the observed $\log_{10}$(IgE) variance in these families.

In several other one-locus segregation studies, evidence for recessive inheritance of high total serum IgE levels has been observed (12,14,17,20). In a one-locus study of several large pedigrees, evidence also was obtained suggesting genetic heterogeneity in the mode of inheritance; the most parsimonious genetic model was different in the different pedigrees (19). From a study of the Amish community, which is genetically isolated and inbred, evidence for codominant inheritance was observed in an early study (18), whereas it was difficult to distinguish between models in a later study (21). Evidence for polygenic inheritance was obtained from a study of large Mormon families (22), who are more representative of the general population than are the Amish.

In each study, evidence was observed for a strong familial component to the inheritance of total serum IgE levels. The differences in the best-fitting model of inheritance and estimates of mean IgE levels and allele frequencies are not unexpected. They reflect differences between populations, ascertainment schemes, and analytic methodology for a trait, where there is substantial overlap between the distributions for individuals with low and high IgE levels. For example, in the one-locus study by Xu et al. (10), the recessive model was the most parsimonious, although the codominant model had a very similar likelihood. However, in a larger study, Martinez et al. (15) were able to estimate means and variances for three distributions, resulting in a codominant model of inheritance. Basically, this result reflects the difficulty in distinguishing between gene carriers (heterozygous individuals, “LH”) and homozygous family members (“LL” or “HH”).

Segregation studies also have been performed in nonwhite populations. A test for genetic heterogeneity across Hispanic and non-Hispanic white families was not significant; there was no evidence for a difference in the mode of inheritance between the two racial groups (15). Although a difference in gene frequency may be observed because of genetic variability in the founding populations, it was not surprising that no difference was found for this common trait.

The fact that evidence for a major gene was obtained from multiple single-locus segregation analysis does not mean that only one locus is involved in regulation of total serum IgE levels. Significant residual variance has been observed, suggesting an additional genetic influence. Two-locus segregation analysis was performed in the Dutch family study, and evidence for a second gene (dominant) was obtained (10).

There have been few segregation studies of allergic or asthmatic phenotypes besides studies involving total serum IgE levels. The evidence for a major gene for bronchial hyperresponsiveness (BHR) is less clear. In a family study of bronchial responsiveness to methacholine, evidence for a major gene was not found, but neither the genetic nor the environmental models could be excluded (23). In another study of the response to inhaled carbachol in 80 families with children with asthma, suggestive evidence for autosomal dominant inheritance with incomplete penetrance was observed (a formal segregation analysis was not performed) (24). In a sample of Dutch families, segregation analysis was performed for BHR to histamine (25). Ninety-two families ascertained through a parent, the proband, with asthma diagnosed approximately 25 years ago, were included in the analysis. Initially, no evidence for a major gene was observed; however, when IgE levels were included as a covariate, evidence for mendelian inheritance of a susceptibility gene for BHR with high penetrance was found. These data suggest that separate, yet interacting, genes for BHR and serum IgE levels may exist.

For the asthma phenotype, segregation analysis based on questionnaire data was performed and showed a strong familial polygenic component, suggesting the presence of multiple genes (26). This finding is not surprising and supports the complex genetic regulation in asthma and allergy. Unfortunately, there are limitations for segregation analysis in common disorders, because modeling three or four loci is not computationally feasible with currently available methods.

GENE IDENTIFICATION

General Approaches

Positional cloning has become easier to perform because of technologic developments that facilitate rapid and accurate large-scale genotyping at a reasonable cost (Fig. 62.2). However, identifying susceptibility genes for common disorders is still a major undertaking. Multiple study designs may be used in linkage studies in which families are used for positional cloning and gene identification. These approaches range from studies of only affected siblings to studies of multigenerational pedigrees. Families may be from a general heterogeneous population or a more genetically homogeneous or isolated population (possibly inbred) with a recent or more distant founder effect. As previously discussed, it may be useful to study associated phenotypes, such as total serum IgE levels, in all family members, as well as to evaluate the presence of symptomatic allergic disease. It also is important to consider the effect of age on the phenotype when studies are performed on individuals from different generations. For example, a young child may not have developed these disorders or traits, or it may not be possible to phenotype accurately a parent with a history of childhood asthma, who is now a smoker with evidence of chronic obstructive lung disease.

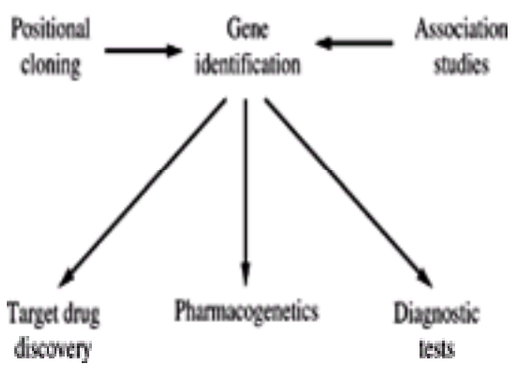

Figure 62.2. Approaches to identifying genes in allergic disorders.

A commonly used approach for gene-mapping studies is to ascertain and characterize affected siblings (or other types of relative pairs). In this study design, parents do not have to be phenotyped, and it is optional as to whether DNA samples are obtained from them. This design is often used for genome screening to identify chromosomal regions likely to contain susceptibility genes. For genomic screening, a large number (usually more than 300) of informative polymorphic DNA markers that span each chromosome at regular intervals (approximately 10 cM) are genotyped and used to define chromosomal regions linked to allergic or asthmatic phenotypes. This study design is useful because siblings are of a similar age and share similar environmental exposures compared with other types of relatives. It may be difficult to phenotype a parent because of other confounding factors or loss of phenotypic expression with age; and the environmental influences present when a parent developed the disorder may be quite different from the children's environmental exposures. This approach has been used by the U.S. Collaborative Study on the Genetics of Asthma (CSGA), in which families were ascertained through an affected sib pair [both with a doctor's diagnosis of asthma, the presence of asthma symptoms, and BHR (or reversibility if it was not appropriate to perform hyperresponsiveness testing)]. This was not strictly a sib-pair design because parents and other siblings were phenotyped and genotyped, and some families were extended to include additional relatives (27).

Large multigenerational pedigrees also may be studied and ascertained through one or more probands. For common disorders, different susceptibility genes may enter the pedigree through multiple individuals (spouses as well as the original founders). For example, a nuclear family (parents and children) may be extended to include a first cousin with asthma; however, it is possible that the genetic susceptibility is different in the first cousin and is due to genes transmitted from the parent married into the pedigree. Although these families are still useful in practice, it is difficult to allow for bilineal families in the analysis.

Studies of isolated and inbred populations, in which the relationships between the nuclear families are known and may be traced back to a small number of founders, are very useful; for example, the Old Order Amish in Pennsylvania (21) and the Hutterites in South Dakota (28). A subpopulation of the Hutterite community has been studied for asthma, atopy, and related phenotypes by Ober et al. (28). The family relationships are well documented, and, although individuals may leave the community, no one enters it. Thus the gene pool has remained closed over multiple generations. However, even in a closed population, multiple genes may be segregating for common diseases. The Pennsylvania Amish are a similar inbred population who have been studied for atopy and total serum IgE levels (21).

It also may be useful to study nuclear families from homogeneous populations with a known founder effect, even if the actual relationships between the families are not known. For example, homogeneous populations have been studied in northern Holland (29). These populations are especially useful for fine mapping studies, because it is more likely that a shared haplotype (small chromosomal region around a disease gene) will be detected over a small region, limiting the number of potential genes to be examined. Similar to genetic studies in inbred populations, the susceptibility genes that are present in a homogeneous population may be less common in larger heterogeneous populations. Thus it is essential to study different populations to replicate findings and to determine differences in gene frequencies.

Results of Genome Screens

In one of the first genomewide screens, 80 white families were studied and genotyped for 253 autosomal markers. Analyses were performed for multiple phenotypes related to atopy and asthma (30). Both log IgE and skin-test response showed evidence for linkage to chromosome 11, and log IgE levels showed linkage to chromosome 16. Evidence for linkage to log of the slope of bronchial responsiveness to methacholine was observed for chromosomes 4 and 7, with *p* values <.0005 (D4S426, D7S484). Evidence for linkage for log IgE levels also was obtained for chromosome 7, but none of the measures of the atopic phenotype (total serum IgE levels, skin tests, or total eosinophil count) showed evidence for linkage to chromosome 4. Both log IgE and skin-test response were linked to chromosome 11, and log IgE levels showed linkage to chromosome 16. The marker spacing used in this early study was broader, and the sample size was smaller (80 families) than those used in some of the later studies and, thus, may have decreased the power to detect genes with smaller effects.

A genome screen to map loci for asthma was performed by the Collaborative Study of the Genetics of Asthma using 320 markers at approximately 10-cM spacing in 261 affected sib pairs from different racial groups (African Americans, whites, and Hispanics) ascertained through two siblings with asthma (27). Affected family members met the same criteria for asthma as the probands, which included a physician's prior diagnosis of asthma, the presence of asthma symptoms, and evidence of BHR or reversibility of airflow obstruction. Evidence for linkage was seen in the African-American families for 5p15 (p = .001) and 17p11.1-q11.2 (p = .0015). In the Hispanic families, evidence for linkage to 2q33 (p = .0005) and 21q21 (p = .004) was seen, with preliminary evidence for 12q14-24.2 (p = .03). In the observed white families, the following regions were detected with *p* values <.01: 11p15, 12q14-24.2, 12q21.3-qter, 14q11.2-13, and 19q13. Two other regions had *p* values <.02: 5q23-31 and 6p21.3-23. Although multiple regions were detected in the white families, none had *p* values <.001. It is important to note that these are the results from the first 40% of the sib total sample and must be verified in the total sample.

A genome screen for asthma and BHR (approximately 300 markers) was performed in the inbred Hutterite population (361 individuals as the primary sample and 292 individuals in a replication sample) (28). The analysis was performed for four phenotypes: “strict” asthma, BHR, asthma symptoms and “loose” asthma (symptoms and/or BHR). Evidence for linkage was observed in both the primary sample and the replication sample for four chromosomal regions: 5q23-q31, 12q15-q24, 19q13, and 21q21 (p = .00091 for D5S1480, .0025 for D12S375, .010 for D19S178, and .033 for D21S1440 for the total sample).

In a multicenter German study, a genomewide scan with 351 markers was performed for 97 families with 156 affected sib pairs (31). Evidence of linkage to four regions of the genome (chromosome 2pter, 6p21.3, 9q, and 12q13) was obtained for clinical asthma D2S2298 (p = .0074), D6S291 (p = .0081), D9S1784 (p = .0073), and D12S351 (p = .0103). For BHR, similar results were found for chromosomes 2 and 9.

In the sample of 200 Dutch families, one- and two-locus segregation analysis was performed and provided evidence for at least two major genes that regulate total serum IgE levels. A genomewide screen was performed in these families for log IgE with variance component analysis, a method appropriate for quantitative trait mapping. LOD scores greater than 2 were obtained for chromosomes 3, 5q, 7, and 12 (10). In addition, a genomewide screen was performed for the BHR phenotype. The strongest evidence for linkage was observed on chromosome 5q in the same area reported previously with a candidate gene approach. Evidence for linkage also was observed for chromosome 3p (LOD = 2.8). Preliminary evidence was seen for three other chromosomes (1, 2, and 7) (32).

Multiple regions of the genome have been observed in several of the genomewide screens, and some of these regions also have been found in candidate gene studies. Replication of results is important because few studies are of adequate size to detect the multiple genes probably present for genetic susceptibility to asthma and allergy. Linkage to chromosomes 5q, 11, and 12q has been observed in multiple studies. Additional regions, including 6p and several others, have been observed but not replicated in other studies. However, even for replicated findings, it is not clear that the same regions are being detected in the different studies. For some chromosomes, the markers reported in different studies are at a significant distance from each other (for example, 30 cM). Possibly there are multiple susceptibility genes in a region. In addition, because of the genetic complexity of these diseases, it is difficult to model the disease accurately in the analysis, which makes it difficult to map the gene location accurately (Table 62.4).

Chromosome 5q	
	Total serum IgE and bronchial hyperresponsiveness mapping to 5q, a region with multiple proinflammatory candidate genes
Chromosome 6p	
	Associations between responses to specific allergens and HLA haplotypes
	Linkage of the asthma phenotype
Chromosome 11	
	Patented gene isolated from small inbred island population
Chromosome 12q	
	Linkage of allergic and asthma phenotypes to multiple chromosomal regions

HLA, human leukocyte antigen.

TABLE 62.4. Proposed Chromosomal Locations for Susceptibility Genes for Asthma and Allergy

Chromosome 5q

Multiple studies, including studies in the Amish, Hutterite, and Dutch populations, have shown evidence for linkage to 5q for allergic and asthmatic phenotypes. This region on 5q contains many candidate genes involved in the regulation of IgE and the development or progression of inflammation associated with allergy and asthma [cytokines interleukin (IL)-3, IL-4, IL-5, IL-9, IL-13, and granulocyte/macrophage colony-stimulating factor (GM-CSF); b_2-adrenergic receptor; glucocorticoid receptor]. In the inbred and Amish population, linkage to chromosome 5q in the region of several of the cytokines was observed for regulation of total serum IgE levels (21). In the Dutch study, significant evidence for linkage was observed for total serum IgE levels, BHR, and asthma. In the white families in the CSGA study, preliminary evidence for linkage to this region of 5q was observed for the asthma phenotype (27). Strong evidence for linkage to 5q (D5S1480) was observed in the Hutterites (another inbred population) for the “loose” definition of asthma, which included individuals with asthma, BHR, or symptoms of asthma (28). Mapping to a region that contains many candidate genes makes it difficult to perform fine mapping studies because polymorphisms in several of these genes may have small effects.

Chromosome 6p

Multiple HLA haplotypes have been correlated with several measures of the allergic phenotype in a number of studies (33,34). Linkage to chromosome 6p has been obtained for a related phenotype (eosinophil count) in a genome screen of atopic families (30). In the CSGA white population, there was evidence for linkage to 6p for the asthma phenotype (27). The German genomewide screen for asthma-susceptibility loci also detected evidence for linkage to this region (31). Because many individuals with asthma are allergic, it is not clear whether the evidence for linkage to this region is due to the major histocompatibility complex (MHC) and its influence

on the allergic response or whether other susceptibility genes for atopy and asthma map to this region.

Chromosome 11q

Evidence for linkage of a broadly defined allergic phenotype to markers on chromosome 11q was first described in 1989 (35). In a later study, evidence was obtained that sequence variants in the FceRIb gene on 11q increases the risk for developing allergy and possibly even asthma (36). The sequence variant detected in their families (Leu181) has not been observed in several other studies including the Dutch family studies (37). However, there is continued evidence for linkage of the atopic phenotype to this region. Recently a patent was released reporting linkage to chromosome 11p (near the centromere) and the identification of two novel susceptibility genes found in an isolated inbred population of approximately 350 individuals living on a small south Atlantic island (Tristan da Cuhna). These genes are similar to members of the *ets* family of transcription factors. According to this patent, at least one polymorphism in one of these genes is associated with asthma. Until this finding is evaluated in other, more heterogeneous populations, it is not clear whether variations in this gene may explain the published linkage to this chromosome. This discussion illustrates the difficulties in accurately mapping the susceptibility gene and determining sequence variants that may confer a moderate increase in susceptibility.

Chromosome 12q

There is a broad region (possibly two regions) on 12q with strong evidence for linkage in multiple studies for several phenotypes associated with allergy and asthma, as well as for clinical asthma. Evidence for linkage for both asthma and total serum IgE levels was found in an Afro-Caribbean population, and for total IgE levels, in the Amish population (38). In the CSGA, evidence for linkage was observed for the asthma phenotype in whites (p = .004) and Hispanics (p = .026) but not in African Americans (27). In a sample of randomly ascertained families and a sample of families ascertained for multiple members with asthma, evidence for linkage was seen for both asthma and allergic phenotypes (39). Although several candidate genes map to this region, including interferon-g, nitric oxide synthase 1, and mast cell growth factor, polymorphisms in these genes do not appear responsible for the observed linkage. As with the other chromosomal regions, evidence for linkage is found over a very wide region of the chromosome, and possibly there are two linked regions and multiple susceptibility genes.

Candidate Gene Associations

Association studies are performed to compare the frequency of a specific polymorphism (sequence variant) in a candidate gene in individuals with and without the disease phenotype. It is important to determine *a priori* the rationale for performing such a study, because there are many candidate genes for most of the common disorders. For example, it is reasonable to test for an association with a sequence variant with a known function that is biologically important. It is also important to demonstrate significant evidence at an appropriate statistical level. For most candidate gene studies, conflicting reports make it difficult to determine the significance of the findings. To add to this complexity, another explanation of an observed association is linkage disequilibrium (i.e., the observed polymorphism is physically close to the sequence variant important in the disorder, and there is co-inheritance of both polymorphisms). Therefore the polymorphism tested may not be the relevant functional polymorphism.

Two candidate genes (CD14 and IL13) that have significant evidence for association to allergic phenotypes map to the region of 5q where linkage has been detected. CD14 is a multifunctional receptor expressed primarily on the surface of monocytes, macrophages, and neutrophils. It also is present in soluble form in serum and in bronchoalveolar lavage fluid after segmental allergen challenge (40). Baldini et al. (41) identified a polymorphism in the promoter region of the CD14 gene (C to T transition at position –159) and found that among 317 whites, TT homozygotes had lower total serum IgE levels than did the CT and CC genotypes together. However, the difference was significant only among skin-test–positive individuals (p = .004; n = 163). In addition, the mean number of skin tests was significantly decreased in TT homozygotes compared with the mean number in the combined CC/CT groups (p = .001). No association was observed among skin-test–positive Hispanics, although the sample size was considerably smaller (n = 43). In the Dutch population, a similar association was found (42). The C allele acting in a recessive manner (CC vs. CT or TT) was associated with an increased number of skin tests in all probands and spouses (p = .008) (42). These data suggest that CD14 may have a role as a modifier gene by modulating allergic phenotypes that affect disease expression (severity), but this gene is probably not involved in genetic susceptibility to allergy.

IL-13 is a cytokine produced by T-helper subtype 2 (Th2) cells capable of inducing isotype class-switching of B cells to IgE (43). Association studies with IL-13 polymorphisms have been performed with various atopic phenotypes in several populations. A promoter polymorphism was identified at position –1055 adjacent to the nuclear factor of activated T cells (NFAT) site and reported to be associated with allergic asthma in a Dutch population (44). In addition, an Arg130Gln polymorphism in exon 4 has been shown to be associated with high total serum IgE levels (45,46), atopic dermatitis (45), and asthma (47) in German (45,46), American (46), British (47), and Japanese (47) populations. Results in the Dutch population reveal significant associations between either asthmatic or atopic phenotypes for the two single-nucleotide polymorphisms described earlier, plus a single-nucleotide polymorphism in the 3' untranslated region (UTR) of the gene (46).

Other examples of association studies with allergic phenotypes are the studies of the IL-4 receptor and its multiple polymorphisms (48,49,50,51 and 52). In the first study, an association for the Gln551Arg polymorphism with atopy (defined as an elevation in total serum IgE levels) was observed (48). This variation also was reported to produce a gain of receptor function. It has now become evident that multiple polymorphisms in this gene are in strong linkage disequilibrium with each other. From studies on the Hutterite population, the 406Cys allele was strongly associated with atopy based on skin-test responsiveness (52). Multiple pair-wise haplotypes were strongly associated with asthma; because the Hutterites are an inbred founder population, there is strong linkage disequilibrium between many of the polymorphisms. Even in the families from the United States CSGA, strong linkage disequilibrium and different haplotypes from those in the Hutterite population provided significant evidence for an association with asthma and atopy (52). Results in our Dutch population revealed a significant association between three polymorphisms (E375A, C406R, and S478P) and allergic phenotypes such as increased levels of total serum IgE levels (³100 IU/mL) and more than one positive skin test. In each case, the common allele (E375, C406, and S478) was associated with the allergic phenotype (p = .001–.04). The most significant results were observed with the E375A, C406R, and S478P polymorphisms in individuals with at least one positive skin test and total serum IgE levels ³100 IU/mL, presumably the “most allergic” group in this population (unpublished data). Because it is not clear from the multiple studies which polymorphism or combination of polymorphisms is responsible for the observed association, t here may be additional important variants in the gene that are in linkage disequilibrium with those already studied. Because polymorphisms within a gene are often in linkage disequilibrium, this example illustrates the difficulty in determining the role of a given polymorphism in a candidate gene that has a modest effect on risk.

Candidate gene studies may be performed to determine the effect of a postulated susceptibility gene or to examine the potential effect of a disease modifier gene. An important example of an association study with clinical implications is in the area of pharmacogenetics. The response to a given therapy varies among patients with asthma, and part of this difference may be due to genetic variability. For example, there are differences in response to b-agonist therapy among patients with asthma, which may be partially due to genotypic differences for polymorphisms in the b_2-adrenergic receptor gene (53). There are several known functional polymorphisms in this gene, which maps to chromosome 5q, including those at codons 16, 27, and 164 (54,55 and 56). b_2-Agonists are the most common therapeutic agent used to treat asthma. It has been suggested that regular use of inhaled b-agonists may be associated with adverse events in asthma. It is possible that differences in pharmacologic responses to b-agonist therapy among individuals with asthma may be affected by polymorphisms in this receptor. Israel et al. (55) reported that individuals who are homozygous for arginine at position 16 (Arg/Arg) showed a decline in respiratory function when treated with regular b_2-agonist therapy. This decline increased during a 4-week run-out period. Findings such as these are important because they may explain clinical observations related to adverse pharmacologic responses and may be used to identify a subgroup of individuals who are likely not to respond as well as others to a specific therapy.

SUMMARY OF PROGRESS

Once linkage is confirmed in complex genetic disorders such as asthma or clinical allergic disease, finding the actual gene is a complex process (11). It remains to be seen whether there are single mutations that have a major effect on genetic susceptibility to asthma, or whether even within a major gene, there are multiple common sequence variants. Clearly, multiple genes influence the atopic and asthmatic phenotype (Table 62.5). This is expected, given the frequency of the disease and the failure to detect a strong major gene effect in family studies. Nevertheless, significant progress has been made in the last decade. Several regions of the genome have been identified for susceptibility loci to asthma and BHR (Table 62.4). Future research will be focused in five major areas: (a) completion of genomewide searches in different populations, (b) replication of previously reported linkages, (c) development of multilocus models to investigate the role of multiple susceptibility loci, (d) fine mapping of all the region(s) of interest, and (e) studies of candidate gene association. Determining the specific role of each gene in the development of atopy and asthma, as well as closely associated phenotypes, will result in improved understanding of phenotype-to-genotype correlations. These combined genetic, molecular, and clinical studies will lead to improved therapies and better techniques for early diagnosis of allergy and asthma. By understanding the basic genetic mechanisms that lead to the development of allergy and asthma, new therapeutic interventions will be developed that will modify the development and clinical progression of these common disorders.

• Evidence for multiple susceptibility genes with ethnic differences. Similar regions detected in a restricted and an inbred population
• Different types of populations are useful to narrow regions for positional cloning and gene identification
• Conditional analyses show evidence for regions similar to those previously reported and for gene/gene interaction
• Multiple susceptibility genes probably have sequence polymorphisms (common variants), probably not severe mutations, that influence the phenotype
• Current challenge is to identify these genes and investigate gene-environment interactions

TABLE 62.5. Summary of Progress in the Genetics of Allergy

Acknowledgment *We were supported by NIH grants HL49602 and HL48341.*

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63 ALLERGIC DISEASES

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Allergic rhinitis is caused by mucous membrane exposure to inhaled allergenic materials and is mediated by specific immunologic mechanisms. The characteristic symptom complex includes sneezing, nasal congestion, and watery discharge as well as conjunctival itching. Bronchoconstriction may accompany these symptoms. This discussion of allergic rhinitis is concerned mainly with the airborne allergens described in Chapter 61. These reactions are triggered initially by immunoglobulin E (IgE) antibodies. There follows a late-phase inflammation, presumably directed by cytokines secreted by T-helper type 2 (Th2) lymphocytes.

ALLERGEN EXPOSURE

The nose is an efficient (nearly 100%) filter for particles 10 µm or greater in diameter. Efficiency drops progressively with particle size; removal is 80% for 5-µm particles and approaches zero for 1- to 2-µm particles (1). The epithelium is squamous at the entrance but changes to ciliary just posterior to the anterior tips of the nasal turbinates. A large portion of large particulate matter deposits in the anterior nonciliated area as a result of high linear velocity, a change in direction of flow, and filtration by hairs. Smaller particles are removed on the ciliary epithelium by impingement against bends in the air stream created by the convoluted passages and also are removed to some unknown degree by electrostatic precipitation (2). Particles deposited on the ciliary epithelium become entrapped in the mucus blanket and are moved to the pharynx for swallowing in 2 to 15 minutes.

Particulate allergens such as pollens vary in size from 10 to 100 µm, making them ideal for filtration by the nose. Mucosal response to deposited allergens is rapid; sneezing often occurs 30 to 60 seconds after inhalation of pollen, because presensitized cells are readily accessible to pollen antigens (3). Mast cell–basophil mediators are readily found in nasal secretions within minutes of exposure, concomitant with symptoms. The mediators identified include histamine, mast cell tryptase (4), prostaglandin D_2 (PGD_2), and leukotrienes (5); there is significant concordance among the mediators. The amount of mediators generated is proportional to the amount of allergen in the challenge. Vascular permeability increases rapidly, as mediators are accompanied by serum proteins including albumin and kininogen (a substrate for kinin formation) (6). Kinins concomitantly found in secretions are generated from this source. Mast cells are the main source of mediators on new exposure. Whereas many of these mediators can come from several cell types, mast cell tryptase measured by radioimmunoassay is specific and can be found regularly in nasal secretions shortly after antigen challenge (4,7).

Mediators are no longer detectable in secretions 30 minutes after a single challenge (3). After the early reaction, symptoms return in many patients 3 to 11 hours later, and most of the mediators reappear in secretions for a time (8). Among mast cell mediators, PGD_2 is notably absent in this “late-phase” reaction, suggesting basophils as a prime source.

CELLULAR FINDINGS

Twenty-four hours after challenge, large numbers of neutrophils and eosinophils have accumulated in the tissues (9) and secretions (10). Intraepithelial mast cells increase in number in the mucosa but not in the secretions (11). Basophils also increase in number in secretions (12) as well as during natural exposure (11,13). One day after challenge, $CD4^+$ T-helper cells are increased in number in the submucosa (9). At the same time, the number of cells expressing messenger RNA (mRNA) for interleukin (IL)-3, IL-4, IL-5, IL-13, and granulocyte/macrophage colony-stimulating factor (GM-CSF) are increased (14,15). The likely sources of these proinflammatory cytokines are T cells of the Th2 type, which are polarized to secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (16). The principal cells expressing IL-4 and IL-5 have been identified as $CD3^+$ T cells (17,18). Fourteen percent to 22% of clones of T cells from nasal and bronchial biopsy sites after challenge showed allergen specificity and exhibited a Th2 profile (16). Presumably, they are stimulated by antigen-presenting cells presenting peptide sequences from the antigen to specific receptors on these T-cell clones. Cells that can process antigen for presentation to T cells are abundant in the nose. CD-1 (T6), human leukocyte antigen (HLA)-DR–expressing dendritic cells are readily found in the mucosa (19). They increase in number during seasonal rhinitis (20). Macrophages positive for CD68/EBM11 and HLA-DR, and thus capable of presenting antigen, also are found in the submucosa and increase in number during seasonal rhinitis and after allergen challenge (21).

Granulocytes enter tissues by sticking to vascular endothelium and subsequent diapedesis. In nasal biopsies 24 hours after allergen challenge, leukocyte adhesion molecules on vascular endothelium, E-selectin, and vascular cell adhesion molecule 1 (VCAM-1) are increased. VCAM-1 has corresponding ligands on eosinophils, basophils, lymphocytes, and monocytes but not on neutrophils (22). Epithelial cells also generate chemokines, particularly the CC chemokines (23,24) with chemotactic activity for eosinophils and T lymphocytes. After challenge, mRNA coding for RANTES (regulated on activation, normal T-cell expressed and secreted), monocyte chemotactic protein (MCP)-3, MCP-4, and eotaxin is expressed mainly by macrophages, T cells, and eosinophils (25). Furthermore, IL-16, a specific chemoattractant for $CD4^+$ cells (T cells, eosinophils, and macrophages) is upregulated after allergen challenge (26). Presumably, the ingress of effector cells into challenged tissue is the result of secretion of these cytokines by resident Th2 lymphocytes. The specific effector substances secreted by eosinophils, basophils, and T lymphocytes are discussed in Chapter 22, Chapter 23, and Chapter 18, respectively, along with their roles in disease.

RELATIONSHIP OF IMMUNOLOGIC REACTIVITY TO SYMPTOMS

There is a strong correlation between the sensitivity of mediator-containing cells and the severity of disease during natural pollen exposure. The antigen concentration required for 50% histamine release from the basophils of an allergic individual defines the cell sensitivity, which is stable from season to season in most untreated adults. It varies, however, 10,000-fold from patient to patient. The severity of a patient's allergic symptoms during the ragweed season is directly related to the sensitivity of his or her cells to ragweed Amb a 1 (27,28). Thus the leukocyte system provides a prognostic measure of the severity of the disease that is useful in selecting matched groups for clinical trials. Serum levels of specific IgE antibodies also parallel the severity of symptoms on natural exposure in untreated ragweed-sensitive patients; and leukocyte histamine release and serum levels of IgE antibodies also have a close correlation (29,30).

NONSPECIFIC ENHANCEMENT OF ALLERGIC REACTIONS

Immunologic mechanisms explain many of the manifestations of simple allergic rhinitis. Nevertheless, mucosal hyperemia, swelling, and hypersecretion can be induced by noxious stimuli that have nothing to do with allergy. Additional processes, both physical and emotional, can therefore contribute to the picture of allergic rhinitis.

Some of these stimuli may act through mediator release triggered by nonimmunologic mechanisms. For instance, Togias et al. (31) found that patients subject to nasal stuffiness and hypersecretion on exposure to cold reacted in the laboratory to nasal challenge with cold (3 to 10°C), dry (10% relative humidity) air not only with characteristic symptoms but also with the appearance of mast cell–basophil mediators in secretions.

A ragweed-sensitive patient with normal nasal function responds to pollen exposure out of season with modest swelling and hyperemia. During the hay fever season, when the mucosa is already swollen and hyperemic, an additional exposure to pollen elicits a severe reaction (32). Exposure of ragweed-sensitive patients on successive days to pollen in sufficient amount to invoke symptoms induces reactions similar in severity with less pollen each day. This *priming* effect is nonspecific and local; repeated exposure to one pollen induces overreactivity to another immunologically unrelated pollen; and if only one nostril is exposed, the unexposed nostril does not become primed. Biopsies of the mucosa after such repeated exposures show eosinophil infiltration and basement membrane changes (33,34). The increased symptomatic response is accompanied by an increase in the level of histamine in secretions, but not of PGD_2, suggesting that an increased number of basophils may contribute to the response (35).

Emotional stimuli act in the same way. Persons exhibiting nasal hyperfunction in response to pollen exposure respond with further reactions when subjected to a

conflict situation. The nose can react in only one way to a variety of stimuli, and these stimuli may act in concert (32,36).

Nevertheless, the severity of seasonal rhinitis is clearly related to allergen exposure. In the laboratory, inhalation of ragweed pollen produces both symptomatic responses and inflammatory mediators in secretions in proportion to the amount of pollen inhaled. The minimal amount of pollen required to elicit a response varies from 10^3 to 5×10^5 pollen grains, depending on the patient (37). A similar quantitative relationship between exposure and severity of symptoms may be demonstrated during natural pollen exposure. As a season of exposure progresses, the severity of symptoms gradually increases in relation to airborne pollen concentration. Although sometimes attributed to the appearance of other allergens (such as mold spores), this phenomenon may equally well represent either specific immunologic enhancement of sensitivity or nonspecific priming.

Over a 4- or 5-year period, annual monitoring of patients' seasonal disease with symptom diaries shows little change if there is no immunologic intervention. The symptoms may gradually fade in severity with aging. Spontaneous remissions are uncommon but well documented. We have observed a small number of young and middle-aged adults who spontaneously lost clinical sensitivity to ragweed pollen as well as histamine-releasing activity of basophils and skin test reactivity despite continuing seasonal exposure to ragweed during treatment with only antihistamines.

CHRONIC RHINITIS, HYPERPLASTIC SINUSITIS, AND NASAL POLYPS

Symptoms of rhinitis can occur year round because of exposure to dust mites, animal danders, mold spores, and other allergens perennially present in the environment, but in many cases, no relationship can be established between allergic exposure and symptoms. Hyperfunctioning noses present a similar appearance; hence, pale, swollen membranes do not establish the diagnosis of allergy. Patients with perennial rhinitis frequently have chronic thickening of the sinus membranes (hyperplastic sinusitis) and polyps of the nose or sinuses (38). With hyperplastic sinusitis, the pathologic change is an irregular thickening of the antral membrane, frequently associated with mucous polyps within the sinus or with cysts containing mucoid fluid. The epithelium loses its cilia and is thickened and hyalinized, and the tunica propria is edematous and infiltrated by lymphocytes, plasma cells, and eosinophils (39). Nasal polyps most frequently arise from within the ethmoid or maxillary sinuses and project into the nasal cavity. The polyp is made up chiefly of edema fluid with sparse fibrous cells and few mucous glands. There is infiltration by eosinophils, mononuclear cells, and plasma cells.

Associated allergic diseases are common. Among 200 patients with hyperplastic sinusitis; 165 patients had asthma, 25 had hay fever, 33 had eczema, and some had other allergic conditions. One half of the patients showed positive wheal and erythema skin reactions to recognized allergens, but many of these reactions were not considered clinically significant. A considerable number of patients were sensitive to aspirin (40).

mRNAs for some of the same cytokines present in tissues of individuals with seasonal allergic rhinitis have been found in patients with chronic rhinitis and sinusitis. These include GM-CSF, IL-3, IL-4, IL-5, and IL-13 in patients with allergies. Individuals with nonatopic sinus disease have increases in mRNA for the same cytokines with the exception of IL-4 (41,42). Furthermore, they have less IL-5 and more GM-CSF than do allergic individuals (43). VCAM-1 is upregulated in both groups. Increased numbers of B cells accompanied by increased IgE heavy-chain transcripts are found only in allergic patients (44). These studies appear to confirm the allergic pathogenesis of many cases of sinus disease. There remain many patients with disease that is not demonstrably allergic and has an obscure pathogenesis, even though it involves some of the same pathways.

Nonallergic sinus disease may be a common term for a number of conditions. Two genetic disorders, cystic fibrosis and primary ciliary dyskinesia (Kartagener syndrome), are associated with persistent sinus disease. Furthermore, a survey of 147 consecutive patients with chronic rhinosinusitis found 11 to carry a mutation in the cystic fibrosis gene, an incidence significantly higher than that of the general population (45). Whether other mutations may confer susceptibility to chronic rhinitis or sinusitis is unknown. Patients with antibody deficiencies usually have sinus disease, along with infections elsewhere.

DIAGNOSIS

Most important in diagnosis is the skill of the physician in eliciting the history (time, place, and circumstances of occurrence of symptoms) and correlating it with the biology of allergens (46,47 and 48). A suspected diagnosis should be confirmed with an objective test for specific immediate sensitivity conferred by IgE antibodies. The long-accepted method is direct skin testing with diluted extracts of the suspected allergens. The two principal means used are (a) the intradermal method, in which 0.01 to 0.05 mL is introduced with a fine hypodermic needle between layers of the skin; and (b) the prick test, in which a superficial wound or scratch in the outermost layer of the skin is overlaid with a drop of antigen solution. A positive reaction is revealed by a wheal with a surrounding flare developing over a 15- to 30-minute period. The intradermal test yields positive reactions at lower concentrations, as a greater volume of antigen is introduced. A study of *Bacillus subtilis* antigen revealed a 1,000-fold difference in sensitivity between the two methods; when this factor was applied, there was excellent correlation between the two tests (49).

Skin tests have been criticized on several counts: (a) They are at times positive to one or more common allergens in the absence of allergic symptoms or are negative when the clinical impression is positive or when provocative tests with the allergen evoke a reaction; (b) The degree of positivity may vary from one anatomic site to another; (c) The range of error is no less than threefold and may be tenfold in clinical practice; (d) Extracts may vary in potency from one batch to another or may deteriorate on storage; and (e) Use of antihistamines or sympathomimetics may influence the outcome. Many of these problems are solvable by adequate attention to technique and to the quality of reagents used (50,51 and 52).

In vitro methods of detecting IgE-mediated reactions include both immunoassays for serum IgE antibodies, such as the radioallergosorbent test (RAST), and biologic tests, such as histamine release from isolated, washed, viable leukocytes. Several studies attest to their diagnostic usefulness in hay fever, asthma, and anaphylaxis (53). Only a few patients with positive RAST and skin tests have leukocytes that do not release histamine *in vitro* (51).

TREATMENT

Three principal therapeutic methods are used to alleviate allergic rhinitis: avoidance of exposure; pharmacologic treatment to minimize or counteract symptoms; and immunologic treatment, which attempts to alter the immune response to allergens.

Avoidance of Exposure

Avoidance of exposure to specific allergens is a simple and direct concept that is often difficult for patients to carry out. Patients with hay fever may take vacations to areas free from pollen for the duration of pollen exposure. The ragweed plant, for instance, occurs principally in central and eastern North America and is not present in high mountains, on the West Coast, or in southern Florida.

House dust mites are a major allergen for perennial rhinitis as well as asthma. When the degree of exposure is uncertain, dust samples should be collected from the mattress and bedroom and sent to a laboratory for determination of dust-mite allergen. Levels >2µg allergen per gram of dust indicate that control measures are in order. The measures commonly recommended to decrease mite exposure in the home involve mostly the sleeping room and are listed in Table 63.1.

House pets are another source of allergen that can be eliminated, although removal of a dog or cat from the home does not result in rapid reduction of allergen exposure. Cat allergen levels, for instance, may remain elevated for 20 weeks or more. Aggressive cleaning can cause allergen levels to decline more rapidly (54).

Enclose pillows, mattresses, and box springs in zippered mite-proof (vinyl) covers
Remove carpeting
Avoid heavy curtains or venetian blinds; launder curtains frequently
Substitute wooden or plastic furniture for upholstered furniture
Wash blankets in hot (>130°F 55°C) water every 2 weeks
Avoid wall pennants, macrame hangings, and other items that collect dust
Clean drawers and closets with a damp cloth
Clean floors with an oiled or damp dust mop
Keep all clothing in a closet with the door shut

TABLE 63.1. Techniques for Dust-Mite Reduction

Mold growth is controlled mainly by keeping humidity low (less than 50% relative humidity), particularly in cellars, and eliminating point sources of moisture such as plumbing leaks. Cockroaches are controlled by extermination. The effectiveness of the measures taken may be checked by laboratory analysis of a dust sample (55).

Inhaled air can be efficiently cleaned of allergens. A laboratory worker sensitive to animal dander may almost eliminate exposure by wearing a mask with an adequate filter. Mechanical filters associated with a blower clean the air in closed environments if they have sufficient capacity to recirculate the air frequently, as multiple passes are required for effective cleaning. Electrostatic precipitators and high-efficiency particulate air (HEPA) filters are more efficient, effectively cleaning the air of 99% or more of particulates in a single passage through the device. Even so, adequate recirculation is needed because of the inevitable addition of outside air. Such air cleaners do not remove allergens from the dust in carpets or furniture, which provide reservoirs of fresh allergen whenever they are disturbed (56).

Drug Treatment

The antihistamines in use for rhinitis are H_1-receptor antagonists, which inhibit the smooth muscle and blood vessel effects of histamine but do not inhibit H_2 effects, such as gastric secretion. These drugs antagonize the manifestations attributable to histamine but not those due to other mediators. They are most often administered orally, but preparations for topical administration in the eye or nose also are available. Antihistamines may be categorized in two classes: the classic antihistamines and the newer, nonsedating antihistamines. Classic antihistamines also are anticholinergic, antiserotoninergic, and anti-a-adrenergic. In addition, they are highly liposoluble and readily cross the blood–brain barrier. As histamine may be a neurotransmitter, antihistamines may cause sedation by inhibition of central nervous system histamine. They may, however, cause occasional patients to become restless, nervous, and sleepless; overdoses may cause seizures. The newer antihistamines are more strict H_1 inhibitors and penetrate the central nervous system less readily; they therefore do not cause sedation in therapeutic doses. They have prolonged half-lives and require less frequent dosing.

Somnolence and incoordination are common side effects of classic antihistamines, but can be an advantage for nighttime use when the discomforts of rhinitis cause sleeplessness. Parasympatholytic activity accounts for the occasional undesirable side effects of dry mouth, difficulty in voiding, and impotence, but is useful in the treatment of hay fever, as it tends to reduce nasal hypersecretion (57). The newer, nonsedating antihistamines rarely cause side effects.

Patients with seasonal allergic rhinitis may have a gratifying initial response to antihistamine treatment, particularly early in the season when pollen counts are low. However, as the season progresses and the exposure to higher concentrations of pollen is prolonged, symptoms are often inadequately controlled. Nasal itching and rhinorrhea tend to be relieved better than nasal obstruction or headache due to sinus congestion. Perennial rhinitis is less successfully relieved by antihistaminics than is the seasonal condition. When antihistamines are unsatisfactory, other methods of treatment are needed.

Orally administered sympathomimetic drugs such as ephedrine, phenylpropanolamine hydrochloride, and pseudoephedrine hydrochloride are sometimes useful in relieving nasal obstruction. These drugs occasionally increase blood pressure levels in hypertensive patients; they may also increase the heart rate and cause excitement. They are often used in combination with antihistamines to control nasal congestion better and to counteract partially the drowsiness produced by antihistamines. Topical use of sympathomimetic amines with a-adrenergic action causes vasoconstriction and shrinkage of edematous nasal mucosa. Their disadvantage is rebound vasodilatation (“aftercongestion”) when the vasoconstriction effect has worn off; and prolonged topical use may result in chronic rhinitis.

An atropine-like drug (ipratropium) applied locally limits the portion of the hypersecretion of mucus that is under neural control. In some patients, it may lead to excessive dryness of the mucous membranes; in others, it is a useful but minor addition to management.

Glucocorticoids in sufficient doses ordinarily effect complete relief of allergic rhinitis in 12 to 36 hours. Although the antiinflammatory action in the allergic reaction is incompletely understood, glucocorticoids are able to inhibit basophil (but not mast cell) mediator release *in vitro*, inhibit expression of adhesion molecules important in granulocyte migration, and inhibit the late phase of a nasal-challenge reaction to pollen extract more readily than the immediate phase (58,59). Oral prednisone in daily doses of 15 to 25 mg nearly always produces amelioration of symptoms in seasonal rhinitis. Once improvement is achieved, the smallest dose that will continue to keep the patient comfortable is commonly 5 to 15 milligrams per day or every other day. When allergen exposure becomes minimal, the drug may be discontinued. The relatively short period of administration (4 to 6 weeks) and the small doses required in most instances avoid the commonly feared side effects of prolonged steroid administration. Doses of 10 to 25 mg of prednisone per day often relieve chronic perennial rhinitis and shrink nasal polyps, sometimes completely. Recurrence of obstruction and recrudescence of polyps are usual when the drug is stopped, however. Prolonged, continuous use of glucocorticoids to control symptoms introduces undesirable side effects.

Nasal sprays of steroids active locally on mucous membranes appear to be a suitable alternative. The steroids (beclomethasone, flunisolide, triamcinolone, budesonide, fluticasone, and mometasone) ameliorate the symptoms of both seasonal and perennial rhinitis without detectable suppression of adrenal activity. If the dose is increased to several times the recommended dose, evidence of mild adrenal suppression appears. Minimal local side effects consist of transitory nasal burning after instillation and infrequent nosebleeds. Although originally recommended for twice-daily use, many of these agents are available in forms that are effective when used once daily. Some patients with severe disease (either acute or chronic) find that twice-daily use is necessary for relief (60).

Cromolyn sodium instilled nasally 5 or 6 times a day as a 4% solution ameliorates the symptoms of allergic rhinitis without side effects. A related compound, nedocromil, in a 1% solution appears to be more effective (61). Comparisons of the efficacy of topical steroids and cromolyn sodium for the treatment of allergic rhinitis showed the steroids to have a modest advantage (62,63). A locally sprayed antihistamine solution (azelastine) used twice daily can also produce some symptomatic relief. Eyedrops that relieve conjunctival symptoms include mast cell stabilizers (cromolyn, lodoximide, ketotifen), antihistamines (pheniramine, levocobastine, emedastine), and nonsteroidal antiinflammatory drugs (keterolac).

Immunologic Therapy

Immunologic treatment for hay fever was introduced in 1911 by Noon (64) and Freeman (65). They used subcutaneous injections of sterilized grass pollen extracts and gradually increased doses in advance of the expected season. Although often referred to as hyposensitization or desensitization, *immunotherapy* is a more appropriate term for this procedure.

Immunotherapy uses aqueous sterile filtered extracts of the specific allergens that cause symptoms in the patient. The extract is first administered in minute doses in weekly or biweekly injections, and the dose is gradually increased as tolerated by the patient. The end point of the dosage is commonly determined by the occurrence of local or systemic allergic reactions to the injections or by patient satisfaction with relief of symptoms. Reduction of mucous membrane or skin sensitivity has been little used in practice as an indicator of adequacy of dosage. Maintenance doses are continued every 2 to 6 weeks for 3 years or more.

EFFICACY

With unaltered aqueous extracts, satisfactory relief of symptoms has been obtained in allergic rhinitis due to grass pollen, ragweed pollen, birch pollen, mountain cedar pollen, cat dander, and house dust mites (66,67). Immunotherapy with extracts of other pollens, molds, danders, and inhalant allergens may be clinically effective by analogy but has not been subjected to controlled study.

Where studied, clinical efficacy depends on dose. With ragweed pollen extract, hay fever symptoms are progressively less severe as the dose is increased up to an, as yet, poorly defined maximum (68). A final dose of whole extract equivalent to about 10 to 20 µg Amb a 1 per injection is near the maximum. Larger doses probably provide little additional protection. From both clinical and immunologic evidence, it seems likely that the maximal or final dose is more meaningful than the cumulative annual dose. Controlled studies of the efficacy of ragweed pollen (37,69), grass pollen (70,71), cat (72,73), and dust-mite (74,75) extracts have been performed with extracts whose content of major allergen has been estimated. The final (or maximal) dose that demonstrated efficacy was similar for these disparate allergens and ranged from 5 to 20 µg of allergen. These numbers provide a rough guide to the target dosage of an immunotherapy regimen. Although major allergen content is not ordinarily included on the label in the United States, data can often be obtained from the manufacturer.

Symptoms are alleviated but are infrequently completely cured. Clinical relief is specific for the allergen administered and does little or nothing for symptoms from immunologically unrelated allergens (76). Accurate and complete diagnosis is therefore mandatory for best results, as a good result with treatment with one or several species of allergens could be overshadowed by continuing symptoms from an allergen not included. Highly purified major allergens have been effective in immunotherapy trials (68,77). Recombinant allergenic proteins, although biologically active, have yet to be tried in controlled trials.

In two studies, some patients relapsed within 6 months to a year after discontinuation of immunotherapy, providing a rationale for long-term administration of booster injections (78,79). How long to continue immunotherapy, however, has been studied rarely. In a study of continuing immunotherapy for grass pollen hay fever, treatment was stopped after 3 to 4 years. Amelioration of symptoms continued through 3 additional years, and there was a sustained reduction in late-phase skin

responses (80).

MODE OF ACTION

During immunotherapy, carefully performed assays to determine titers show the intradermal skin test wheal and erythema to be somewhat suppressed (81,82). Immediate mediator responses to nasal challenges are suppressed in both the magnitude of response and the threshold for any response (37,83). More striking are changes in the late-phase response, particularly in the skin. Nearly every person receiving immunotherapy has an almost complete suppression of the late-phase response to intradermally administered allergen (83,84,85 and 86). With mountain cedar pollinosis, suppression of the late cutaneous response correlated significantly with dose of extract, postseasonal levels of IgG1 and IgG2 antibodies, and improvement in symptoms (85). Although not everyone with ragweed pollinosis has a late-phase mediator response to nasal challenge as it is usually carried out, those individuals who did have a late-phase response no longer exhibited one after immunotherapy (83).

These changes are accompanied by multiple immunologic changes. Whereas exposure by the transmucosal route leads mostly to antibodies of the IgE type, adequate parenteral immunization results in a striking increase in IgG antibodies (28,87). These antibodies block histamine release from basophils (88). There is a significant correlation between the dose of allergen administered and the IgG antibody response (28,87). Although patients with high serum IgG antibody levels more often have less severe symptoms after immunotherapy than do patients with low IgG antibody levels ($p < .02$), the correlation is not useful for predicting the clinical response in a particular individual (87). With prolonged immunotherapy, there is an IgG antibody subclass switch from IgG1 to IgG4. IgG4 antibodies form small, nonprecipitating immune complexes because of functional monovalency (89,90), which may help to account for the lack of immune complex disease during immunotherapy.

Specific IgG and secretory IgA antibodies in nasal secretions also have increased levels after immunotherapy and appear to be locally generated. Levels of IgA or IgG antibody in secretions showed no correlation with symptoms reported after natural exposure (91).

Immunotherapy by the usual regimen raises the level of serum IgE antibodies before IgG antibody levels begin to increase. This increase in IgE may be associated with the higher incidence of untoward reactions while the dose of allergen is being increased. Eventually IgE antibodies decline to levels below baseline after a year or more (92). With allergens such as ragweed that involve seasonal exposure, the usual postseasonal increase in IgE antibody levels no longer occurs (30,93). At best, however, the specific IgE antibody levels remain in the range found in sensitive patients, so that it is difficult to attribute improvement to these modest changes.

Basophil reactivity to *in vitro* antigen challenge may decline dramatically in some patients and become almost nonexistent. This change has no relationship to serum IgE and may represent a nonspecific change in cell reactivity (94). Although this change may be significant for the patients who develop it, it is not induced in many patients and cannot account for the effects of immunotherapy in most people.

Various indirect measures of lymphocyte activity in the presence of allergen are reduced in patients undergoing immunotherapy in comparison with those in untreated control subjects (95,96). These activities are now attributed to T cells. In cultures of CD4 lymphocytes from peripheral blood, IL-4 production is reduced after antigen stimulation (97), even though it may be temporarily increased early in the course of treatment (98). This finding indicates eventual downregulation of antigenically specific Th2 activity.

Biopsies of late-phase skin reactions from untreated patients show infiltrating T cells with CD3 and CD4 markers. These cells are significantly reduced in number in biopsies from patients receiving clinically efficacious immunotherapy. Eosinophil infiltration in these biopsies was less in the treated patients but not significantly so (99). The increased number of eosinophils in nasal secretions after a nasal allergen challenge also is reduced but not to a significant degree (83). The number of eosinophils in nasal secretions during natural seasonal exposure is, however, significantly reduced in treated patients (100). In patients successfully treated for grass pollen allergy, biopsies of skin and nasal mucosa 24 hours after a challenge show increased mRNA for interferon-g (IFN-g) and IL-2 but no change in mRNA for IL-4 or IL-5 (101). Another study of skin biopsies showed increased numbers of macrophages positive for IL-12 mRNA (102). Because IL-12 is an inducer of Th1 activity, these studies indicate an upregulation of Th1 lymphocytes. The change in balance between Th2- and Th1-lymphocyte–directed activities could explain the considerable increase in IgG antibodies and the modest decline in IgE antibodies.

UNTOWARD REACTIONS

There are three types of allergic reactions to pollen extracts administered subcutaneously. Early in treatment, there is usually a local reaction: a wheal and erythema at the site of injection that resembles a positive reaction to a skin test. It appears in 10 to 30 minutes and subsides in a few hours. A second kind of local reaction appears as an itching erythematous swelling of the subcutaneous tissues without wheal formation, beginning 2 to 4 hours after the injection. These reactions commonly reach a maximum in 18 to 24 hours, without necrosis, and subside in a day or two. IgE antibodies participate in late (2 to 24 hours) skin reactions. Such reactions are a minor problem unless a too rapid progression in dosage leads to a large, uncomfortable swelling (103).

In contrast, “systemic” reactions, although relatively uncommon and usually mild, are a potentially serious consequence of therapy, as they represent anaphylaxis and may be fatal (104). They require prompt management with epinephrine and other measures (Chapter 64). Administration of glucocorticoids for several days after a severe systemic reaction may prevent recurrences of hives and angioedematous swellings, but these drugs have no effect on the immediate anaphylactic response itself. Long-term ill effects such as immune complex disease or polyarteritis have been suspected in individual cases, but two prospective studies failed to find that circulating immune complexes are any more common in patients receiving immunotherapy than in patients with similar allergies who are not receiving immunotherapy (105,106).

STANDARDIZATION OF ALLERGENIC EXTRACTS

The two historic methods used for labeling extracts for therapy are wt/vol (dry weight of starting material/volume of extracting fluid) and the protein nitrogen unit (1 PNU = 0.00001 mg protein nitrogen, as determined by precipitating protein with phosphotungstic acid and performing a Kjeldahl nitrogen analysis on the precipitate) (107). Neither is necessarily related to allergenic activity or immunizing activity in immunotherapy, and, in practice, neither provides adequate information to the physician (108,109).

Direct skin testing of allergic patients is often used for setting units of direct biologic relevance. Two methods have achieved acceptance: (a) In the histamine equivalent in prick testing (HEP) system, the concentration of allergen extract is adjusted to elicit a wheal in the skin-prick test equivalent to that produced by a histamine hydrochloride solution of 1 milligram per milliliter. A preparation of this strength is said to contain 1,000 biologic units (BU) per milliliter (110). The HEP system is used in the European Union. (b) An intradermal bioassay uses testing of allergic patients with threefold dilutions and establishes a best-fit linear regression line from four serial dilutions. In each patient, the dilution producing a 50-mL sum of erythema response (D_{50}) is calculated. The mean D_{50} in a population of 15 maximally sensitive individuals defines the potency of a reference. Potency is denominated in Bioequivalent Allergy Units (BAU) (111). The Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration has prepared standards labeled in BAU that can be compared with commercial materials by RAST inhibition. Mite, grass pollen, and cat extracts are currently standardized in this way. In addition, for ragweed and cat, in which major allergens have been identified and isolated, standard sera are furnished for a radial immunodiffusion assay of the allergen. A ragweed pollen extract labeled 100,000 U/mL must contain 200 to 400 µg Amb a 1 per milliliter. A combination of RAST inhibition, isoelectric focusing, and crossed immunoelectrophoresis also serves as laboratory controls for allergenic products.

An Allergen Standardization Subcommittee of the International Union of Immunologic Societies has developed standard extracts of short ragweed, timothy grass, dog dander, birch, and *Dermatophagoides pteronyssinus*, and has prepared large batches of glass-sealed ampules labeled in arbitrary units. These materials have been accepted by the World Health Organization as international standard preparations (ISPs).

Isolation, cloning, and production of recombinant proteins representing specific allergenic components along with development of monoclonal antibodies make it possible to quantitate specific active proteins in crude extracts and to offer an additional opportunity for accurate standardization in mass units of one or more major allergens (see Chapter 61).

ALTERNATE METHODS

Techniques to shorten the time required to reach full doses or to reduce the risk of serious allergic reactions have been tried repeatedly. A “clustered” regimen gives several injections at about 30-minute intervals at each visit. The number of visits is reduced, but the time required to reach a therapeutic dose and the rate of systemic reactions are not changed (112). A “rush” program reaches full doses in 4 days with a rate of systemic reactions not dissimilar to that noted with slower programs (113). The administration of concentrated aqueous extracts orally in the form of capsules containing extract or drops diluted with water produces a low rate of systemic reactions. The controlled studies that have demonstrated satisfactory efficacy with birch and ragweed extracts required very large doses, about 100 times that used in injection regimens (114,115).

Extracts may be physically or chemically modified to delay absorption or reduce allergenicity. In the United States, only alum-precipitated extracts are available to

physicians; these extracts offer a modest advantage in achieving effective doses with fewer injections (116). In Europe, clinically significant results have been obtained with stabilized alum-precipitated preparations of grass pollen and cat extracts (117,118). Modification by cross-linking protein molecules with aldehydes increases the average molecular weight of the constituents of a crude extract greatly and results in reduced allergenic activity (119,120). “Allergoids,” extracts modified by formaldehyde, are in common use outside the United States (121) and have been introduced in an alum-precipitated form. Other modifications include conjugation of the extract to alginate, methoxy-polyethylene glycol coupling, and tyrosine-precipitated, glutaraldehyde-modified extract.

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64 ANAPHYLACTIC SYNDROME

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Systemic anaphylaxis is an acute allergic reaction that involves the release of mediators from mast cells, basophils, and secondarily recruited inflammatory cells, which may occur within a few minutes to a few hours after exposure to a triggering agent. The discovery of anaphylaxis (155) is an instructive example of the manner in which scientific advances occur when a curious fact is interpreted by a prepared mind. The story of how Portier and Richet discovered, during a Mediterranean cruise, that injection of an extract of “sea anemone” (the tentacles of jellyfish) into a dog was tolerated the first time but induced death when reinjected in the same amount several weeks later is part of the early folklore of immunology. The term *anaphylaxis*, as proposed by Portier and Richet, means loss of “protection”; but these investigators (155) also recognized that (a) anaphylaxis is acquired and (b) it is the opposite of “prophylaxis.” A further step was taken by Arthus (6), who realized that the “toxin” does not have to be a toxin; any nontoxic foreign protein can induce anaphylaxis.

Over the last century, numerous antigens have been reported to be associated with anaphylaxis. These include serum therapy used in the treatment of diphtheria, tetanus, meningococcemia, and tuberculosis and that used for treatment of renal and hepatic allograft rejection (14,24,97,132,157,203,205), antibiotics such as penicillin (3,45,64,66,76,90,94,95,96,101,102,103,105,116,151,164,214), therapeutic proteins and biologics (35,82,113,202), polysaccharides (9,55,120), and ingested antigens (12,22,43,48,57,80,83,127,138,146,169,217).

Exposure to environmental allergens, such as latex, seminal fluid, and insect or crotalid venoms accounts for additional cases of anaphylaxis (65,104,134,152,189,191,218). Anaphylaxis resulting from stings by insects of the order Hymenoptera (see Chapter 69) is a persistent problem: serious systemic reactions have been estimated to occur in 0.4% of the population (13,106,195). The appearance of systemic reactions to the sting of the imported fire ant has expanded the significance of sensitivity in the order Hymenoptera (114,159). The diverse group of proteins and haptens that elicit the typical clinical sequence of anaphylaxis is continually increasing with the introduction of new diagnostic and therapeutic agents. One estimate places the rate of fatal anaphylaxis among treated patients at 0.001% (204) and another at 0.002% (76).

Because the life-threatening clinical manifestations of anaphylactic shock in humans can arise from obstruction of the upper or lower airways with secondary vascular collapse, or from primary circulatory failure without antecedent respiratory difficulty, the anaphylactic response is best considered a syndrome. Defining anaphylaxis as an adverse immunologic syndrome not limited to an immunoglobulin E (IgE)-mediated event avoids the need for the term *anaphylactoid reaction,* which sometimes is used to describe shock-like reactions from presumed non-IgE-dependent mechanisms. Indeed, studies with IgE-deficient mice are consistent with the hypothesis that non-IgE-mediated signal pathways also can initiate systemic reactions resembling anaphylaxis (140). Although presently the mechanism is unknown in humans, in the mouse, this process possibly may be mediated through IgG1 and the Fc receptor III for IgG1 (Fcg RIII) (31,36,131).

CONSTITUTIONAL FACTORS AND ETIOLOGY

Whether underlying factors such as age, sex, race, occupation, or geographic location predispose a person to anaphylaxis is unclear and remains controversial, except insofar as they may provide exposure to a particular antigen. The atopic diathesis may represent a risk factor for anaphylaxis, although there is room for disagreement (82). In one study, 5 of 17 patients who died of anaphylaxis after penicillin treatment had asthma (94); in another study, skin-sensitizing antibodies to penicillin developed in 12 of 15 atopic patients after penicillin treatment compared with 31 of 110 nonatopic persons (102). In a familial drug study, children of parents known to be allergic to an antibiotic were found to have a 15-fold higher relative risk for allergic reactions to antibiotics than children without such histories (35). In contrast, a multicenter study with more than 1,000 patients disclosed no correlation between penicillin reactions and a family or personal history of allergy (60,74). With respect to age differences, the risk for drug allergy and the concomitant risk for anaphylaxis appear less common and less severe in infants and in elderly persons; perhaps because of immaturity and involution of the immune system, respectively, at these two extremes of human development (35). Women appear to have an approximately 35% higher incidence and an approximately 20-fold higher risk for adverse cutaneous reactions to different agents and for systemic reactions to radiocontrast media, aspirin, muscle relaxants, and latex than men (35,108). A syndrome has been described entitled *multiple drug allergy* in which patients report allergic reactions to multiple antibiotics (209). This entity may reflect an underlying predisposition—genetic or otherwise—to respond immunologically to *haptens*, simple chemicals that are not antigenic in themselves but become antigenic after the chemical or one of its metabolites forms a stable bond with a host protein rather than an underlying predisposition to react to specific classes of drugs (108).

The dose, duration, frequency, and route of administration may affect the propensity to develop sensitization. Although sensitization may occur by any route, topical and oral routes of exposure appear to have the greatest and lowest risks, respectively. In the sensitized person, however, reexposure to antigen through the oral route may lead to prolonged and potentially severe symptoms as a result of slow or delayed absorption of antigen. Single, intermittent doses are less likely to induce an allergic response than high doses with prolonged parenteral exposure. More frequent exposures are more likely to result in sensitization than those separated by years.

Proteins, polysaccharides, and haptens (Table 64.1) are known to elicit systemic anaphylactic reactions in humans as defined by one or more of the following criteria: (a) a confirmatory postmortem examination that reveals laryngeal edema, acute pulmonary hyperinflation, or a positive wheal-and-flare response; (b) *in vitro* demonstration of IgE to the eliciting material; and/or (c) hypotension, concomitant with other manifestations of anaphylaxis, such as characteristic cutaneous, respiratory, or gastrointestinal involvement. Primary hypotensive responses without antecedent respiratory problems are a predominant reaction pattern with certain agents such as protein hormones or penicillins (81,110).

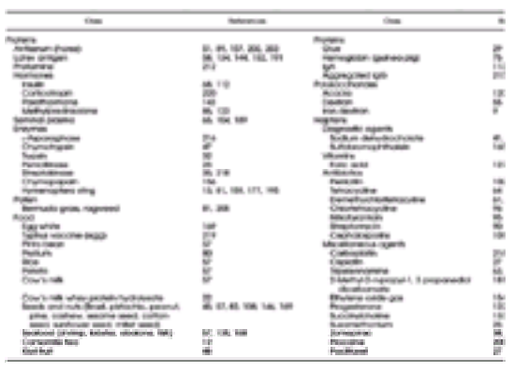

TABLE 64.1. Materials Producing Anaphylactic Reactions in Humans

Substances that cause anaphylaxis are diverse, and some of the more common and clinically prevalent etiologic agents are summarized here. A historically noteworthy protein class known to elicit anaphylaxis is exemplified by horse antihuman lymphocyte globulin (ALG) used as an adjunct for immunosuppression of allograft recipients. ALG elicited systemic anaphylactic reactions in 11 of 53 patients (89) and in 6 of 48 patients (24) treated by two groups. The high incidence of systemic reactions as well as the augmentation of immediate-type skin test reactivity during treatment with ALG (24) is particularly relevant in view of the virtual absence of serum sickness in this patient population (132,202). In these studies, nearly all recipients of ALG had an antibody response of the IgM class to horse proteins. It may be that the failure of these patients to make such a response in the IgG class accounts for their lack of serum sickness as well as for their propensity to demonstrate an IgE-type response.

Food allergens are a common cause of anaphylaxis and are responsible for up to one third of cases in some series (108). Food allergy and anaphylaxis resulting from food reactions appear to be more prevalent in the pediatric population. The most frequent offenders are peanuts, true nuts, fish, shellfish, milk, and eggs (82). Cross-reactivity with other foods in the same group is possible. Anaphylactic reactions to foods almost always occur within 1 hour of ingestion. Symptoms may involve

the gastrointestinal, cutaneous, ocular, respiratory, and/or cardiovascular systems.

Of the heterologous proteins in clinical use, the most significant addition of anaphylactogenic agents in the past decade may be the latex antigens (82,152,191) (see Chapter 73). These water-soluble allergens are proteins in latex sap used to make latex rubber articles. Anaphylaxis has been reported after intraoperative contact with rubber gloves (58,134) or contact with latex catheters used during a barium enema (144). The incidence of anaphylaxis to latex allergens is increased in health care personnel, in children with spina bifida and genitourinary abnormalities, and in workers with occupational exposure to latex. Reactions appear to be more common in atopic persons. The incidence of reactions to latex appears to have increased over the last decade, although it is not clear whether this increase is due in part to better reporting and diagnosis of the problem, to changes in the manufacture of latex, or to an overall increase in the number of persons with atopy. Evidence of IgE-mediated sensitivity to foods (as evidenced by skin prick testing or *in vitro* tests) is common in latex-sensitive patients (> 50% of patients in some series). Nonetheless, only a small fraction of patients allergic to latex have clinical reactions on food ingestion (27% of patients with positive skin test responses in one series) (10). Clinical and *in vitro* cross-reactivity has been reported between latex and avocado, banana, chestnut, potato, tomato, and kiwi fruit.

Anaphylactic reactions to purified heterologous proteins such as hormones (35,68,112,220) can be attributed to unrecognized contaminants or denaturation when positive skin tests are obtained with natural but not synthetic materials (163), but this is not invariably the case. The best studied example addresses recombinant human insulin. Antibody of the IgE class in patients experiencing anaphylaxis to insulin has been shown to react against human synthetic insulin and insulin prepared from recombination of polypeptide subunits (149). Patients are more likely to experience anaphylaxis if therapy is interrupted.

An unusual association of recurrent unexplained anaphylaxis with menses has been reported. These episodes are observed in some women before the onset of menses and appear to be due to hypersensitivity to progesterone. The term *catamenial anaphylaxis* has been applied to this form of anaphylaxis (122,192).

Systemic anaphylactic reactions from insect stings of the order Hymenoptera (see Chapter 69), contained in the superfamilies Apoidea (bees), Vespoidea (wasps, hornets, yellow jackets), and Sphecoidea (solitary wasps), are well recognized. Identical reactions secondary to ant stings of the superfamily Formicoidea have been documented. Two South American species of fire ants introduced into the southern United States early in the twentieth century, *Solenopsis invicta* and *Solenopsis richteri*, are known for their aggressive behavior, and their stings also elicit a reaction (114,159). Other biting insects, such as deer ticks and mosquitos, occasionally cause anaphylaxis (218). Triatoma, indigenous to the American Southwest, is a nocturnal blood-sucking arthropod whose bite also may produce anaphylaxis.

Among the haptens, benzylpenicillin, semisynthetic penicillins, and cephalosporins are best characterized and continue to be problematic clinically. Penicillin reactions occur at a frequency of 1 to 5 per 10,000 administrations of penicillin, and fatalities occur at a rate of approximately 1 per 50,000 to 100,000 courses (108). The benzylpenicilloyl group of benzylpenicillin has been established as a major (in abundance only but not in terms of predisposition to immediate hypersensitivity reactions) haptenic determinant (145), and there is extensive cross-reactivity with a-aminobenzyl (ampicillin) penicilloyl haptens. Only slight cross-reactivity is observed with dimethoxyphenyl (methicillin) or oxacilloyl (oxacillin) penicilloyl haptens (101). Allergenicity to the major determinant is less predictive of immediate hypersensitivity reactions than that to the minor determinant (35). In addition to benzylpenicilloyl-specific antibody, penicillin therapy thus may induce the formation of antibodies for minor determinants derived from benzylpenicillin (103). These penicillin minor determinants constitute the remaining 5% of penicillin metabolites and include penicilloate, penicilloyl-amine, benzylpenicilloyl-*N*-prophylamine, and penilloate. IgE antibodies to penicillin minor determinants are responsible for most anaphylactic reactions associated with penicillin use (35,82,108). If skin test results for penicillin with both major (penicilloyl) and minor determinants (penicillin G and others) are negative, 97% to 99% of patients (depending on the reagents used) will tolerate penicillin administration without risk of an immediate reaction (82). Conversely, a patient with a positive history and a positive skin test response to penicillin has a 50% or greater chance of an immediate reaction if penicillin is readministered.

Four reaction patterns associated with penicillin hypersensitivity have been described. These patterns—immediate, accelerated, late, and recurrent—are characterized by urticaria with or without systemic manifestations and skin-sensitizing IgE antibodies of varying specificities (102,106). By definition, only the immediate reaction associated with skin-sensitizing antibody to minor determinants of benzylpenicillin would ordinarily be recognized as anaphylactic, that is, according to the criteria of rapidity of onset after antigen exposure and association with vascular and respiratory difficulties. The less spectacular nature of the response associated with skin-sensitizing antibodies of benzylpenicilloyl specificity (i.e., accelerated and late urticarial reactions) is possibly due to modification by neutralizing antibody or to the requirement for metabolism to the eliciting determinant or both. IgG and IgM anti-penicillin antibodies appear responsible for penicillin-associated hemolytic anemia, nephritis, and serum sickness.

Cephalosporins also give rise to a major determinant, the cephaloyl group (66), and to minor determinants that cross-react with those of benzylpenicillin (101). First-generation cephalosporins may pose a greater risk than second- or third-generation cephalosporins. Indeed, patients with a history of urticaria or anaphylaxis to penicillin are three to four times more likely to react to a cephalosporin, although the risk of a reaction to a cephalosporin in this setting is less than 10% (3,82). For related antibiotic classes, carbapenems (e.g., imipenem) should be considered cross-reactive with penicillin. Aztreonam, a monobactam, rarely cross-reacts with penicillin (35,82).

Aspirin and nonsteroidal antiinflammatory drugs (NSAIDs) and radiographic dyes are two common classes of agents that can elicit adverse reactions reminiscent of systemic anaphylaxis. Both groups, however, can act through non-IgE mediated mechanisms. The lack of skin-test reactivity to *N*-acetylsalicyloyl or salicyloyl determinants (175) in persons who react to aspirin and their clinical intolerance to structurally unrelated NSAIDs (170) indicate a need to identify the non-IgE-dependent mechanism(s) involved. For iodinated organic contrast agents, although some adverse reactions have been indistinguishable from anaphylaxis by clinicopathologic criteria (28,139,150), skin testing has not yielded predictable results (49), and adverse responders have not been shown to have specific IgE. Severe reactions are noted in about 0.01% of patients undergoing urographic studies (4); such reactions may occur more frequently with the doses used for angiography (139) and less frequently when agents of lower osmolarity are employed (109). Risk factors for adverse reactions to radiocontrast materials (RCM) consist of a history of asthma or atopy, cardiovascular disease, and use of b-adrenergic blocking agents or angiotensin-converting enzyme (ACE) inhibitors (82). Clinically, these reactions tend to be characterized by hypotension with pruritic urticaria rather than by primary respiratory distress. Patients who have experienced previous adverse reactions from administration of RCM are at increased risk for a repeat reaction, with estimates of this risk ranging from 16% to 44% for procedures with high osmolality RCM (82). Direct histamine release may be responsible in part for the reaction because RCMs, such as methylglucamine diatrizoate, release histamine from suspensions of human peripheral leukocytes (126) and degranulate mast cells in the hamster cheek pouch after topical application (99). The presumption that adverse reactors are unduly susceptible to direct histamine release has not been substantiated by analysis of plasma histamine levels (20). The capacity of radiographic dyes to activate the alternative complement pathway *in vitro* and even *in vivo* suggests another mechanism for the adverse reactions, but the *in vivo* hypocomplementemia has not been associated with clinical manifestations (5).

In addition to RCM, adverse systemic reactions experienced during diagnostic or surgical procedures may be caused by a number of other etiologic agents, which can make identification of the inciting factor(s) difficult. Furthermore, it may be difficult to differentiate between immune and nonimmune mast cell-mediated reactions and pharmacologic effects. Although anaphylactic reactions to local anesthetics have been reported, IgE-mediated systemic anaphylaxis to these agents appears unusual. Local anesthetics are classified into groups: esters (aminobenzoate and benzoic acid subtypes), amides, ethers, and ketones. In patients who have had reactions to an ester-type anesthetic, an amide should be considered for provocative or graded dose testing; however, in those with a reaction to an amide, another amide should be tested because cross-reactivity among amides has not been documented. Other etiologic agents of adverse reactions during operative procedures include thiopental (IgE-mediated), opioid analgesics (direct mast cell mediator release), neuromuscular blocking agents such as *d*-tubocurarine or succinylcholine (IgE-mediated in some cases; also histamine release by direct cross-linking of mast cell surface IgE molecules), protamine (both IgE-mediated and non-IgE-mediated reactions), ethylene oxide (IgE-mediated), methylmethacrylate (bone cement, nonimmunologic mechanism), and, far less frequently, additives such as parabens or sulfites.

Transfusion reactions characterized by hypotension on receipt of a few milliliters of whole blood or intravenous immune globulin have been noted in IgA-deficient persons and are attributed to the presence of IgG or IgE antibodies that recognize IgA (113,176,207). Selective IgA deficiency occurs with a frequency of about 1:700 (121), and about 40% of those affected manifest such class-specific anti-IgA (176). These patients are skin test-negative to plasma, buffy-coat cells, erythrocytes (176), and even IgA (137). The acquired hypocomplementemia in some patients with agammaglobulinemia during an adverse reaction to g-globulin administration has been attributed in some instances to the direct action of aggregates in the preparation. Newer preparations of intravenous g-globulin are relatively free of this complication. Complement activation is also thought to follow the formation of complexes of recipient IgG and donor IgA (215). Urticaria and fever have been noted in transfusion reactions to allotype-specific antibodies (53).

Anaphylaxis has been described in the setting of administration of avian-based vaccines and has been attributed to the egg protein in the vaccine as well as to hydrolyzed gelatin, sorbitol, and neomycin (82). True anaphylactic reactions to vaccines, however, are rare, including vaccines that may have minute quantities of avian protein (measles, mumps, yellow fever, and influenza). Two studies performed in egg-allergic children (n = 140 and n = 54, respectively) who received the measles, mumps, and rubella (MMR) vaccine indicated that at least 97.5% of these children tolerated the vaccine without significant difficulty (44,79). Furthermore, in a total of 17 studies, MMR vaccine was safely given as single dose to 1,209 patients with a positive skin test to egg. Whether the same safety profile would be seen in patients with underlying asthma is unknown, although asthmatic children with a positive skin test to influenza vaccine and a history of egg sensitivity were safely given influenza vaccine with use of a desensitization procedure (78).

The incidence of anaphylaxis during allergy skin testing or immunotherapy is low. The risk factors for the development of systemic reactions to allergen immunotherapy have been identified. As summarized in the American Academy of Allergy, Asthma and Immunology's Joint Task Force on Practice Parameters for “The Diagnosis and Management of Anaphylaxis,” these risk factors consist of the following: (a) unstable glucocorticoid-dependent asthma; (b) strong allergic reactivity, based on diagnostic tests such as immediate hypersensitivity skin tests; (c) a history of previous systemic reactions to allergen immunotherapy; (d) starting a new vial of allergen

extract; (e) asthmatic symptoms immediately before receiving an injection of allergenic extract; (f) concomitant treatment with b-adrenergic blocking agents; (g) administration of pollen extracts, especially during high environmental exposure periods to aeroallergens; and (h) a rate of increase in the dose of allergenic extract that is too rapid for the patient's degree of sensitivity (82). Other factors that may impact on increased risk for immediate hypersensitivity are use of nonstandardized extracts that may have significant lot-to-lot potency differences; administration of extract at the time of exacerbation of allergy symptoms, asthma symptoms, or during an upper respiratory infection; or administration of extract in the setting of fever from any cause. Patients receiving ACE inhibitors may be at increased risk for anaphylaxis (173,187), with at-risk patients having significantly lower levels of renin and of angiotensin I and II than those who do not experience repeated episodes of anaphylaxis with immunotherapy (70,71 and 72).

CLINICOPATHOLOGIC CHARACTERISTICS

The time course of appearance and the perception of symptoms and signs differ among individual patients, but the hallmark of the anaphylactic reaction is the onset of an associated manifestation within seconds to minutes after introduction of the antigen, generally by ingestion or injection. In general, the magnitude of the anaphylactic response parallels the magnitude of the stimulus. Thus, more severe reactions occur closer temporally to the time of exposure to the inciting agent. Respiratory distress may originate in the upper or lower airway or both. Laryngeal edema may be experienced as a “lump” in the throat or hoarseness of voice or stridor. Bronchial obstruction is associated with a feeling of tightness in the chest or even audible wheezing.

A particularly characteristic feature of anaphylaxis is an eruption of well-circumscribed, discrete, cutaneous wheals with erythematous, raised, serpiginous borders and blanched centers. These urticarial eruptions are intensely pruritic, diffusely distributed or localized, and may coalesce to form giant hives. These lesions seldom persist beyond 48 hours. A localized, nonpitting, deeper edematous cutaneous process—angioedema—also may be present asymptomatically or with a burning or stinging sensation. Flushing, headache, and rhinitis represent bothersome, but non–life-threatening symptoms. Gastrointestinal manifestations include nausea, vomiting, cramping abdominal pain, and diarrhea that is occasionally bloody. Uterine cramping also may occur. Reactions may be more severe in patients receiving b-adrenergic-blocking drugs (201).

Life-threatening manifestations most commonly involve respiratory distress secondary to upper airway obstruction from angioedema of the tongue, oropharynx, or larynx or secondary lower airway obstruction from bronchospasm. In fatal cases with bronchial obstruction, the lungs are greatly hyperinflated (15,81,185,205). The microscopic findings in the bronchi, however, are limited to increased luminal secretions, peribronchial congestion, submucosal edema, and eosinophilic infiltration. The angioedema associated with death by mechanical obstruction occurs in the epiglottis and larynx (81,205), but the process also may occur in the hypopharynx and to some extent in the trachea. Upper airway edema and bronchial obstruction with hyperinflation of the lungs can be found in about 60% and 50% of deaths, respectively, from anaphylaxis. On microscopic examination, edema is presumed by the wide separation of the collagen fibers and the glandular elements, with vascular congestion and eosinophilic infiltration also present. In some fatal cases, both upper and lower airway involvement may be marked. A tissue feature that may distinguish persons who die of systemic anaphylaxis from other mechanisms of acute death consists of a prominence of eosinophils in pulmonary capillaries, splenic and liver sinusoids, and lamina propria of the respiratory tract (34,81,205).

Cardiovascular symptoms, which are often severe and refractory to treatment, include hypotension, arrhythmias, and hypovolemic shock. The major factors causing cardiovascular abnormalities include an initial loss of intravascular fluid and vasodilation. These events may be followed shortly thereafter by vasoconstriction and myocardial depression. Persons who die of vascular collapse without antecedent hypoxia caused by respiratory insufficiency exhibit visceral congestion (34,81,164) but no major shift in the distribution of blood volume. Whether the associated electrocardiographic (ECG) abnormalities (151), with or without infarction, noted in such patients reflect a primary cardiac event or are secondary to a critical reduction in plasma volume (67) is not established. Left ventricular contractility, however, is depressed with IgE-mediated anaphylaxis (30). Involvement of the cardiovascular system constitutes the second most common cause of death in anaphylactic syndromes (218).

Increased peripheral vascular resistance represents a normal hemodynamic response to improve cardiac output during anaphylaxis. This physiologic change involves both secretion of catecholamines, such as epinephrine and norepinephrine, and activation of the angiotensin system, resulting in conversion of angiotensin I to angiotensin II and increased secretion of these two agents. In some patients experiencing anaphylaxis, peripheral resistance is abnormally elevated because of this response. In other patients, despite elevated levels of these vasopressor substances, systemic vascular resistance falls. Indeed, it has been suggested that a failure to mobilize these compensatory mechanisms may place individuals at risk for anaphylaxis and perhaps a worse outcome from such an event. This hypothesis appears to be supported by at least one study in which Hymenoptera-sensitive patients were shown to have lower levels of baseline plasma angiotensin I and II than normal controls (71). An inverse correlation between angiotensin levels and the severity of anaphylaxis was noted, with more severe symptoms in patients with lower levels.

IMMUNOPHARMACOLOGIC FEATURES

The Prausnitz–Küstner reaction is a classic observation used to illustrate the importance of the role of IgE in the pathogenesis of at least some types of anaphylaxis. An example of this reaction was observed after serum obtained from the heart blood of a patient dying of systemic anaphylaxis was transferred intradermally into a normal recipient. Rechallenge of the recipient 24 hours later with the original antigenic reagent at the same site resulted in the development of the wheal-and-flare reaction (75). Indeed, antigen-specific IgE has been identified in the serum of the patients who have undergone a systemic anaphylactic reaction (Table 64.1) using a number of different laboratory methods (149,189,195). Fixation of IgE to human basophils has been demonstrated by autoradiography (77), and the presence of IgE on mast cells in human skin and respiratory tissues has been established by immunofluorescence with specific anti-human IgE (91). The presence of functional IgE on mast cells of human lung (91) and nasal polyp (84) tissues has been demonstrated by the capacity of anti-IgE to generate and release the mediators of immediate hypersensitivity from these tissues. The location of mast cells in the perivenular areas of connective tissues, as well as intraepithelially in the portio vaginalis uteri and the gastrointestinal and respiratory systems, affords their membrane-bound IgE ready access to potential antigens and makes their products available to a wide variety of cell types, including fibroblasts, vascular endothelial cells, nerve cells, glandular epithelial cells, smooth muscle cells, and other cells of the immune system (46,222).

A wealth of signal transduction research in the area of IgE-mediated immediate hypersensitivity reactions has shed light on important molecular signaling events that regulate the various downstream pathways that ultimately result in mast cell mediator release and cytokine production. Cross-linking of the high-affinity Fc receptor for IgE (FceRI) has been generally thought to occur between the Fab portions of IgE and multivalent antigens. The possibility of additional mechanisms resulting in FceRI aggregation also have been discussed, namely, that anaphylaxis may be triggered through antibodies directed against the a chain of FceRI or through antibodies directed against IgE or IgG-IgE complexes bound to FceRI (118). Regardless of the specific site(s) on FceRI involved, and regardless of the mechanism, aggregation of FceRI initiates intracellular signaling cascades, leading to mast cell degranulation, *de novo* synthesis, release of phospholipid and arachidonic acid-derived mediators, and induction of cytosolic gene expression (59). Tyrosine phosphorylation of the FceRIb and FceRIg immunoreceptor tyrosine activation motifs (ITAMs) on the b and g chains of FceRI is the first molecular alteration observed after receptor aggregation. This event is followed by an increased association of $p56^{lyn}$ with FceRIb and binding of $p72^{syk}$ to FceRIg. Subsequent transphosphorylation of FceRIg-bound $p72^{syk}$ produces an increase in $p72^{syk}$ catalytic activity with resultant activation of multiple downstream signaling pathways, including activation of phospholipases (PLCg1 and PLCg2) and of G proteins (Ras and Pyk2), regulation of protein phosphorylation [phosphokinase (PK) A, PKC, PI-3-kinase, mitogen-activated protein (MAP) kinase], and dephosphorylation [src homology 2 domain-containing phosphatase 1 (SHP-1), SHP-2, *src* homology 2 domain-containing polyinositol 5-phosphatase (SHIP), CD45, protein tyrosine phosphatase (PTP) 1C, PTP1D], and ultimately calcium mobilization. Enzymatic activity of Janus kinase 3 (JAK3), a member of the Janus family of protein-tyrosine kinases that is expressed in mast cells, is enhanced by IgE receptor/FceRI cross-linking (117).

Evidence indicates that there is a divergence of downstream pathways with respect to activation of molecules necessary for the rapid release of mediators during degranulation, such as histamine and heparin; pathways involved in the synthesis of newly formed lipid mediators, such as arachidonic acid metabolites; and pathways that regulate gene expression of cytokines. The biologic sequelae of these events (see Chapter 27) include the release and generation of mediators, some preformed and stored in secretory granules and others synthesized *de novo*. These include histamine, tryptase, chymase, heparin, prostaglandin D_2 (PGD_2), leukotriene C4 $(LTC)_4$, thromboxane B_2, platelet-activating factor (PAF), cytokines [interleukin (IL)-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, IL-16; tumor necrosis factor-a (TNF-a)], growth factors [granulocyte–macrophage colony-stimulating factor (GM-CSF)], stem cell factor (SCF), and chemotactic factors [macrophage inflammatory protein (MIP-1a, MIP-1b), and monocyte chemoattractant protein (MCP-1)] (18,26,56). Many of these mediators play an important role in increasing vascular permeability, inducing smooth muscle contraction, promoting tissue edema and mucus secretion, and regulating the influx of inflammatory cells. In addition, many of these mediators activate other inflammatory pathways. Tryptase has kallikrein activity and can activate the complement cascade as well as cleave fibrinogen. PAF can activate the clotting cascade and promote disseminated intravascular coagulation. Heparin inhibits clotting. Chemotactic factors have the ability to induce migration of eosinophils and other inflammatory cells and thus to intensify and prolong reactions. Resulting cytokine production by T-lymphocytes may drive or predispose an individual to future anaphylactic episodes. This possibility has been illustrated both in an ovalbumin (OVA)-specific T-cell receptor transgenic mouse model, where orally ingested antigen was shown to elicit a T-helper cell type 2 (Th2) response from a subpopulation of T cells that produced high levels of IL-4 and promoted IgE secretion (186) and also by IL-4 mRNA induction by peripheral blood lymphocytes specific for b-lactam antibiotics in a patient with immediate hypersensitivity to b-lactam antibiotics (54). Taken together, these various physiologic processes result in the acute clinical sequelae pathognomonic of anaphylaxis and may be associated with protracted symptoms and/or multiphasic responses.

The physiologic effects of individual chemical mediators released during anaphylaxis overlap. It is thus difficult to attribute specific clinical manifestations to any one substance. Because histamine infusions produce many of the symptoms seen during anaphylaxis, histamine is generally recognized as one of the principal mediators of anaphylaxis. Vascular collapse after the sting of a Hymenoptera occurs with an elevation of blood histamine and mast cell tryptase (177,193). Elevations in blood

histamine occur with experimentally induced episodes of physical allergy in patients with cold-induced urticaria–angioedema (88), exercise-induced cholinergic urticaria (196), and exercise-induced anaphylaxis (limited) (183). In the latter case, cutaneous mast cell degranulation also was demonstrated with detailed ultrastructural studies (184). Although clinically important, histamine release is insufficient to account for the vascular collapse in patients with systemic mastocytosis, and H1 and H2 antihistaminics fail to prevent all such attacks. Also, attacks of vascular collapse appear to subside with administration of aspirin. It has been suggested that this occurs via inhibition of the formation of PGD_2 (161).

In vivo systemic histamine administration (213) and endogenous release by chemical histamine releasers (100) lead to urticaria and angioedema. These manifestations of the anaphylactic syndrome thus are customarily attributed to histamine. Thus, vascular collapse without antecedent hypoxia or myocardial ischemia may be due principally to the release of histamine and the generation of PGD_2.

Similarly, the sulfidopeptide leukotrienes are considered the most likely mediators of the marked peripheral airway effects, which present as “acute emphysema.” In normal humans, the potencies of LTC_4 and LTD4 are 3 logs greater than that of histamine, and that of LTE_4 is 1 log greater in terms of compromising airflow as assessed by expiration from 30% of vital capacity, a measurement reflecting the effects on peripheral airways in preference to central airways (210,211). In experimental animal models, the sulfidopeptide leukotrienes are potent constrictors of coronary arteries (8), and thus they also may be involved in instances of vascular collapse of central origin with ECG findings of myocardial ischemia. The mast cell degranulation associated with a wheal-and-flare response to specific antigen in atopic persons is followed by the incremental local accumulation of eosinophils (7), presumably through the action of factors chemotactic for eosinophils or those that promote eosinophil adhesion (91). Logically, the same mechanism would account for the tissue eosinophilia observed at postmortem examination of persons who died of systemic anaphylaxis (81).

Whereas the importance of other mast cell and basophil mediators has not been as clearly shown in anaphylaxis, recent data indicate that nitric oxide (NO) may play a role in this acute inflammatory reaction. NO is constitutively produced by vascular endothelium, and both mast cells and vascular smooth-muscle cells can be stimulated to produce this molecule (107). NO synthesis is induced by numerous factors, including histamine, LTC_4, bradykinin, substance P, and hypoxia (119). NO acts on the peripheral vasculature to increase permeability and induce smooth-muscle relaxation, resulting in hypotension (86,129). Animal models of anaphylaxis indicate that NO may play an important role as a signaling molecule between chemical mediators produced early in the anaphylactic response and the cardiovascular system (128,130,166).

DIFFERENTIAL DIAGNOSIS

The diagnosis of an anaphylactic reaction depends largely on an accurate history revealing the onset of the appropriate signs and symptoms within minutes after the responsible material is encountered. Various disorders may present with clinical manifestations not unlike anaphylaxis and hence constitute the differential diagnostic list for this syndrome (Table 64.2) (52,125,160,218). The vasovagal reaction is perhaps the most common diagnosis that is confused with anaphylaxis. It presents with symptoms of pallor, profuse sweating, weakness, bradycardia, hypotension, and occasionally syncope (218). Because bradycardia is an early manifestation of hypovolemic shock, heart rate cannot be used reliably to differentiate a vasovagal reaction from anaphylaxis. Indeed, the heart rate has been reported to be low, normal, or elevated during anaphylaxis.

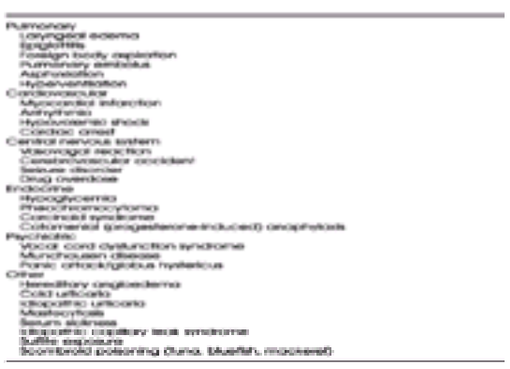

TABLE 64.2. Differential Diagnosis of Anaphylaxis

When only a portion of the full syndrome is present, such as isolated urticaria, sudden bronchospasm in a patient with asthma, or vascular collapse after intravenous administration of an agent, it is difficult to exclude a nonimmunologic, toxicologic, or idiosyncratic response. Even the presence of the full syndrome does not ensure an IgE-dependent mechanism. Intravenous administration of chemical mast cell–degranulating agents (100) can elicit generalized pruritic urticaria, angioedema, and a sensation of retrosternal oppression. In patients receiving an intravenous infusion of morphine, a drug known to release histamine from mast cells, peripheral vasodilation, hypotension, and a marked elevation in blood histamine have been noted (162). NSAIDs such as indomethacin sometimes precipitate a life-threatening episode of obstruction of the upper or lower airways in persons with asthma or an attack of urticaria and angioedema in atopic persons without asthma accompanied by occasional vascular collapse, which is clinically reminiscent of anaphylaxis. The mechanism does not appear to represent an acquired immunologic response, however, in that the patients experience similar episodes on ingesting NSAIDs that do not possess common antigenic determinants and do not stimulate production of detectable specific IgE (170). NSAIDs inhibit the enzyme cyclooxygenase with a potency that parallels their ability to provoke an adverse reaction resulting from increasing arachidonic acid metabolism by lipoxygenase-dependent pathways. Mast cells may be involved in an idiosyncratic activation, as evidenced by the documentation of mast cell tryptase and histamine release in aspirin-sensitive individuals with asthma after oral challenge with aspirin (17), so as to include leukotriene generation.

It is still not clear whether the systemic reaction to iodinated contrast agents is by direct mast cell or basophil degranulation or by a non-IgE immunologic mechanism. Adverse reactions to the contrast agents generally lack the post-event febrile period characteristic of a transfusion reaction, however. Patients with recurring systemic anaphylaxis that clinically is not due to a recognized antigen, including food allergens (199), and in whom the non-IgE-mediated causes of similar reactions have been excluded, are labeled as manifesting idiopathic anaphylaxis (39,111,182). Regardless of the mechanism, each of the previously discussed acute adverse responses is best managed by therapy appropriate for IgE-mediated anaphylaxis.

Other differential diagnostic possibilities may require alternative or additional maneuvers. Low levels or abnormal function of C1 inhibitor, a plasma regulatory protein, is associated in hereditary angioedema with episodic attacks of nonpitting edema that may involve any external body surface as well as mucosal surfaces. Attacks may affect the airway with respiratory obstruction. In the absence of oropharyngeal angioedema or other diagnostic historical facts, the diagnosis of laryngeal edema should be confirmed by visualization and the exclusion of laryngeal spasm, foreign-body aspiration, and tumor (148). The diagnosis of a carcinoid syndrome, caused by enterochromaffin cell tumors that secrete vasoactive substances such as serotonin, is favored by the finding of excessive 5-hydroxyindoleacetic acid (5-HIAA) in the urine, right-sided valvular disease, and blockade of flushing by administration of somatostatin (octreotide) (125). The superimposition of a myocardial infarction on an anaphylactic reaction also has been recognized (106,177,218) and requires an approach appropriate for that condition. Detection of skin-sensitizing antibodies, as evaluated by direct skin testing, is a sensitive, often useful method for the diagnosis of IgE hypersensitivity to a given moiety but carries the small risk of inducing an anaphylactic reaction in the patient being tested (69). Furthermore, the finding that IgE may be produced mucosally and exported to the lumen under the control of IL-4 indicates that serum and skin-based assays occasionally may be inadequate for investigating the role of IgE in hypersensitivity syndromes (31). This possibility needs to be taken into account in skin test–negative patients with a strongly positive recent history for anaphylaxis. Accordingly, several other approaches are available for evaluating hypersensitivity to an allergen. Active histamine release from peripheral leukocytes of patients has been used to confirm anaphylactic sensitivity to nuts, seafoods, and other foods (57) and to Hymenoptera venoms (195). Passive sensitization of peripheral leukocytes from normal donors has been used to establish that the antibody of the Hymenoptera-sensitive donor was of the IgE class (195). These latter approaches, however, often are available only at research centers and thus are rarely routinely used. If purified antigen is available, a solid-phase immunoadsorbent radioimmunoassay or enzymatic assay may be used to demonstrate the presence of specific IgE (143,149). For patients with suspected food allergy, double- or single-blinded, placebo-controlled food challenges may be performed with the suspected food. Such testing should be performed in a medical facility where personnel are experienced in this procedure and are prepared to treat anaphylaxis (82). Finally, elevated plasma histamine, urinary histamine metabolites, or plasma/serum tryptase levels are retrospective supportive findings in favor of a mast cell–mediated disorder such as anaphylaxis (42,52,73,82). Plasma histamine levels are maximal 5 to 15 minutes after an anaphylactic episode and return to baseline by 30 to 60 minutes. Use of plasma histamine levels may be clinically problematic because blood specimens must be processed immediately to prevent spontaneous basophil histamine release and the resulting artifactually elevated histamine levels in unseparated blood. Urinary histamine and its metabolites are elevated for a longer time, and measurements of these levels may be useful (82).

b-Tryptase is a neutral protease stored in mast cell secretory granules that is secreted by human mast cells after activation (87,177,178,179 and 180). b-Tryptase is usually undetectable in serum at baseline (< 1 ng/mL), but the level increases to reflect the severity of mast cell activation. During anaphylactic reactions, serum levels of b-tryptase have been reported to peak 30 to 60 minutes after the onset of symptoms and then decline, with a half-life of about 2 hours. Lack of correlation between

histamine and tryptase levels has been reported, with some patients exhibiting elevations of only one of these mediators (98). Unlike b-tryptase, a-tryptase is not stored in secretory granules of mast cells but appears to be produced constitutively. In systemic mastocytosis, there is a baseline increase in a-tryptase production (180). Commercially available tryptase assays (UniCAP System, Pharmacia-Upjohn) currently detect both a- and b-tryptase, with a normal range below 11 ng per milliliter. b-Tryptase alone may be detected with the G5 monoclonal antibody; however, this assay system is not currently available for commercial use. The total tryptase (a and b) assay system appears to be less sensitive than the b-tryptase assay for detecting elevated tryptase levels during anaphylaxis. If, however, baseline and acute blood samples are compared, a twofold or greater increase in total tryptase during the acute event provides at least as high a sensitivity as the b-tryptase specific assay (82,87,178,179).

The use of tryptase levels in postmortem sera as an indicator of anaphylaxis may contribute to the exclusion of anaphylaxis as the underlying cause of death in unexplained cases, but caution is warranted because serum b-tryptase levels occasionally are elevated in cases where anaphylaxis is not expected (40,87). In addition, several reports suggest that tryptase levels are more consistently increased in IgE-mediated anaphylaxis and less consistently so in non-IgE–mediated reactions and thus may be contemplated as a surrogate marker to distinguish the two (50,87,158,167). In all these situations, interpretation of serum tryptase levels must take into account the severity of the reaction because milder anaphylactic reactions are not always associated with significantly higher tryptase levels (82,87,142,177).

Other potential surrogate markers to distinguish anaphylaxis from mastocytosis eventually may include soluble CD25 (sCD25) and soluble kit (sKIT, CD117) levels. Both of these shed receptors are increased only modestly during anaphylactic episodes as detected by enzyme-linked immunosorbent assay (ELISA) and are markedly increased in systemic mastocytosis (2). The cellular source of sCD25 in anaphylaxis is likely to be activated T-lymphocytes, in line with the demonstration of activated T cells in Hymenoptera hypersensitivity (16) and downregulation of their surface IL-2 receptors after rush hyposensitization (200). Whether these surrogate markers will be clinically useful or will remain interesting research findings remains to be determined.

PREVENTION AND TREATMENT

A negative history of a previous adverse effect is no guarantee that administration of a pharmaceutical agent will not produce anaphylaxis. Subclinical sensitization may result from a previous uneventful clinical exposure (81), for example, as with the presence of the material in food (102,214) or with contact of a cross-reacting antigen (103,157). In a study of six fatal cases of systemic anaphylaxis (81), no instance was found in which serious difficulty would have been predictable on historical grounds. Yet the constitutional reaction began within minutes of antigen administration, and the patients were dead within 16 to 120 minutes despite emergency medical attention in four cases.

Prevention of the anaphylactic syndrome must take into account the sensitivity of the recipient, the dose and character of the diagnostic or therapeutic agent, and the effect of the administration route on the rate of absorption. Identification and avoidance of causative agents, as feasible, remain the most important steps in the management of susceptible persons. If there is a definite history of an anaphylactic reaction with a particular treatment, even though mild, it is advisable to select another agent or procedure. A skin test should be performed before the administration of certain materials that produce a high incidence of anaphylactic reactions (e.g., horse serum or allergenic extracts) or when the nature of a past adverse reaction is unclear. Patients with known sensitivities, such as to foods or stinging insects, should be taught to recognize the clinical signals of impending anaphylaxis (i.e., a lump in throat, difficulty breathing, lightheadedness, feeling of impending doom) and feel comfortable and skilled in the technique of self-administration of epinephrine. Epinephrine in a syringe or in an autoinjector device should be carried by the patient at all times, with several spares in convenient locations (e.g., at home and at work), and these kits should be renewed on a yearly basis.

In the case of penicillin, not all urticarial reactions imply the same hazard. Thus, skin testing to determine the presence and haptenic specificity of skin-sensitizing antibody may be justified if penicillin is clearly the drug of choice. If the skin-sensitizing antibody has minor determinant specificity to benzylpenicillin, penicillin should be avoided, whereas isolated major determinant specificity to benzylpenicilloyl generally would not contradict penicillin therapy if the clinical indication exclusively warranted treatment with penicillin. An occasional patient with skin-sensitizing antibody of benzylpenicilloyl specificity can manifest immediate reactions (45). In these situations, the skin-sensitizing antibody was of high concentration and binding avidity, whereas the titer of IgG antibody was relatively low.

Because even an intradermal or conjunctival test may produce a serious reaction, a prick test should precede these tests in any high-risk situation. In the event an agent is to be used despite a positive history, a positive skin test, or both, precautionary measures should include the following: (a) having present an intravenous infusion, intubation equipment, and a tracheostomy set; and (b) administration of the material first by prick test or intradermally into the distal portion of an extremity. If this test is tolerated, injection of material intradermally, subcutaneously, intramuscularly, or intravenously may be considered with increasing doses at 20- to 30-minute intervals such that the initial dose by the next route does not exceed the final dose by the previous route. Even with this technique, it may be difficult to be certain when target cell exhaustion has been reached and how long it will persist; in several instances (14,62,203), parenteral injection of the therapeutic dose of the agent has been lethal despite painstaking previous “acute desensitization.” With such “exhaustion” or “pharmacologic” desensitization, it is critical to give the therapeutic agent at regular intervals to prevent reestablishing a sensitized cell pool of hazardous size. Otherwise, because this procedure constitutes a form of “controlled anaphylaxis,” patients are likely to require repeat desensitization in the event of a future requirement for readministration of the known agent.

A different form of response involves the development of antibody of the IgG class that appears protective against anaphylaxis induced by Hymenoptera group venom by interacting with the antigen so that less of it reaches the sensitized tissue mast cells. Indeed, immunotherapy is often efficacious and indicated for the treatment of Hymenoptera hypersensitivity.

The hazards of anaphylactic transfusion reactions in IgA-deficient patients have been circumvented by using extensively washed erythrocytes, whole blood from IgA-deficient persons (113), and even autotransfusion. The protective role of pretreatment with glucocorticoids and H_1 and H_2 antihistamines with or without ephedrine (126, 221) at least 2 hours before radiographic studies in patients with a history of a previous reaction is well documented. Even under such conditions, studies should use a test dose, contrast agents of lower osmolarity; and, in the case of pyelography, a retrograde rather than an intravenous route when possible.

Patients with idiopathic anaphylaxis who experience frequent attacks should be placed on maintenance therapy with oral antihistamines and, in some severe cases, glucocorticoids (e.g., oral prednisone, 20–40 mg daily) until the episodes abate. Glucocorticoids may be tapered thereafter. High-risk patients on b-blocker therapy, including topical medications used in the treatment of glaucoma, should be continued on these medications only if feasible alternatives are unavailable and the drug is medically indicated.

Early recognition of an anaphylactic reaction is essential because death can occur within minutes to hours after the first symptoms appear (81,92). The immediate treatment of anaphylaxis begins with a rapid assessment of the patient's level of consciousness, along with the patient's airway, breathing, and circulation (ABCs). The management of anaphylaxis should take into consideration the severity of the reaction. Epinephrine is the initial drug of choice (Table 64.3) (124). The recommended dose of epinephrine in adults is usually 0.3 mL of 1:1,000 aqueous epinephrine injected subcutaneously, although the dosage may need to be decreased in elderly patients (to perhaps 0.2 mL) or increased in patients receiving b-adrenergic blockers (to 0.5 mL). Epinephrine dosages in children are based on weight, that is, 0.01 mL per kilogram of a 1:1,000 dilution up to a maximum of 0.3 mL of epinephrine injected subcutaneously. If the antigenic material has been injected into an extremity, the rate of absorption may be reduced by prompt application of a tourniquet proximal to the reaction area, administration of 0.2 mL 1:1,000 epinephrine into the site, and removal of an insect stinger, if present, without compression. One study of 17 children with a history of anaphylaxis to food, Hymenoptera venom, or other substances (188) concluded that intramuscular administration of epinephrine may afford greater bioavailability. The mean maximum epinephrine concentration was slightly greater after intramuscular injection, and the time to maximum concentration was almost five times longer with subcutaneous than with intramuscular injection (approximately 30 and 7 minutes, respectively) (141,188). In addition, more than 70% of patients who received intramuscular epinephrine achieved peak epinephrine levels within 5 minutes, compared with only 20% of those who received epinephrine subcutaneously.

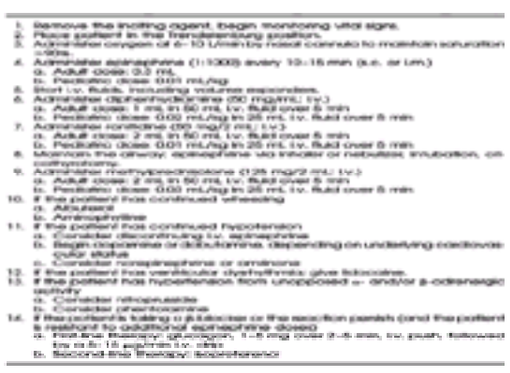

TABLE 64.3. Treatment of Anaphylaxis

Supportive measures also should include establishment of an intravenous infusion to provide for the administration of volume expanders and other medications (21,218). Oxygen via a nasal cannula or intermittent positive-pressure breathing of oxygen with epinephrine via nebulizer or inhaler may be helpful, but either endotracheal intubation or a tracheostomy is required for progressive hypoxia. Ancillary agents such as the H_1 antihistamine, diphenhydramine, or aminophylline are appropriate for urticaria–angioedema and bronchospasm, respectively. H_1 and H_2 actions of histamine may lead to arrhythmias and a widened pulse pressure, suggesting a beneficial effect of H_2 antihistamines (110). Clinical and experimental evidence suggests that treatment with a combination of H_1 and H_2 receptor antagonists may be more effective than treatment with an H_1 antihistamine alone in preventing histamine-induced hypotension (21,42,82,218). Intravenous glucocorticoids are not efficacious for the acute event but should be considered in instances in which amelioration is not complete and subacute bronchospasm or hypotension persists (218).

In addition to fluid resuscitative measures, treatment of refractory hypotension and shock may require pharmacologic intervention. Replacement of serial subcutaneous and intramuscular epinephrine injections with an intravenous bolus, followed by a continuous infusion of a 1:10,000 epinephrine drip, is generally recommended as first-line therapy (21,218). Lack of response to this measure may warrant the addition of an intravenous continuous infusion of dopamine or dobutamine, depending on the patient's underlying cardiovascular status, followed by norepinephrine or amrinone. Established dosages of these vasoactive inotropic medications are provided by the American Heart Association (AHA) guidelines (33). Hypertension resulting from unopposed b-adrenergic and a-adrenergic activity may require prompt treatment with nitroprusside or phentolamine until blood pressure stabilizes. Even with close cardiac monitoring and titration, myocardial infarction is a known associated sequela of systemic anaphylaxis, either from the initial hypotensive insult to the myocardium or from a- or b-adrenergic effects on an already compromised cardiovascular system.

Occasionally, apparent “biphasic reactions” occur; that is, reactions recur hours after the initial reaction (19,37,197). It is therefore recommended that patients experiencing anaphylaxis be kept under observation for up to 24 hours and that glucocorticoids be administered in instances of moderate or severe anaphylaxis to help prevent such delayed-onset reactions. Whether epinephrine acts to prevent mediator release, reverse the action of mediators on target tissues, or both is not established, but its early administration appears to be critical. Epinephrine is still the drug of choice when anaphylaxis occurs in a patient on a b-adrenergic blocker (82,201,218) or in pregnant women experiencing anaphylaxis (218). It is also an important strategy to prevent or lessen the severity of anaphylactic reactions by early self-intervention in high-risk patients.

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65 ASTHMA

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HISTORY

The word *asthma* was first introduced in ancient Greece. It originally referred to the symptom of breathlessness rather than to the disease that is recognized today. Mild breathlessness was termed *dyspnea*; moderate breathlessness, *asthma*; and severe breathlessness, *orthopnea* (1). Hippocrates (460–380 BC) believed that ill health arose from an imbalance among the four humors (body fluids): blood, phlegm, yellow bile, and black bile. Phlegm was thought to originate in the brain, from where it condensed in the nasal cavities via the pituitary gland. Asthma was considered to result from excess phlegm flowing down into the lung (1). Claudius Galen of Pergamum (129–216 AD) elaborated on the hippocratic concept of the humors and dominated medical thinking about the causation of ill health throughout the Middle Ages (1).

Unlike Galen and Hippocrates, a number of early physicians did appear to appreciate at least some of the clinical features of asthma, attempting to differentiate it from other diseases that cause breathlessness. Aretaeus of Cappadocia (81–131 AD) referred to asthma as a disease characterized by paroxysms of breathlessness induced by exercise and associated with cough. His writings were not discovered until 1554 and are considered the best description of asthma from ancient times (2). Moses Maimonides (1130–1204) also referred to the paroxysms of breathlessness in his *Treatise on Asthma* (1190), the earliest known monograph on the condition, and recognized that the disease often improved during adolescence (1).

Galenic dogma was eventually challenged during the Renaissance period by Paracelsus (1493–1541), who vigorously denied the ancient humoral theories and burned Galen's works. His ideas led to the gradual liberation of European medical thinking about the pathogenesis of disease. Johannes Baptiste van Helmont (1579–1644) argued against the ancient humoral theory of asthma on the basis that coughing prevented the descent of phlegm into the lungs. Although he reasoned that the production of phlegm occurred in the airways, he argued that the onset of a paroxysm of breathlessness was too rapid to be explained by the accumulation of phlegm within the lung (3).

Early descriptions of asthma overlap to varying degrees with descriptions of other respiratory and cardiac diseases that cause breathlessness and cough. Sir John Floyer (1649–1734) is generally considered the first to describe clearly and to differentiate asthma from these other conditions in his *Treatise of the Asthma* (1698), the first monograph on the subject written in English (4). In it, he describes the periodic nature of the disease, the typical worsening of symptoms during the night, and the expectoration of bronchial casts of viscid phlegm. He refers to the clinical signs of airflow obstruction, noting hyperinflation of the chest and prolonged expiration associated with wheeze. Like van Helmont, Floyer also regarded asthma as a nervous disorder, and he postulated that involuntary spasms of the bronchi resulted from the contraction of muscle fibers under the influence of the vagus nerve. Opposition to the *spasm* theory of asthma, however, came from those who continued to argue that phlegm was the cause of airflow obstruction in the disease. These opponents included Giovanni Battista Morgagni (1682–1771), who observed thick mucus obstructing the airways in a case of presumed fatal asthma (2).

In 1808, Franz Reisseinen identified the circular smooth-muscle layer in the bronchial wall (1); and in 1830, Eberle postulated that bronchoconstriction was not the result of a convulsion but was instead a reflex action in response to irritating stimuli in the lungs or elsewhere (5). In 1842, Francois Longet demonstrated that electrical stimulation of the vagus nerve could indeed induce bronchoconstriction (1); by the mid-nineteenth century, the spasm theory of asthma prevailed. This conclusion is evident in the writings of Henry Hyde Salter (1823–1871), the greatest authority on asthma in Victorian England. In his treatise *On Asthma: Its Pathology and Treatment* (1860) (6), Salter reinforced the concept of asthma as a disease of the nervous system causing paroxysms of bronchial spasm, grouping cases according to whether he considered them a reflex phenomenon or a nonreflex disorder originating in the brain. He dismissed mucosal edema and tenacious mucus plugs as additional causes of bronchial obstruction on the basis that he could find no evidence of these in the lungs of persons with asthma who had died of other causes. In 1875, Karl Stoerk directly observed the rapid development of mucosal edema and hyperemia throughout the trachea in a patient during an attack of asthma. In 1886, Sir Andrew Clark challenged the spasm theory of asthma, arguing that episodes of mucosal edema with associated increased mucus production could cause paroxysms of airflow obstruction in the absence of bronchial smooth muscle contraction (7). By the end of the nineteenth century, asthma still was considered a nervous disorder, but opinion differed as to whether the cause of bronchial obstruction was bronchospasm or neurogenic vasodilatation.

The observation that many individuals with asthma react to specific environmental factors in an idiosyncratic manner has been documented since the Renaissance period, when increasing recognition was given to the influence of external factors on health. In 1547, Geromo Cardano advised the asthmatic Archbishop of St. Andrews, Scotland, to avoid feather bedding. This resulted in symptomatic improvement and is often cited as the first example of therapeutic allergen avoidance (1). Both van Helmont and Floyer recognized that the inhalation of dust and the ingestion of certain foods could induce asthma in susceptible individuals, and Barnadino Ramazzini (1633–1714) gave the first clear account of occupational asthma in bakers and millers (8). Although the influence of seasonal changes on the disease was appreciated by Floyer, it was John Elliotson who, in 1831, first proposed that pollen might be the environmental factor responsible for inducing asthma in association with hay fever (1). In 1873, Charles Blackley confirmed this idea, postulating that other “proteids” might be important in other forms of the disease (9). By the end of the nineteenth century, the *nervous* theory of asthma suggested that certain inhaled or ingested environmental irritants and proteids could stimulate either reflex bronchospasm or mucosal vasodilatation in susceptible individuals. Recognizing the idiosyncratic nature of the disease, Salter argued that the susceptibility of the individual to asthma reflected an abnormal increase in the irritability of the part of the nervous system affected (6).

Immunologic Highlights

In 1902, Charles Richet and Paul Portier reported that, whereas the extract from sea anemone tentacles had no effect on dogs when given as a single injection, a second injection after a few weeks caused a rapid and fatal reaction. Richet gave the term *anaphylaxis* to this phenomenon, suggesting that the initial injection of toxin had somehow weakened the dog's defense mechanisms and increased its susceptibility to toxin on subsequent exposure (9). In 1904, Theobold Smith reported that fatal anaphylactic shock could be induced by repeated injections of horse serum into guinea pigs, suggesting that nontoxic proteins also could cause anaphylaxis (9).

The following year, Clement von Pirquet and Bela Schick reported the occurrence of a transient fever, rash, and polyarthritis in patients given antiserum for the treatment of major infection. They termed this clinical syndrome *serum sickness* and noticed that a repeated injection of antiserum could induce an immediate and sometimes fatal reaction (9). On the basis of similarities between this observation and the anaphylactic shock described in animals, they argued that serum sickness was a form of anaphylactic reaction in humans. In 1907, von Pirquet introduced the term *allergy* to refer to the altered state of reactivity induced by repeated exposure to foreign substances that is responsible for anaphylaxis. In 1910, John Auer and Paul Lewis demonstrated bronchospasm to be a key feature of anaphylactic shock in guinea pigs, leading Samuel Meltzer to propose the *anaphylactic* theory of asthma later the same year, thus classifying asthma not as a nervous disorder but as an allergic disease (10).

The following year, Leonard Noon demonstrated that persons who were sensitized to grass pollen developed a wheal-and-flare reaction after receiving an injection of a grass pollen extract into the skin, thereby introducing allergen skin testing (9). In 1919, Maximilian Ramirez reported the case of a man who developed asthmatic symptoms on contact with horses shortly after being transfused with blood from a horse-sensitive donor. This condition was associated with a positive skin test to horse antigen, and Ramirez concluded that “anaphylactic bodies,” responsible for mediating anaphylaxis, had been passively transferred via the donor's blood (11). Two years later, Carl Prausnitz and Heinz Küstner renamed this transferable factor *reagin* (9). On the basis of positive skin testing, Coca and Cooke introduced the term *atopy* in 1923 to denote the sensitization of persons to one or more environmental allergens (12). It had already become apparent, however, that not all persons with asthma had positive skin tests, leading to the classification of asthma into *extrinsic* (allergic) and *intrinsic* (nonallergic) groups by Walker in 1918 (13). Nonetheless, the anaphylactic theory continued to dominate the field of asthma research.

In 1929, Sir Henry Dale demonstrated that intravenous administration of histamine could induce an anaphylactic-like syndrome in guinea pigs and rabbits, and he postulated that histamine played a key role in the anaphylactic reaction (9). Evidence in support of Dale's hypothesis came when increased serum levels of histamine were shown to be associated with anaphylaxis in guinea pigs. Furthermore, the newly developed histamine receptor antagonists significantly reduced the severity of the anaphylactic reaction in this animal model (9). In 1952, Riley demonstrated the source of histamine to be the tissue mast cell (14), and the mast cell hypothesis of anaphylaxis and asthma was subsequently proposed. It soon became evident, however, that histamine was not the only soluble factor mediating anaphylaxis. Using the isolated organ bath preparation of sensitized guinea pig lung, Brocklehurst demonstrated that antihistamines failed to prevent a sustained smooth muscle contraction in response to allergen challenge. In 1960, on the basis of the time course of this contractile response, he termed the soluble mediators responsible the slow-reacting substance of anaphylaxis (SRS-A) (15).

Kimishige and Teruko Ishizaka identified reagin as the immunoglobulin E (IgE) subclass of antibody in 1967 (16), and Johansson reported an association between allergic asthma and elevated levels of serum IgE the same year (17). In 1971, Tomoika and Kimishige Ishizaka demonstrated the presence of receptors for IgE on mast cells, later shown to be high-affinity receptors for the Fc portion of the IgE molecule (FceRI). By 1979, the Ishazakas had reported mast cell activation after the cross-linking of these receptors by allergen-IgE antibody complexes, resulting in the release of histamine and SRS-A (18). SRS-A subsequently was identified as the cysteinyl leukotrienes-leukotriene (LT)C_4 LTD_4, and LTE_4, whose structures and synthetic pathways were elucidated in 1983 (19).

In 1986, Mossman et al. (20) published data from a study in mice, which has since had a major influence on our thinking about the cellular and molecular mechanisms underlying allergy. On the basis of differences in patterns of cytokine expression *in vitro*, two distinct subsets of T helper memory cells, termed T helper type 1 (Th1) and Th2, were identified within the $CD4^+$ lymphocyte population. Murine Th1 cells were found to express interleukin-2 (IL-2) and interferon-g (IFN-g), whereas Th2 cells expressed IL-4 and IL-5. Both subsets produced IL-3 and granulocyte–macrophage colony-stimulating factor (GM-CSF). Subsequent studies in humans demonstrated similar subsets of T helper cells (21), termed Th1-like (IFN-g) and Th2-like (IL-3, -4, -5, -6, -9,-13; GM-CSF). Because IL-4 upregulates IgE production (22) and IL-5 promotes eosinophil recruitment (23), Th2-like and Th1-like cells were considered to orchestrate allergic and nonallergic immune responses, respectively. Consequently, the mast cell hypothesis was superseded by the postulated pivotal role of the T cell in asthma.

EPIDEMIOLOGY

Population-based studies provide data on the prevalence, geographic distribution, and natural history of disease. In addition, they play a key role in directing research on the etiology of disease by focusing attention on genetic and environmental risk factors. The outcomes and conclusions drawn from such studies are critically dependent, however, on how the disease has been defined.

Definition of Asthma

The lack of an objective definition for asthma, allowing the unequivocal differentiation of the asthmatic from the nonasthmatic phenotype, remains a fundamental problem (24,25), reflecting the phenotypic heterogeneity of the disease and the current lack of understanding of the processes involved. Such heterogeneity is suggested by the various clinical subgroups (subphenotypes) of asthma that are recognized, including atopic asthma, aspirin-intolerant asthma (AIA), exercise-induced asthma (EIA), occupational non-IgE-dependent asthma, intrinsic asthma, brittle asthma, and cough-variant asthma. This complexity is further increased when variations in disease chronicity, severity, and response to treatment are considered. Thus, far from being a single disease entity, asthma is probably best considered a clinical syndrome comprising multiple variants.

The original clinical definition of asthma can be found in Salter's treatise of 1860, in which he describes the condition as “paroxysmal dyspnea, generally periodic, with healthy respiration between attacks” (6). In 1959, the Ciba Foundation guest symposium redefined asthma as “a widespread narrowing of the bronchial airways which changes in severity over short periods of time, either spontaneously or under treatment” (26). This definition drew a distinction between “reversible” airflow obstruction, corresponding to asthma, and “irreversible” airflow obstruction, subsequently synonymous with chronic obstructive pulmonary disease (COPD). Longitudinal studies have since shown that a significant proportion of persons who have chronic asthma exhibit an increased rate of decline in lung function over time, leading to the progressive acquisition of a fixed component of airflow obstruction (27). Furthermore, asthmatic and COPD populations overlap in peak expiratory flow (PEF) variability (28). Thus, similarities in lung function in the clinical syndromes of chronic asthma and COPD have added to the diagnostic difficulty.

Bronchial responsiveness refers to a change in airway caliber in response to a variety of nonspecific physical or chemical stimuli; it was first introduced as a concept by Dautrebande and Philpott in 1941. An exaggerated bronchoconstrictor response, termed *bronchial hyperresponsiveness* (BHR), subsequently was found to be a feature of asthma, leading to the incorporation of BHR into the definition of the disease by the American Thoracic Society in 1962 (29). More recently, fiberoptic bronchoscopy has allowed new insights into the pathogenesis of asthma, with the recognition that persistent airway inflammation is a consistent feature of all forms of the disease (30,31). Consequently, the combined World Health Organization (WHO) and National Heart, Lung, and Blood Institute (NHLBI) working group of 1995 defined asthma as “a chronic inflammatory disorder of the airways giving rise to recurrent respiratory symptoms associated with widespread, variable, and reversible airflow obstruction and bronchial hyperresponsiveness” (32). Chronic Th2-mediated airway inflammation has since been shown to be a feature of both atopic and nonatopic asthma (33,34 and 35).

In clinical practice, the diagnosis of asthma equates to a medical opinion that is based on a balance of probabilities, taking account of the medical history, physical examination, measures of lung function, and response to treatment. Although such an assessment may be feasible for each subject in a small-scale study, this is generally not the case when the study is population-based. Thus, epidemiologists have used symptom questionnaires, but the validity of this method continues to be a subject for debate (24). In attempting to improve diagnostic accuracy, many epidemiologists have combined symptom questionnaires with “objective” measures of bronchial responsiveness or PEF variability.

Bronchial Hyperresponsiveness and Peak Expiratory Flow Variability

The measure of lung function that has received the greatest attention as an objective diagnostic marker for asthma has been BHR. Physical stimuli used to assess bronchial responsiveness include exercise and cold (dry) air; chemical stimuli include histamine, methacholine, and adenosine monophosphate. The histamine challenge and methacholine challenge are the methods most widely used to provide a measure of bronchial responsiveness; they are expressed as the concentration (or dose), termed the PC_{20} (or PD_{20}), of histamine or methacholine that is required to produce a 20% fall in FEV_1 (forced expiratory volume in 1 second). The threshold used to define BHR is arbitrary, but values of 8 mg per milliliter or less are generally used, and both chemicals give comparable results. When defined this way with histamine, one study (36) found BHR to have a sensitivity of 97% for physician-diagnosed asthma, suggesting that 97% of the asthmatic population have BHR. The specificity of the test was only 71%, however, suggesting that 29% of the nonasthmatic population also have BHR by this definition. Others have reported similar results (37). Thus, a negative test may be considered useful for excluding asthma, but a positive test has limited use as a diagnostic marker for the disease because BHR is also associated with atopy, COPD, increasing age, and female gender in the nonasthmatic population (24). Although some authorities advocate a measure of bronchial hyperresponsiveness in addition to a standardized symptom questionnaire when defining asthma in population-based studies (38), others argue that the diagnostic accuracy of a standardized symptom questionnaire *alone* is probably little different (24,25).

In contrast, there has been relatively little interest in the measure of airflow variability as an objective diagnostic marker for asthma, despite the fact that the various definitions of asthma have variable airflow obstruction as a unifying feature. When defined as PEF variability exceeding the 90th percentile of a distribution, one study (39) involving children found variable airflow obstruction to have a specificity of 91% for physician-diagnosed asthma but a sensitivity of only 36%. Similar levels of sensitivity have been found when 90% specificity is taken as a cutoff in other studies involving children and adults (37,40). Numerous issues relating to the

measurement and analysis of airflow variability have yet to be resolved (41).

The correlation between PEF variability and bronchial responsiveness is not strong (28,40), perhaps indicating that these two measures of lung function relate to different pathophysiologic processes in the diseased lung. Whereas PEF variability exhibits a stronger correlation than BHR with respect to cough and wheeze in the past week, BHR shows a stronger correlation than PEF variability with respect to such symptoms over a period of 12 months (37), and exacerbations of asthma do not appear to be associated with changes in BHR (42). Furthermore, BHR not only correlates with reduced FEV_1 (24), but it also is associated with impaired growth in FEV_1 in children and accelerated decline in FEV_1 in adults (27). Thus, BHR might be of use as a prognostic marker, helping to identify persons with asthma who are at increased risk of chronic and severe disease, whereas PEF variability might simply be a marker of current disease activity. Longitudinal studies also indicate that asymptomatic BHR is an independent risk factor for the development of asthma (43,44), suggesting that BHR may represent an intermediate phenotype between normality and the clinical disease (45). The debate surrounding the significance of BHR probably will continue until its underlying mechanisms are better understood.

Prevalence, Morbidity, and Mortality

Despite difficulties relating to the definition of asthma and changes in the International Classification of Diseases (ICD), it is generally agreed that prevalence rates for early-onset asthma and atopy have increased throughout the world since the 1960s, probably twofold or threefold (46). In contrast, the prevalence of late-onset disease, presenting for the first time at age 40 or older, appears not to have increased over the last 20 years, at least in elderly persons (47).

To obtain data allowing for more reliable comparisons between countries, as well as with future time points, efforts have been made to establish international collaborative studies with standardized methodology. These include the International Study of Asthma and Allergies in Childhood (ISAAC) (48) and The European Community Respiratory Health Survey (ECRHS) (49), which focused on adults between the ages of 20 and 44 years. ISAAC phase 1 assessed 721,601 children, aged 6 to 7 and 13 to 14 years, from 56 countries throughout the world. The results indicate a prevalence of asthmalike symptoms in the past 12 months of 2% to 32% and a previous history of physician-diagnosed asthma of 1.5% to 28%, with little difference between the two age groups. Countries with a prevalence of less than 10% were found mainly in Asia, North Africa, Eastern Europe, and the Eastern Mediterranean regions. In contrast, countries with a prevalence of more than 20% included the United States, the United Kingdom, New Zealand, Australia, and Latin America.

Although asthma can present at any age (50), 80% to 90% of all cases are thought to present with symptoms before the age of 5 years (51), and the mean annual incidence for wheezy illness in childhood is believed to be greatest before the age of 3 years (52). Longitudinal studies have shown that, of the cases of wheezy illness presenting under the age of 5 years, only a third persist into later childhood as established cases of asthma (52,53), and up to half of these cases resolve by adulthood (54). These studies suggest that, although persistent asthma appears to affect between 5% and 12% of the general population, at least three times as many people experience sporadic asthmalike symptoms at some point during their lives. During childhood, the male-to-female ratio for physician-diagnosed asthma is approximately 2:1. After puberty, however, the disease is more common in the female population, suggesting that hormonal factors may play an important role (55).

The ISAAC study found that the proportion of children with severe asthma compared with the total number of children with asthmalike symptoms varied little between countries, suggesting that factors influencing the prevalence of the disease might also affect the severity. This finding is consistent with observed increases in hospital admission rates (56), reflecting the rise in the prevalence of asthma in westernized societies. In the United States alone, the cost of treating asthma is an estimated $6 billion per annum, half of which is spent on hospital care and 80% of which is spent on the 20% of patients at the severe end of the disease spectrum (57). There is also concern relating to the apparent increase in asthma-related deaths observed in most developed countries, with mortality rates presently standing at between 1.0 and 1.5 per 100,000 of the general population. This increase in the asthma mortality rate appears to be real and cannot be explained by changes in ICD coding or death certification (58).

Role of Atopy

Since 1910, the anaphylactic theory of asthma has dominated research into the disease, largely supported by data from population-based studies. These data suggest that about 90% of persons under the age of 30 years who have asthma are atopic and that atopy is the strongest risk factor for the disease, with an odds ratio of between 10 and 20 (59). Furthermore, longitudinal studies have shown atopy to be a risk factor for the persistence of early childhood wheeze into late childhood (52,53) and for the persistence of childhood asthma into adulthood (54).

Although originally atopy was defined on the basis of positive skin testing to allergen, it was recognized that a negative test did not necessarily exclude atopy because it was still possible to be allergic to proteins not present on the panel of test allergens. After the introduction of tests allowing the measurement of total serum IgE, the definition of atopy was revised. Consequently, population-based studies challenged the traditional classification of asthma into extrinsic and intrinsic groups by demonstrating an association between elevated levels of total serum IgE and all forms of asthma, irrespective of skin-prick test results (60,61). This finding led to the suggestion that atopy and allergic airway inflammation were in fact pathognomonic for asthma, further supporting the anaphylactic theory. In the case of “intrinsic” asthma, it was suggested that the allergens responsible were unidentified environmental or endogenous proteins (60). Consequently, the pathogenesis of asthma was viewed in terms of a serial model for allergic disease in which the exposure of genetically susceptible individuals to allergen induces a presymptomatic state of atopic sensitization, characterized by a Th2-mediated inflammatory response to allergen, with progression to clinical asthma on continued allergen exposure. This model, however, raises a fundamental question: What are the mechanisms whereby Th1 and Th2 immune responses are programmed into long-term immunologic memory? Furthermore, it fails to take into account the observation that only 20% of the atopic population develop asthma, with additional subsets presenting with atopic eczema or allergic rhinitis. This finding raises a second fundamental question: What are the mechanisms by which the Th2-mediated inflammatory response to allergen localizes to a mucosal surface? In the case of asthma, it is postulated that gene-environment interactions not only skew the programming of immunologic memory toward the Th2 phenotype but also determine target-organ selectivity, localizing the allergic inflammatory response to the airway mucosa. The persistence of asthma in lungs transplanted from asthmatic donors to nonasthmatic recipients, but not the converse, strongly suggests that determinants of target-organ localization reside within the target organ itself (62).

Although it is generally accepted that atopy plays an important role in asthma pathogenesis, a number of epidemiologic observations have challenged the dominance of the anaphylactic theory, questioning the contribution made by IgE-dependent mechanisms to the development of the disease. In parallel with asthma, evidence suggests that the prevalence of atopy has increased throughout the world since the 1960s and that almost 50% of the population from westernized societies demonstrate sensitization to one or more environmental allergens, with lower prevalence rates observed in less developed countries (59). Most epidemiologic studies, however, found no evidence to suggest an association between the prevalence of asthma and atopy (defined as skin-prick test positivity) when measured in the same population at different times or in two or more different populations at the same time (63).

There is also concern about the validity of using total serum IgE to define atopy. Although levels of total serum IgE correlate with skin-prick test positivity, this correlation is stronger if measures of allergen-specific serum IgE radioallergosorbent test (RAST) are used instead. Furthermore, because the level of serum IgE used to define atopy is purely arbitrary, it is considered likely that the prevalence of atopy has been overestimated significantly in many population-based studies (63). Indeed, many epidemiologists now argue that atopy should be defined either in terms of RAST or skin-prick test positivity to common environmental allergens (64). By this definition, the correlation observed between elevated levels of total serum IgE and nonatopic asthma led to the suggestion that coinherited genetic factors might increase a person's susceptibility to both asthma and increased IgE production (65). In this case, IgE may not necessarily play a causal role in asthma, but it may simply be a marker of the chronic Th2-mediated airway inflammation that underlies the disease. Family-based studies suggest, however, that the tendency to produce increased amounts of IgE is just one of a number of inherited factors associated with the inheritance of asthma, and its ability to predict the development of asthma is limited (66). In cases of late-onset asthma, the prevalence of atopy is no greater than that in the age-matched nonasthmatic population, and atopy diminishes as a risk factor, especially toward the severe end of the disease spectrum (67). Thus, factors other than atopy are likely to play an important role in the development of asthma.

Intermediate Phenotypes

Population-based studies provided data on which hypothetical models of asthma pathogenesis have been based. These models suggest that, as a consequence of gene-environment interactions, chronic Th2-mediated airway inflammation and BHR develop, giving rise to an asymptomatic “asthmatic state.” This state then provides the context in which progression to clinical asthma might occur (45). Exactly how these two intermediate phenotypes interact with one another is not completely understood (Fig. 65.1). Although airway inflammation is considered an important mechanism underlying BHR, asymptomatic BHR and markers of allergic inflammation are independent risk factors for the development of asthma (43). It is postulated that children who “outgrow” their asthma retain the asthmatic state and are at risk of asthma relapse in later life, as lung function declines. Failure to recollect the childhood illness may give rise to the diagnostic label of late-onset asthma (55).

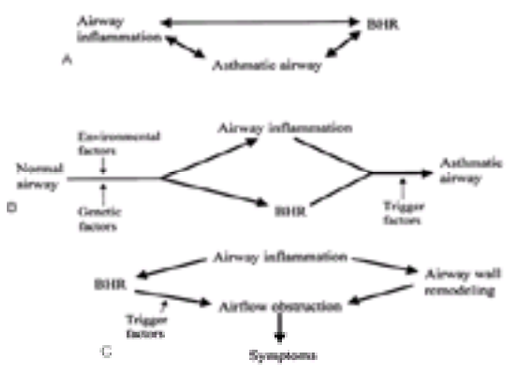

Figure 65.1. Hypothetical models of asthma. **(A)** Airway inflammation and BHR interdependence.**(B)** Steps involved in transforming a normal airway into an asthmatic airway.**(C)** Airway inflammation as the dominant driving force in the creation of the asthmatic state. (Reprinted from Banik AN, Holgate ST. Problems and progress in measuring methacholine metacholine bronchial reactivity. *Clin Exp Allergy* 1998;28(Suppl 1):15–19, with permission.)

In the absence of an objective definition for asthma, epidemiologists and geneticists have used intermediate phenotypes in the design of their studies, using atopy (RAST and skin test positivity) and total serum IgE as surrogate markers for chronic Th2-mediated airway inflammation. Despite the close relationships between BHR, skin test positivity, RAST, total serum IgE, and asthma (60,61), these phenotypes are not interchangeable, and it cannot be assumed that the gene–environment interactions responsible for the intermediate phenotypes are the same as those responsible for asthma. Thus, reliance on intermediate phenotypes carries with it a risk that important etiologic factors other than atopy might go unrecognized.

Genetics of Asthma and Atopy

The tendency for asthma to cluster within families was recognized by Salter in his treatise of 1860 (6) and later was confirmed by familial aggregation studies (68). Although suggestive of a genetic basis for asthma, these studies are not conclusive because an alternative explanation could be the familial sharing of environmental factors. Stronger evidence for a genetic contribution to the etiology of asthma has come from the study of inheritance in twins. Twin studies are based on the fact that monozygotic twins have identical genotypes, whereas dizygotic twins share on average only half their genes. Thus, diseases with a genetic component show higher rates of concordance in twins that are monozygotic than in those that are dizygotic. This pattern of inheritance is observed not only for asthma but also for BHR, atopy, and increased levels of total serum IgE, strongly suggesting a genetic basis for both the disease and intermediate phenotypes (69). In the case of atopy, the genetic component appears to be independent from that determining the target-organ selectivity of allergic disease (68) as well as from that determining BHR (70). This finding is consistent with data from segregation studies that indicate that asthma is a complex multigenic disorder, probably involving a number of subtle polymorphisms whose expressions are dependent on interactions with other genes (epistasis) as well as with the environment (69). Estimates of the contribution that genetic factors make to asthma susceptibility range from 35% to 70%, depending on the population and design of study (71). The two main methods used in attempts to identify asthma and atopy susceptibility genes are the *genome scan* (positional cloning) and *candidate gene approach* (fine mapping) (69,71).

Genome Scan

Genome scans use a panel of microsatellite DNA markers that label the entire human genome at known positions, typically 5 to 10 cM apart. Linkage studies are conducted within families or inbred populations, correlating the inheritance of genetic material, identified by the markers, with the phenotype of interest. The closer a microsatellite marker is to a disease-related gene, the less chance there is that they will be separated at meiosis. Linkage is measured by the lod score (log of the odds ratio of the probability of linkage to the probability of no linkage), and a lod score greater than 3.6 is considered significant (72). Thus, genome scans identify candidate susceptibility loci, within which susceptibility genes might reside. It is currently not possible, however, to narrow the focus to less than 1 cM (1 million base pairs), corresponding close to 100 genes. Numerous genome scans for asthma and atopy have now been completed (Table 65.1) (73,74,75,76 and 77), each reporting evidence for linkage to multiple loci. Most of these studies suggest candidate susceptibility loci within chromosomal regions 5q, 6p, 11q, 12q, and 13q. Although other regions have been highlighted by one or two studies, there is concern regarding statistical power because all these studies involved relatively small numbers of families (i.e., 100–500) (72). In an attempt to address this problem, efforts have been made to pool data (78); however, no candidate susceptibility genes for asthma or atopy have emerged from genome scans to date.

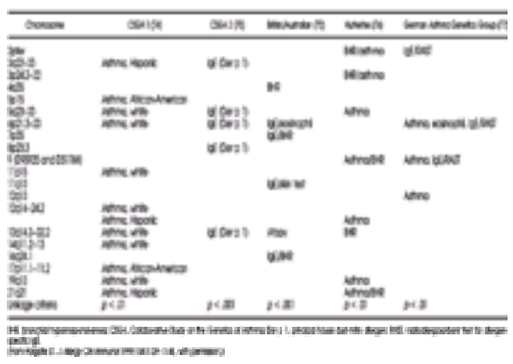

TABLE 65.1. Summary of Results from Published Whole-Genome Scans in Asthma

Candidate Gene Approach

The candidate gene approach involves direct testing for linkage or association between the allele (gene polymorphism) and phenotype of interest. Whereas linkage studies are performed on families or inbred populations, association studies generally are undertaken on populations of unrelated persons and compare the frequency of the candidate allele in those expressing the phenotype of interest with its frequency in those not affected. Candidate genes are chosen on the basis of cell and molecular models of asthma and the considered likelihood that they are important determinants of disease susceptibility, disease modification (chronicity and severity), or response to drug therapy (*pharmacogenetics*). A positive association does not necessarily imply a causal relationship because the candidate gene might be in linkage disequilibrium with a genuine disease-causing gene. Alternatively, a positive association might also occur if the candidate gene and disease both occur with increased frequency in an ethnic group. Thus, to prevent false-positive results, association studies should be performed in relatively homogeneous populations. Although numerous candidate genes have been described (Table 65.2), no gene has yet been identified as being involved in the inheritance of asthma with any certainty. Much interest has focused on the IL-4 gene cluster on chromosome 5q31-33, encoding the Th2 cytokine array, IL-3, IL-4, IL-5, IL-6, IL-9, IL-13, and GM-CSF. Importantly, the promoter regions of all human Th2-like genes share a common motif (CTTGG|.|.|. CCAAG), which is not present in the IFN-g promoter (79). This fact would be consistent with a mechanism for coexpression of Th2-like genes and has stimulated interest in the transcription factors involved.

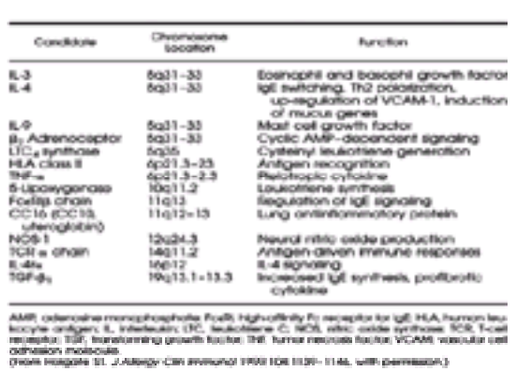

TABLE 65.2. Candidate Genes in Asthma and Allergy and Their Chromosomal Locations

Environmental Factors

Although genetic factors, at least in part, might account for geographic and racial differences in the prevalence of asthma and atopy, they fail to explain the rate of increase in the prevalence of these conditions over the past three to four decades, strongly suggesting that environmental changes are primarily responsible. When considering which environmental factors are involved, it is important to determine whether they increase the risk of developing asthma, influence disease chronicity and severity, or trigger acute exacerbations. The gradient of increasing prevalence observed for asthma and atopy from developing to developed countries led to the *westernization hypothesis* (80), suggesting that environmental factors predisposing to the development of asthma might be identified from among those closely associated with westernized living conditions.

Aeroallergens

Historically, allergens received greatest attention in asthma research. Population-based studies indicated that of all environmental allergens, common indoor aeroallergens are most closely associated with asthma (81). In temperate climes, the principal allergen is the fecal protein excreted by house dust mites (82), *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. In regions with low humidity, other indoor allergens predominate and are more closely associated with the disease. These include aeroallergens derived from domestic pets (83), cockroaches (84), and the fungal spores of *Alternaria alternata* (85). Food allergens (86) and pollens (81) are rarely associated with asthma.

Population-based studies suggest that infancy is the optimum time for aeroallergen exposure to induce atopy in later life, supporting the concept of a “sensitization window” (87). Although the risk of developing atopy is dependent on allergen dose and duration of exposure, individual susceptibility varies, and no minimum dose threshold has yet been identified (88). Repeated inhalation of low-dose allergen increases bronchial responsiveness in atopic, nonasthmatic persons (89) and induces asthmatic symptoms in atopic persons who have preexisting asymptomatic BHR (44). In addition, Sporik et al. (90) demonstrated a dose-dependent relationship between exposure to house dust-mite allergen (Der p 1) during the first 2 years of life and the likelihood of developing asthma up to the age of 11 years. These observations were used to support the hypothesis that aeroallergens play a causal role in the pathogenesis of asthma. Increased disease prevalence reflects, at least in part, higher levels of exposure to house dust mites associated with westernized housing (91), characterized by wall-to-wall carpeting, central heating, and energy-conservation measures. Martinez (92) argued against this hypothesis, suggesting that if a causal relationship between aeroallergens and asthma existed, the likelihood of developing asthma should not be dependent on the age at which prior sensitization occurs. He cited a study by Peat et al. (93) in which children who had become atopic to aeroallergens after the age of 8 years were shown to be at less risk of developing asthma or BHR than those who had become sensitized before this age. These children were at no greater risk than their nonatopic peers were over a 4-year period. The epidemiologic evidence used to support the allergen hypothesis was recently critically reviewed and considered to be equivocal (94), adding to the argument that factors other than atopy are likely to play an important role in the development of the disease.

In established asthma, allergen exposure can increase BHR and clinical disease activity (95). Numerous studies also indicate a positive correlation between the level of allergen sensitization and the degree of asthma chronicity and severity (88). Aeroallergens may precipitate acute exacerbations of the disease (96).

Infection

A major achievement of modern society has been a steady reduction in childhood infection rates. Smaller family size, improved sanitation, vaccination programs, and antibiotics have all contributed. Along with the use of detergents and antiseptics, these measures have made the westernized environment relatively sterile. The inverse correlation observed over time between infection rates and the prevalence of asthma and atopy (97,98) led to the *hygiene hypothesis* (99), which postulates that exposure to certain infectious agents during infancy reduces the risk of allergy by promoting the maturation of Th1-like responses, shortening the sensitization window. A gradient of infection rate might therefore explain why the risk of allergy declines in the last-born child as sibling numbers increase (100). In addition, the hygiene hypothesis, at least in part, might account for the observed gradients of increasing allergy prevalence from low to high socioeconomic class (101), from rural to urban regions in Africa (102), and from East to West Germany shortly after reunification in 1989 (103). Evidence in support of this hypothesis includes the finding that the level of childhood exposure to lipopolysaccharide (LPS, endotoxin), a component of the gram-negative bacterial cell wall, is inversely related to the risk of allergic disease (104). Furthermore, a reduced risk of allergy has been associated with measles infection during infancy in Africa (105) and with strongly positive tuberculin tests, indicative of exposure to *Mycobacterium tuberculosis* in Japanese children (106). The hygiene hypothesis, however, is more closely associated with atopy than it is with asthma (107). For example, despite increases in the prevalence of atopy and hay fever associated with the westernization of former East Germany from 1991/1992 through 1995/1996, prevalence rates for asthma and BHR have remained essentially unchanged (46). Thus, other environmental factors are likely to be responsible for localizing Th2-like responses to the airway mucosa in genetically susceptible persons.

Wheezy illness, usually the result of a viral respiratory tract infection (RTI), affects up to 30% of all children under the age of 5 years, and one third of these children will develop atopic asthma in later childhood (52). In children under the age of 3 years, the respiratory syncytial virus (RSV) is the most common cause of lower RTI (108), and the minority of young children who have elevated levels of serum IgE and RSV-specific IgE at the time of infection are at greatest risk of persistent wheeze into late childhood (109). Some studies suggest that RSV bronchiolitis in early life is associated with an increased risk of developing atopy (110), and it is postulated that such infection also may induce asthma by promoting airway mucosal Th2-like responses in genetically susceptible persons (111). A recent cohort study of children in the community, however, found that lower RTI with RSV before the age of 3 years did not increase the risk of developing atopy (108). Furthermore, although RSV infection in early childhood increased the risk of developing persistent wheeze during the first 10 years of life, no difference in asthma prevalence was observed at age 13 years between children who had and had not been infected with RSV before the age of 3 years. These researchers concluded that RSV plays no role in the causation of atopy and argue that the virus is unlikely to be causal factor for atopic asthma because this condition most commonly affects older children and adolescents (108). Children infected with RSV in early life had a significantly reduced FEV_1 at age 11 years. This reduction occurred independently of asthmalike symptoms and reversed to a nonsignificant value with bronchodilator therapy, suggesting an association between RSV infection in early life and altered regulation of airway smooth muscle tone. The significance of this finding is presently unclear (108). Although some studies suggest that RSV bronchiolitis in early life is a risk factor for the development of BHR, others argue that this risk is dependent on the coexistence of atopy (112). Other respiratory tract viruses, however, can induce BHR in persons who do not have asthma (113), and a history of viral RTI in the previous 5 years is reported to be a risk factor for the development of asthma in persons with preexisting BHR (44).

In established asthma, viral RTI is the most common cause of acute exacerbation, accounting for 80% to 85% of childhood cases (114) and 44% of cases in adults (115). In both groups, the common cold virus (*rhinovirus*) is most frequently responsible (114,115). Viral respiratory tract infection also has been implicated in most asthma deaths (58).

Air Pollution

Epidemiologic studies fail to support a causal role for outdoor oxidant air pollutants (sulfur dioxide, nitrogen dioxide, ozone) in the pathogenesis of asthma or atopy. No evidence of an increase in the levels of these pollutants has been found in the United Kingdom since the 1970s (80). Furthermore, although prevalence rates for asthma and atopy were greater in West Germany at the start of reunification, air pollution levels were higher in East Germany (103). Some evidence suggests that diesel exhaust particles may increase the risk of sensitization to aeroallergens (116), and this finding may be of relevance in view of the increasing numbers of diesel cars on European roads. In established asthma, there is little doubt that outdoor air pollution increases asthma morbidity rates (80), in association with increased airway inflammation and BHR (117,118). This fact, at least in part, reflects a synergistic interaction between air pollutants and aeroallergens (119,120).

Tobacco smoke is the most common indoor air pollutant, and there is evidence to suggest an increased risk of atopy and asthma in children whose mothers smoked during pregnancy (91). Maternal smoking during pregnancy is associated with reduced lung function, increased bronchial responsiveness, and higher risks of wheezy illness in infancy (91). In children with wheezy illness in early life, continued cigarette smoke exposure is associated with a progressive deterioration in lung function and increased bronchial responsiveness (91). Other studies, however, suggest that maternal cigarette smoking is *not* a risk factor for the persistence of wheeze into late childhood (52). Although active cigarette smoking in adolescence and adulthood increases bronchial responsiveness (121), no studies have directly linked this increase to the development of late-onset asthma. Active cigarette smoking is associated with an increased risk of asthma relapse at age 33 years, after a prolonged period without symptoms (122).

Diet

The westernization hypothesis postulates that dietary changes contributed to the rising prevalence of asthma (80). Epidemiologic studies demonstrated a general decline in consumption of antioxidant vitamins A, C, and E; and it is suggested that this circumstance increased the susceptibility of the airway mucosa to oxidant

stress from air pollutants and activated inflammatory cells (80). In addition, there has also been a trend toward the increased consumption of w-6 fatty acids, found in vegetable oil, relative to the consumption of w-3 fatty acids, found in fish oil and butter (91). In view of evidence suggesting proinflammatory and antiinflammatory actions for w-6 and w-3 fatty acids, respectively, it is postulated that an altered balance in their consumption increased the susceptibility of the airway mucosa to the damaging effects of allergens, viruses, and air pollutants (91). Finally, a decline in breast-feeding and the increased use of formula feeds have been postulated to play a causal role in asthma and atopy (91). Evidence in support of all these dietary hypotheses remains controversial.

Occupation

About 200 workplace-related compounds are now known to be capable of inducing asthma in susceptible persons (Table 65.3), and it is estimated that occupational asthma accounts for up to 7% of late-onset disease (50). The responsible compounds are inhaled, and they may be divided into two groups based on their molecular weight (50). The high-molecular-weight compounds are proteins that induce asthma through immunologic mechanisms that generally involve the production of antigen-specific IgE. Such compounds are more likely to induce asthma in persons who have preexisting atopy. In contrast, the low-molecular-weight compounds act as haptens (chemical sensitizers), promoting an immunologic response against self-antigens within the airway mucosa. Generally, this is not associated with increased IgE production, and preexisting atopy appears not to be a risk factor (50). The prevalence of occupational asthma in workers exposed to these chemical sensitizers depends on the compound involved and the level of exposure. For example, whereas 5% of workers exposed to toluene diisocyanate develop asthma (123), more that 50% of workers exposed to complex platinum salts are affected, with greatest risk observed among cigarette smokers (124).

Compounds	Industry/Personnel
Metal salts	
Platinum	Metal refining
Wood dusts and vegetables	
Oak	Carpenters, woodmill workers
Mahogany	
Western red cedar (plicatic acid)	
Grain (mites, fungi)	Millers, bakers
Flour	
Industrial chemicals	
Isocyanates	Polyurethane, paint, adhesives
Phthalic acid anhydride, trimellitic anhydride	Epoxy resins
Aminoethylethanolamine and colophony	Soldering
Ethylenediamine	Rubber industry
Paraphenylenediamine	Dye industry
Persulfates	Hair bleach
Pharmaceutical agents	
Penicillins	Pharmacists
Cimetidine	
Biologic enzymes	
Bacillus subtilis	Washing powder
Pancreatic enzymes	Pharmacists
Animal secretions	
α-Globulin in rat urine	Laboratory workers, veterinarians
Parathymin in mouse urine	
Locusts	
Animal dander	

TABLE 65.3. Examples of Compounds that Induce Occupational Asthma

CLINICAL PRESENTATION

Symptoms and Signs

The clinical syndrome of asthma is characterized by paroxysms of breathlessness, wheeze, chest tightness, and cough. Physical signs include tachypnea, a prolonged expiratory phase, and diffuse polyphonic wheeze on auscultation. If the patient is asymptomatic, clinical examination may be unremarkable. As the severity of chronic asthma increases, daytime and nocturnal symptoms become more frequent. In the minority of patients who have chronic severe disease, symptoms are continuous, activities of daily living are limited, and acute exacerbations are common.

Factors that suggest atopic asthma include onset of symptoms before the age of 30 years, a personal or family history of eczema or hay fever, and the recognition that paroxysms are triggered by allergen exposure. About 80% of patients also experience exercise-induced symptoms, and exercise may be the only trigger in a minority of cases. Symptoms typically develop 5 to 10 minutes after the exercise is stopped and resolve within half an hour. A refractory period of 1 hour may follow, during which further exercise fails to induce more symptoms.

Patients who develop symptoms after the age of 40 years are likely to have intrinsic disease, although occupational asthma should be considered if symptoms relate to the workplace. Of patients with asthma, 5% to 30% experience symptoms within 2 hours of ingesting aspirin or other nonsteroidal antiinflammatory drugs (NSAIDs). AIA classically occurs in female patients with late-onset, nonatopic disease, associated with nasal polyps and vasomotor rhinitis. In cough-variant asthma, cough is the predominant or only symptom.

Pathophysiology

Widespread, variable, and reversible airflow obstruction characterize asthma. Spirometry provides a measure of FEV_1 and forced vital capacity (FVC), and airflow obstruction is objectively defined as an FEV_1:FVC ratio of less than 70%. Although PEF correlates with FEV_1, its measurement tends to underestimate the degree of airflow obstruction and fails to differentiate obstructive from restrictive ventilatory defects. The severity of airflow obstruction is objectively assessed by comparing measured FEV_1 or PEF with predicted values, based on the normal distribution of lung function in the population, taking into account age, height, gender, and ethnicity. If FEV_1 is 80% or less of the predicted value, the degree of reversibility can be assessed by repeating the measurement 10 to 15 minutes after inhalation of a standard dose of short-acting b_2-agonist. Provided the increase in FEV_1 exceeds 200 mL, reversibility of 15% or more is generally accepted as being diagnostic for asthma (32). If airflow obstruction is present and reversibility in response to short-acting b-agonist is not observed, however, reversibility in response to a course of systemic glucocorticoids (prednisolone, 30–40 mg daily for 14 days) should be assessed in an attempt to differentiate chronic asthma from COPD. This course also allows the personal best FEV_1 and PEF to be identified.

In chronic asthma, moderate disease is defined as an FEV_1 or PEF 60% to 80% of that predicted, with mild disease above 80% and severe disease below 60% (32). In cases of acute asthma, it is recommended that measured FEV_1 or PEF be compared with the patient's personal best value, if this is known. An acute moderate exacerbation then is defined as a percentage best (or predicted) FEV_1 or PEF of 60% to 80%, with acute severe disease below 60% (32). The cutoff percentage values for FEV_1 and PEF used to define mild, moderate, and severe asthma are arbitrary, and the correlation that exists between perceived severity of asthma symptoms and degree of underlying airflow obstruction is poor (125).

If spirometry is normal, assessment of daily PEF variability may help to confirm a diagnosis of asthma. PEF is measured at least twice a day (morning and night), before and 10 to 15 minutes after a standard dose of short-acting b-agonist. Daily PEF variability of 20% or more, typically with dips in the early morning, is generally accepted as being diagnostic for asthma (126). Monitoring of PEF has its limitations (41), however, and PEF variability may not be present in cases of mild intermittent asthma. Nonetheless, the degree of PEF variability tends to reflect disease severity, with mild persistent asthma associated with values of 20% to 30% and moderate to severe disease associated with PEF variability greater than 30% (32). In severe chronic disease, PEF variability may be lost. In contrast, brittle asthma is characterized by extreme variability in PEF, with precipitous drops in lung function increasing the risk of severe and life-threatening exacerbations (127). PEF monitoring may help with the diagnosis of occupational asthma (128).

For safety reasons, challenge tests are generally not used in routine clinical practice. Exercise testing may be of value in the diagnosis of EIA. In a standard 6-minute running protocol, falls in FEV_1 or PEF of at least 15% and 20%, respectively, measured 5 to 15 minutes after exercise, are considered diagnostic (32). Measurement of airway responsiveness by histamine or methacholine challenge may help to exclude asthma, but its use is largely confined to research.

In asthma, airflow obstruction occurs as a consequence of airway lumen narrowing. Turbulent airflow generates wheeze, and breathlessness results from increased gas trapping and decreased dynamic lung compliance, increasing the work of breathing. In severe disease, the situation is further complicated by hypoxemia due to ventilation–perfusion mismatch (129). Narrowing of the airway lumen results from a combination of bronchial smooth-muscle constriction, airway-wall thickening, and mucus hypersecretion.

Allergen Challenge Model

After its introduction by Lowell and Schiller in 1948, the inhaled-allergen challenge test became a model for the study of allergen-induced airflow obstruction and allergic airway inflammation. In response to an inhaled dose of allergen, sensitized persons with asthma exhibit a decline in FEV_1 during the first 20 minutes, with

gradual recovery over the following 2 hours. This marks the early asthmatic response (EAR). Approximately half of the persons who have asthma also exhibit a late asthmatic response (LAR), corresponding to a second fall in FEV_1 at 6 hours (Fig. 65.2). Although this usually resolves within 12 hours, an associated increase in BHR may last several days or weeks (130).

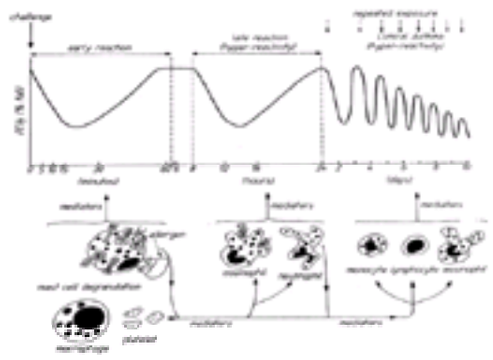

Figure 65.2. Mechanisms of the allergen-produced early and late reactions and the acquired increase in bronchial responsiveness.

Early Asthmatic Response

Inhibition of the EAR by inhaled disodium cromoglycate (131) suggests that this response is primarily mediated by mast cells, resident in the airways. Cross-linking of FceRI by allergen-IgE antibody complex induces mast cell activation (18), resulting in the release of preformed mediators, most notably histamine, tryptase, and chymase (132). Mast cell tryptase liberates secondary mediators, C3a and bradykinin, from precursor proteins. In addition, activated mast cells synthesize and release platelet-activating factor (PAF) and metabolites of arachidonic acid, including LTA_4, LTB_4, and prostanoids (prostaglandin D_2, thromboxane A_2) (133,134). LTA_4 gives rise to the cysteinyl leukotriene LTC_4, which is rapidly metabolized to LTD_4 and then to LTE_4 (19).

Evidence suggests that mast cell–derived cysteinyl leukotrienes are the principal mediators that cause airflow obstruction during the EAR. Cysteinyl leukotrienes induce contraction of isolated human bronchial smooth muscle with 1,000- to 5,000-fold more potency than histamine, and antagonists of the LTD_4/LTE_4 ($CysLT_1$) receptor inhibit the EAR by 50% to 90% (135). Significant reductions in the EAR by antagonists of the H_1-receptor and thromboxane receptor suggest that histamine is the principal mediator during the first 7 minutes of the EAR, whereas prostanoids act synergistically with the cysteinyl leukotrienes during the remainder of the EAR (136). The contributions of PAF, C3a, and bradykinin to early allergen-induced bronchoconstriction appear to be relatively minor, and the role of LTB activity in asthma is questionable (137).

During the EAR, airway mucosal edema and mucus hypersecretion augment airflow obstruction. The cysteinyl leukotrienes, prostanoids, histamine, PAF, C3a, and bradykinin are all vasoactive mediators, increasing capillary permeability and local blood flow and causing plasma exudation and airway wall thickening. In addition, the cysteinyl leukotrienes (138), prostanoids (139), histamine (140), and PAF and chymase (141) stimulate the secretion of mucus by glands in the submucosa. Mucus and exuded plasma not only tend to occlude the airway lumen, but they also may cross-react and increase mucus viscosity (142), thereby promoting mucostasis. Thus, the EAR can be accounted for by allergen-induced mast cell activation and mediator release.

Late Asthmatic Response

In contrast to the EAR, the LAR is associated with increased numbers of activated eosinophils in both the bronchial mucosa (143) and bronchoalveolar lavage (BAL) fluid (144), suggesting an influx of eosinophils from the vascular compartment to the airway lumen. Activated eosinophils release the cysteinyl leukotrienes (145), and CysLT receptor antagonists inhibit the LAR by 25% to 90% (135). These observations suggest that principal mediators causing airflow obstruction during the LAR include eosinophil-derived cysteinyl leukotrienes.

Exercise-induced Asthma

Non-IgE-mediated mast cell activation may also account for EIA, reproduced by hyperventilation. Increasing inspired air humidity reduces the severity of hyperventilation-induced airflow obstruction, whereas changes in inspired air temperature have no effect, provided humidity is kept constant (146). This finding suggests that the stimulus for EIA is airway water loss. It is postulated that, as a consequence, transient hyperosmolarity of the epithelial lining fluid triggers local mast cell activation (147). Observations in support of this hypothesis include hyperosmolar-induced mast cell activation *in vitro* and increased urinary levels of LTE shortly after exercise-induced bronchoconstriction (136). In addition, antagonists of the H_1-receptor and the $CysLT_1$ receptor inhibit exercise-induced bronchoconstriction by 30% to 50% and 60% to 70%, respectively (136). Why certain persons should be susceptible to exercise-induced airway water loss is not clear, but it is suggested that this circumstance may reflect an inability of the airway epithelium to condition-inspired air as minute volume increases (147).

Aspirin-intolerant Asthma

Evidence suggests that the cysteinyl leukotrienes also play a major role in AIA. In activated mast cells and eosinophils, arachidonic acid metabolism may take one of two main pathways. Arachidonic acid may be converted by 5-lipoxygenase (5-LO) to LTA_4 and LTB_4. LTA_4 is then converted by LTC_4 synthase (LTC_4S) to the cysteinyl leukotrienes. Alternatively, prostanoids are generated from arachidonic acid by cyclooxygenase. The expression of LTC_4S by mast cells and eosinophils of persons with AIA is increased fivefold above that of persons with aspirin-tolerant asthma, correlating with increased production of cysteinyl leukotrienes (148). The gene for LTC_4S resides on 5q35, and studies suggest an association between AIA and a polymorphism for the LTC_4S promoter (149). It is postulated that by inhibiting cyclooxygenase, NSAIDs promote metabolism of arachidonic acid via the lipoxygenase pathway, augmenting the production of cysteinyl leukotrienes, especially in cases of AIA with overexpressed LTC_4S. This hypothesis is consistent with observed increases in urinary levels of LTE_4 after NSAID ingestion (150) and inhibition of AIA by $CysLT_1$ receptor antagonists (135).

Bronchial Hyperresponsiveness

Although allergen, exercise, hyperventilation, and aspirin challenge models all have provided some insight into the mechanisms underlying acute airflow obstruction in asthma, epidemiologic evidence suggests that variable and reversible airflow obstruction, characteristic of chronic asthma, occurs in the context of an exaggerated bronchoconstrictor response to nonspecific stimulation, that is, BHR. Postulated models for BHR remain controversial, and mechanisms underlying this disorder of airway function are likely to be multiple and complex, involving neuromuscular, inflammatory, and geometric factors.

The caliber of the airway lumen is dependent on a balance between the force generated by airway smooth muscle (ASM) and the opposing mechanical load from structural elements of the airway wall and surrounding lung parenchyma (151). It is postulated that BHR results from a primary abnormality of asthmatic ASM, giving rise to the generation of excessive force when stimulated. *In vitro* studies, however, failed to demonstrate an intrinsic difference in length–tension properties between asthmatic and nonasthmatic ASM (152). Despite this, ASM hypertrophy and hyperplasia still might be capable of generating excessive force *in vivo* (153,154).

Whereas deep inspiration fully reverses induced bronchoconstriction in persons without asthma, this response is only partial or absent in persons who have asthma, suggesting that resting ASM tone may be abnormally increased in asthma (155). In addition, airway responsiveness can be increased in persons without asthma simply by changing the pattern of breathing and avoiding deep inspiration (156). Thus, BHR may be more a problem of limited ASM relaxation than of exaggerated ASM contraction (156). Either way, it is postulated that BHR results from altered autonomic regulation of ASM tone (157).

Autonomic Regulation of Airway Function

Airway smooth muscle tone is primarily regulated by the parasympathetic nervous system. Cholinergic nerves innervate ASM, and muscarinic receptor activation by acetylcholine induces ASM contraction and bronchoconstriction (157). Airway cholinergic nerves also release cotransmitters, including vasoactive intestinal polypeptide (VIP) and nitric oxide (NO). These provide the inhibitory limb of the nonadrenergic, noncholinergic (i-NANC) nervous system and attenuate cholinergic neural

bronchoconstriction by functionally antagonizing acetylcholine (158). Indeed, the i-NANC nervous system is the only neural bronchodilator pathway known within the human airway because ASM lacks direct sympathetic innervation (157). b_2-adrenergic receptor (b_2-AR) activation by circulating catecholamines or inhaled b2-agonists can induce ASM relaxation and bronchodilation. In addition to regulating ASM tone, parasympathetic nerves also innervate submucosal glands, stimulating mucus secretion (157).

Sensory autonomic nerves relay signals from C-fiber and rapidly adapting receptors (RARs), intimately associated with the airway epithelium (159). These polymodal irritant receptors respond to a range of stimuli, including air pollutants, epithelial lining fluid hyperosmolarity, bradykinin, histamine, PAF, and prostanoids; and they mediate protective vagal reflexes, including cough (159), mucus hypersecretion, and increased ASM tone (bronchial responsiveness) (160). Stimulation of RARs and C-fiber receptors is thought to account for the sensation of chest tightness (157), and neural sensitivity to epithelial lining fluid hyperosmolarity is postulated to be a parallel mechanism for the induction of EIA (147).

The neurotransmitters of sensory C-fibers are tachykinin neuropeptides, including substance P and neurokinin A. In addition to conducting signals to the central nervous system, C-fiber stimulation can also elicit a local axon reflex, releasing substance P and neurokinin A within the airway wall. This provides the excitatory limb of the NANC nervous system (e-NANC) and augments cholinergic neural bronchoconstriction by stimulating tachykinin receptors expressed on ASM (157). Thus, the NANC nervous system appears to modulate parasympathetic function, and it is postulated that increased ASM tone and BHR result from low i-NANC and/or high e-NANC activity (157).

Role of Airway Inflammation

A large body of evidence supports the hypothesis that BHR is a direct consequence of airway inflammation. In the allergen challenge model, increased bronchial responsiveness develops between the EAR and LAR (130) in association with eosinophil recruitment into the airways (143,144). Positive correlations have been reported between the degree of bronchial responsiveness and the numbers of (a) eosinophils in induced sputum (161); (b) activated eosinophils, mast cells, and Th2-memory cells in BAL fluid (162,163 and 164); and (c) activated eosinophils, mast cells, $CD4^+$ T-memory cells, and $CD8^+$ T cells in bronchial biopsies (165,166). In addition, airway inflammation and increased bronchial responsiveness can be induced by inhalation of IL-5 (167), tumor necrosis factor-a (TNF-a) (167), sulfur dioxide (117), nitrogen dioxide (118), ozone (168), toluene diisocyanate (169), and rhinovirus (170). Whereas IL-5 inhalation induces airway eosinophilia, inhalation of TNF-a, ozone, or toluene diisocyanate induces airway neutrophilia.

Increased i-NANC neurotransmitter degradation as a consequence of airway inflammation might account for reduced i-NANC activity and exaggerated cholinergic reflex bronchoconstriction in asthma. Mast cell tryptase degrades VIP, and inflammatory cell-derived reactive oxygen intermediates may increase the clearance of NO. A complete absence of VIP immunoreactivity has been described in asthmatic airways, but no reduction in the i-NANC bronchodilator reflex is observed in persons who have mild asthma (157). Despite this, low i-NANC activity still may play a role in more severe disease.

Normally, tachykinins of the e-NANC system are inactivated by neutral endopeptidase (NEP, CD10), a membrane-bound metallopeptidase predominantly expressed by airway epithelial cells. Increased epithelial damage (171,172) and shedding (173) are characteristic features of asthma, correlating with BHR; and associated loss of NEP is postulated to cause increased e-NANC activity and exaggerated cholinergic reflex bronchoconstriction in asthma (157). Alternatively, viral RTI, oxidant air pollutants, and IL-1b may downregulate airway epithelial NEP expression (157). This hypothesis is supported by the observed inverse relationship between airway epithelial NEP expression and bronchial responsiveness in persons with asthma (174) and increased levels of substance P in BAL fluid and induced sputum from persons with asthma compared with levels in normal controls (157). In addition, increased numbers of substance P-immunoreactive nerves have been observed in asthmatic airways (175).

It is also postulated that increased e-NANC activity mediates so-called *neurogenic inflammation* (157). Animal models have shown that tachykinins can increase capillary permeability and local blood flow, causing plasma exudation. In addition, the e-NANC nervous system may modulate inflammatory cell recruitment and activity (176). The contribution of neurogenic mechanisms to chronic airway inflammation in asthma remains unclear, but the limited evidence available suggests that it is not important in mild asthma (157). Neurogenic inflammation could play a more significant role in severe disease, however, including brittle asthma.

Bronchial hyperresponsiveness might result from increased cholinergic and NANC neural activity resulting from higher levels of RAR and C-fiber receptor activation. This may result from greater exposure to inhaled stimuli as a consequence of airway epithelial damage and tight junction disruption (177). Alternatively, the threshold for activation of these irritant receptors might be reduced by inflammatory mediators, such as PAF (157). The ability of inhaled bradykinin to induce asthma symptoms in asthmatic individuals suggests that this inflammatory mediator may be an important activator of RAR and C-fiber receptors in the disease. Indeed, inhibition of the bronchoconstrictor response to inhaled bradykinin by antagonists of the tachykinin receptor suggests that the actions of bradykinin occur largely via stimulation of cholinergic and NANC reflexes (178).

Models also have been proposed in which airway inflammation induces BHR through mechanisms that do not involve altered autonomic neural function. For example, endothelin-1 is a potent bronchoconstrictor, and its release by airway epithelial cells is increased in association with airway inflammation in asthma (179). Alternatively, airway epithelial damage and tight junction disruption could promote allergen access to mast cells and antigen-presenting cells (APCs) (177), inducing a stronger inflammatory response with the release of greater amounts of cysteinyl leukotrienes, IL-5, and TNF-a. In addition, TNF-a and IL-1b may directly increase the contractile response of ASM (180), and airway edema may unload (mechanically uncouple) ASM, allowing greater contraction for the same degree of stimulation (151).

Geometric Factors

It is postulated that BHR is the consequence of increased airway wall thickness, with a greater reduction in airway caliber occurring for the same degree of ASM shortening (181). In addition, partial occlusion of the airway lumen with mucus and inflammatory exudate could further amplify the bronchoconstrictor response. In cases of fatal asthma, postmortem studies consistently demonstrate prominent thickening of the airway wall (182). High-resolution computed tomography (HRCT) has shown that airway wall thickening also occurs in milder forms of the disease (183,184). In chronic moderate to severe asthma, characterized by a fixed component of airflow obstruction, a positive correlation has been observed between airway wall thickness and the degree of BHR (185). In addition, airway wall thickness has been shown to correlate with the severity of chronic asthma, including a decline in FEV_1 (183). Increased airway wall thickness might therefore account for the associations observed in epidemiologic studies between BHR, reduced FEV_1, asthma chronicity, and disease severity (24). Other studies, however, found no correlation between airway wall thickness and bronchial responsiveness or asthma severity (184).

Despite the large number of studies that support the hypothesis that BHR is a direct consequence of airway inflammation, an almost equal number of reports demonstrated no significant correlation between airway responsiveness and airway inflammation (186). Indeed, BHR has been reported in persons with asthma in the absence of any demonstrable airway inflammation (187). Thus, chronic Th2-mediated airway inflammation alone cannot account for BHR in chronic asthma. Although glucocorticoid therapy tends to normalize airway responsiveness in association with suppressed airway inflammation, complete normalization of airway responsiveness is unusual, suggesting that BHR results from a combination of steroid-responsive and steroid-nonresponsive mechanisms (188). The latter may relate to the noninflammatory component of asthmatic airway wall thickening resulting from the relatively fixed structural changes, termed *airway wall remodeling* (189).

Laboratory Tests

Asthma is associated with blood eosinophilia and elevated total serum IgE, but neither of these is diagnostic. Tests for atopy (skin-prick testing and RAST IgE) also add little to the diagnosis but may be of use in the management of asthma, helping to confirm potential allergenic triggers. A positive skin or RAST test does not necessarily imply that an allergen is a trigger for asthma. The likelihood of this must be judged from the patient's history.

Pathology

Fatal Asthma

Postmortem examination of the lungs of patients who have died of acute severe asthma reveals prominent airway wall thickening, a markedly edematous airway mucosa, and occlusion of bronchial lumen with plugs of viscid mucus (182,190). Microscopic examination reveals vasodilatation and marked inflammatory cell infiltration, extending from the airway lumen to the submucosa (Fig. 65.3). The predominant inflammatory cell types are neutrophils (191) and eosinophils, but lymphocytes are also present. Structural alterations are present throughout the airway wall, including extensive airway epithelial damage, goblet cell hyperplasia, subbasement membrane (SBM) thickening (190), ASM hypertrophy and hyperplasia (153), and submucosal mucus gland hypertrophy (192). These structural changes of airway wall remodeling contribute to the increased thickness of the asthmatic airway wall.

Figure 65.3. Cross-section of peripheral bronchus from a person dying of asthma. The sample was immunostained with a monoclonal antibody to eosinophil cationic protein. Marked eosinophil infiltrate, thickening of the subbasement membrane, and a plug of mucus containing cellular debris filling the airway are present.

Mild to Moderate Asthma

Fiberoptic bronchoscopy allows sampling of the bronchial mucosa in persons with asthma. Limitations of the technique include the small size of biopsy and the restricted number of sites that can be sampled. In addition, the depth of biopsy precludes assessment of ASM and deeper structures and provides no measure of airway wall thickness. Despite these limitations, fiberoptic bronchoscopy has revolutionized research in asthma.

Chronic airway inflammation and airway wall remodeling are consistent features of all forms of asthma. Inflammatory cell infiltration of the bronchial mucosa is characterized by a predominance of activated eosinophils (30), but T-lymphocytes (193) and mast cells (194) are also present. Numbers of activated eosinophils, mast cells, and T cells are also increased in BAL fluid from individuals with chronic stable asthma compared with normal controls (195). Disease severity correlates with the numbers of activated eosinophils and $CD4^+$ T cells in the airway lumen (161,196). In contrast, disease severity does not correlate with the numbers of eosinophils in the bronchial mucosa (166,187,194,197). Furthermore, in chronic severe asthma, eosinophil numbers may be increased only marginally in the bronchial mucosa compared with normal controls (198). This finding suggests rapid eosinophil migration through the bronchial mucosa and airway epithelium and has stimulated research into the eosinophil chemotactic activity of sputum (161). In acute severe exacerbations (199) or severe chronic disease (200), the predominant inflammatory cell type may be the neutrophil, but this may be, at least in part, an effect of high-dose glucocorticoid therapy (201).

Features of airway wall remodeling in asthmatic bronchial biopsies include airway epithelial damage (171,172), SBM thickening (171,202), increased numbers of myofibroblasts (203), neovascularization (204), and new autonomic nerve growth (175). Evidence suggests that airway epithelial damage is a consistent feature of asthma and is characterized by the selective loss of columnar cells, leaving the underlying basal cell layer adherent to the basement membrane (171). This circumstance is consistent with the greater degree of epithelial shedding in asthma, with clusters of desquamated columnar epithelial cells forming creola bodies (173). Until recently, it has not been possible to differentiate confidently artifactual epithelial damage from biopsy forceps and tissue processing from true epithelial damage. Recent findings of upregulated expression of CD44 (205) and the epidermal growth factor receptor (EGFR, c-erbB1) (206) in asthmatic epithelium provide evidence of a response to true injury.

Thickening of SBM is characterized by the deposition of types I, III, and IV collagen, fibronectin, and tenascin into the lamina reticularis (202,207). SBM thickness positively correlates with the number of underlying myofibroblasts (203), and these cells are thought to be the source of deposited collagen. In addition, there is evidence of increased collagen deposition into the submucosa of asthmatic airways compared with airways of normal controls (208). Because of the limited depth of bronchial biopsy, SBM thickness has come to be used as a marker of the remodeling process. Relationships between SBM thickness and other structural changes of airway wall remodeling, including airway wall thickness, remain unclear.

Pathologic observations raise a fundamental question: What is the relationship between chronic airway inflammation and airway wall remodeling? It is postulated that airway wall remodeling occurs as part of the repair response to repeated epithelial injury resulting from chronic airway inflammation and insults from environmental factors, including aeroallergens, oxidant air pollutants, and viruses (209). Furthermore, airway epithelial cells have the potential of signaling to inflammatory cells. This finding led to a model of the asthmatic airway in which the damaged and stressed airway epithelium functions as a key regulator not only of the remodeling process but also of inflammatory cell recruitment and local T-cell responses, driving sustained Th2-immune deviation and disease chronicity (209).

Pathologic observations raise a second fundamental question: What is the consequence of airway wall remodeling on lung function, including airway responsiveness? Indirect evidence suggesting alterations in airway function in association with airway wall remodeling already have been hinted at from previously mentioned HRCT studies. In addition, positive correlations have been reported between SBM thickness and both (a) bronchial responsiveness (210,211) and (b) disease severity and increased decline in FEV_1 (210,212). These observations, however, have not been confirmed in other studies (197,202,203). Thus, the functional significance of SBM thickening and airway wall remodeling in asthma remains controversial, but its importance may be reflected in the observation that SBM thickening occurs in childhood (190), even before the development of asthma symptoms (213).

MANAGEMENT

The aims of asthma management are (a) to achieve and maintain symptom control with minimal (ideally no) short-acting b_2-agonist requirement; (b) to prevent acute exacerbations; (c) to maintain (near) normal lung function, with daily PEF variability below 20%; (d) to maintain normal activities, including exercise; and (e) to avoid adverse drug effects. To achieve these objectives, groups of leading respiratory physicians have published national and international guidelines for the management of chronic and acute asthma in children and adults. Guidelines for the management of chronic asthma are based on a stepwise approach (Fig. 65.4) to drug treatment according to disease severity (32). Six classes of asthma drug therapy are currently available: b_2-agonists, anticholinergics, and methylxanthines are bronchodilators, acting primarily on ASM. In contrast, glucocorticoids, chromones, and leukotriene receptor antagonists are antiinflammatory agents. Recognition that asthma is a chronic inflammatory disorder of the airways has promoted early intervention with antiinflammatory drug therapy at step two. The place for leukotriene receptor antagonists in the stepwise approach to asthma management has yet to be determined.

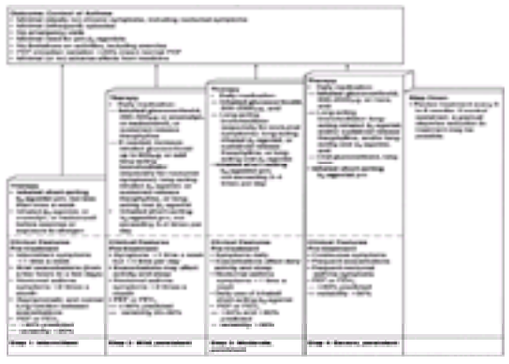

Figure 65.4. Stepwise approach to the treatment of asthma as recommended by the National Heart, Lung, and Blood Institute/World Health Organization (NHLBI/WHO) workshop report. The presence of any one of the features of severity is sufficient to place a patient in that category. Patients should start treatment at the step most appropriate to the initial severity of their condition. Step-up treatment is initiated if control is not achieved at the current step, but first the inhaler technique, compliance, and environmental control (i.e., avoidance of allergens or other trigger factors) should be reviewed. A rescue course of prednisolone may be needed at any time and at any step. Preferred treatments are in bold. (Adapted from *Global Initiative for Asthma (GINA)*. ISBN 91-3042. NHLBI/WHO Workshop Report, 1995, with permission.)

Because asthma is a dynamic condition, with relapses and remissions (Fig. 65.5), drug treatment needs to be stepped up or down as required. Asthma control is achieved most effectively through patient education and guidance, with the development of a partnership in which comanagement is encouraged. This involves a

personalized asthma-management plan (32) and domiciliary PEF monitoring. Objective measurement of airflow obstruction is desirable because subjective perception of asthma severity may be inaccurate. Informed patients then can step up their treatment at the first signs of an exacerbation and seek medical attention in good time. These signs include increased symptom frequency, nocturnal symptoms, a decline in PEF, and increased PEF variability.

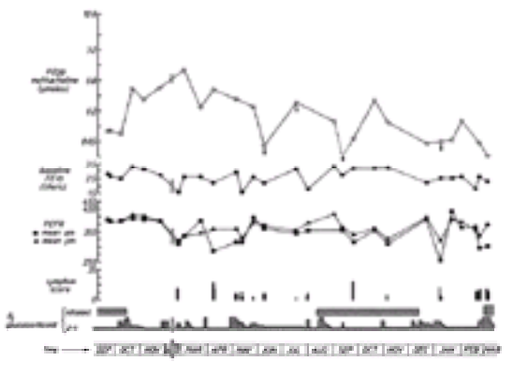

Figure 65.5. One-year diary of symptoms, peak expiratory flow rates (PEFR), forced expiratory volume (FEV), methacholine response (PD_{20}), and treatment in a 16-year-old girl with moderately severe asthma. Although asthma is perceived by the patients as “episodes,” there is evidence for continuous airway dysfunction.

Regular follow-up allows the opportunity to reinforce patient education and guidance, check inhaler technique, monitor lung function, and step down treatment when appropriate. Explaining the rationale behind the various treatment options promotes compliance, especially with inhaled glucocorticoid therapy. Referral to a respiratory physician is indicated when there is diagnostic doubt or difficulty achieving symptom control or maintaining lung function.

Avoidance of Triggers

Identification and avoidance of exacerbating triggers (such as allergens, cigarette smoke, and other air pollutants), drugs (NSAIDs, b-blockers), workplace-related compounds, and psychological stress play an important role in asthma management. Avoiding exercise and cold (dry) air generally imposes inappropriate restrictions, however, and it is often preferable to adjust drug treatment accordingly. Although studies have demonstrated improved symptom control and lung function as well as reduced airway responsiveness resulting from allergen avoidance measures (91,96), a recent metaanalysis failed to find any improvement in lung function in house dust mite–sensitive asthmatic persons (214). This analysis may have overlooked improvements in symptoms and airway responsiveness, however, and it may have included studies in which allergen avoidance was not achieved (215).

Acute Asthma

Acute moderate to severe exacerbations are most commonly the consequence of a rhinovirus RTI (114,115). Although moderate exacerbations (PEF or FEV_1 60%–80% of personal best or predicted) may be managed in the community under close medical supervision, severe exacerbations require hospitalization (Fig. 65.6). Treatment of acute moderate to severe asthma exacerbations involves repeated administration of inhaled b_2-agonist and early introduction of systemic glucocorticoid rescue therapy (216). In acute severe asthma, supplemental oxygen should be given to maintain oxygen saturations above 90% (95% in children). In addition, inhaled anticholinergic (217) and parenteral b_2-agonist (218) therapies should be considered, although the latter may worsen ventilation–perfusion mismatch (219). Intravenous aminophylline provides no additional bronchodilator effect over adequate doses of b_2-agonist (220). It may benefit respiratory drive or respiratory muscle function, however, and prolong or sustain the response to b_2-agonist between doses. Cases of life-threatening asthma should be closely observed in the intensive care unit, where immediate ventilatory support can be provided if necessary.

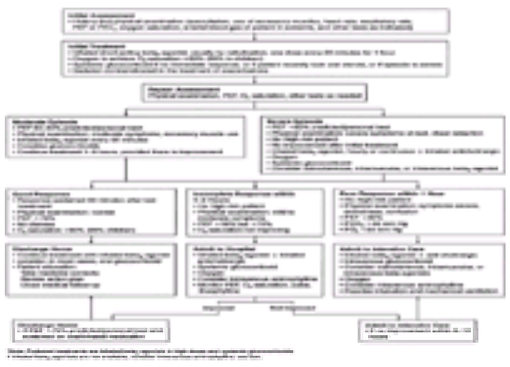

Figure 65.6. Decision tree for managing an exacerbation of asthma in the hospital. (From *Global Initiative for Asthma (GINA)*. ISBN 91-3042. NHLBI/WHO Workshop Report, 1995, with permission.)

Drug Therapies

Glucocorticoids

This class of drug remains the most effective therapy available for asthma. Glucocorticoids reduce symptoms, increase lung function, decrease bronchial responsiveness, and reduce the frequency of acute exacerbations (221,222). In addition, they also can reduce asthma-related mortality (223). The beneficial effects of glucocorticoids are associated with reductions in the numbers of eosinophils and T cells in BAL fluid (224) and of eosinophils, T cells, and mast cells in the bronchial biopsies (225). In addition, glucocorticoid therapy reduces the number of cells expressing mRNA for IL-4 and IL-5 in BAL fluid (224,226) and for IL-4, IL-5, and IL-13 in bronchial biopsies (225,227). In contrast, they increase the number of cells expressing mRNA for IFN-g (225,226) and IL-12 (227) and have no influence on cysteinyl leukotriene production (228).

Glucocorticoids act by binding to the glucocorticoid receptor (GCR), a transcription factor that translocates from the cytoplasm to the nucleus; they bind to specific regulatory DNA sequences, termed *glucocorticoid responsive elements* (GREs), within the promoter regions of target genes (79). Mutual repression of transcriptional activity may occur as a consequence of GCR binding to proinflammatory transcription factors, including nuclear factor-kB (NF-kB), activator protein-1 (AP-1), nuclear factor of activated T cells (NF-AT), and cyclic AMP response element binding protein (CREB) (79). Gene expression is therefore likely to be dependent on the ratio of interacting GCRs and proinflammatory transcription factors present in the nucleus of the cell.

Moderate to severe chronic asthma is characterized by reduced responsiveness to glucocorticoid therapy (229). A spectrum of glucocorticoid responsiveness probably exists, with glucocorticoid-sensitive and glucocorticoid-resistant asthma at opposite ends. True glucocorticoid-resistant asthma, defined as the failure to exhibit significant reversibility in response to high-dose systemic glucocorticoid therapy despite demonstrable reversibility in response to short-acting b_2-agonist, is rare. Instead, most moderate to severe asthmatic persons exhibit relative glucocorticoid resistance, with the term *steroid-dependent asthma* referring to those with severe disease requiring long-term oral glucocorticoid therapy. Glucocorticoid resistance results, at least in part, from a failure of glucocorticoid therapy to suppress Th2-like responses and airway inflammation (224,227). The mechanism that underlies glucocorticoid resistance remains unclear, however (229).

The gene encoding the GCR resides on chromosome 5q31-33, and polymorphisms might play a role in glucocorticoid-resistant disease. One study of six glucocorticoid-sensitive and six glucocorticoid-resistant asthmatic persons, however, found no evidence to support this possibility (230). Reduced GCR binding affinity for glucocorticoids in T cells was reported in a subgroup of glucocorticoid-resistant asthmatic persons and could be induced *in vitro* with a combination of IL-2 and IL-4 (229). In a second subgroup of glucocorticoid-resistant asthmatic persons, GCR binding affinity was normal, but the numbers of glucocorticoid binding sites were reduced (229). Other studies showed glucocorticoid-resistant asthma to be associated with an altered ability of the GCR to bind to GRE (229). Increased expression of

AP-1 in glucocorticoid-resistant asthma led to the hypothesis that excessive stimulation of cells by proinflammatory cytokines induces reduced steroid responsiveness through the interference of GCR function by increased levels of proinflammatory transcription factors (79). Thus, reduced glucocorticoid responsiveness might be expected in cases of more established Th2-mediated airway inflammation. Indeed, it has been observed that delay in the introduction of glucocorticoid therapy for chronic asthma is associated with reduced improvement in lung function and airway responsiveness (231,232). An alternative hypothesis to explain this observation is that early introduction of glucocorticoid therapy prevents the decline in lung function due to airway wall remodeling, secondary to chronic airway inflammation. This hypothesis would be consistent with the finding that glucocorticoid therapy can partially reverse the deposition of collagen, fibronectin, and tenascin into the SBM (207). These observations and hypotheses led to the recommendation that effective antiinflammatory therapy be introduced early in an attempt to prevent or even reverse the development of fixed airflow obstruction. Improvements in lung function and airway responsiveness, however, usually are lost on withdrawal of glucocorticoid treatment (231), and it remains unclear as to whether antiinflammatory therapy simply suppresses asthma or actually modifies its natural history.

Long-term inhaled glucocorticoid therapy (>800 µg daily of beclomethasone or budesonide, and > 400 µg daily of fluticasone) is associated with an increased risk of cataracts, glaucoma, osteoporosis, skin bruising, and adrenal suppression (233). Hence, it is important to prescribe the lowest dose that achieves asthma control. There is no evidence that inhaled glucocorticoid therapy retards growth in children with asthma (233). The use of a spacer device combined with mouth rinsing after glucocorticoid administration may prevent oropharyngeal candidiasis.

Leukotriene Receptor Antagonists

The contributions made by the cysteinyl leukotrienes to many of the features of asthma have been discussed already. Leukotriene receptor antagonists reduce the numbers of T cells, mast cells, and eosinophils in asthmatic bronchial mucosa (234). This class of drug is particularly effective in the treatment of EIA and AIA (135). Clinical trials showed that leukotriene receptor antagonists provide added benefit over glucocorticoid therapy in the treatment of chronic asthma, reflecting the apparent lack of glucocorticoid influence on cysteinyl leukotriene production (135,228). The response to leukotriene receptor antagonists therapy is variable, however, and evidence suggests that this situation may reflect a pharmacogenetic interaction involving 5-LO (235) and LTC_4S (149) promoter polymorphisms. A Churg-Strauss–like syndrome has been reported in association with leukotriene receptor antagonists use, but it probably represents unmasking of this condition as a result of the reduction or cessation of oral glucocorticoid therapy (233).

b_2-Agonists

This class of asthma drug acts primarily on airway smooth muscle, inducing relaxation via activation of the adenylyl cyclase. Although *in vitro* studies suggest that b_2-agonists also have potential antiinflammatory activity (236), there is little evidence of this *in vivo* (237). The gene encoding the b_2-adrenoceptor (b_2-AR) resides on chromosome 5q31-33, and several polymorphisms have been identified (238). Of these, the Gly16 polymorphism has been associated with features of moderate to severe asthma, including nocturnal symptoms, reduced bronchodilator response, and increased airway responsiveness (71). In addition, the Gly16 polymorphism is particularly susceptible to agonist-induced receptor downregulation *in vitro* and may be associated with tachyphylaxis, although this finding is not supported by other clinical studies (71). The Glu27 polymorphism has been associated with childhood asthma and elevated serum IgE levels in asthmatic families; however, the evidence of linkage between b_2-AR polymorphisms and asthma remains controversial (71).

Other Asthma Drugs

Chromones appear to act predominantly by stabilizing mast cells (239) but also may suppress other inflammatory cells, including eosinophils, neutrophils, monocytes, and macrophages (240). Oral theophylline is a relatively weak bronchodilator with a low toxic:therapeutic ratio, requiring monitoring of plasma levels. Like b_2-agonists, there is interest in its antiinflammatory activity, which led to a reappraisal of its role in asthma (241). It is argued that antihistamines, in combination with leukotriene receptor antagonists, are also worthy of reappraisal (136). Methotrexate, cyclosporin, and gold have some glucocorticoid-sparing effect (242), but their use in asthma should be restricted to selected patients who are under the supervision of a respiratory physician.

Specific Immunotherapy

There is uncertainty about the value of specific injection immunotherapy (SIT) in the management of asthma. First, evidence suggests that the role of allergy in the pathogenesis of chronic asthma is less important than once thought. Second, SIT is specific for the allergen injected, and patients with atopic asthma are often multiply sensitized. Third, and most important, patients with asthma are at increased risk of severe adverse reactions with SIT. In a confidential inquiry by the American Academy of Allergy and Immunology into deaths associated with SIT, almost all those who died were being treated for asthma, and the mode of death was catastrophic bronchospasm (243). For these reasons, SIT is not currently recommended for asthma.

PROGNOSIS

Longitudinal studies showed that, of children with established asthma, up to 50% will become asymptomatic by adulthood (54). Factors predisposing to the persistence of childhood asthma into later life include moderate to severe chronic disease, parental history of asthma, strong signs of atopy (including atopic dermatitis or allergic rhinitis), female gender, and active cigarette smoking (54).

Population-based studies suggest that of adults with chronic asthma, 60% have mild, 20% moderate, and 20% severe disease (244). Furthermore, longitudinal studies suggest that 12% of adults with chronic asthma become asymptomatic, with normal lung function, within 25 years (245). Thus, most patients with asthma have a good prognosis. Poor prognostic indicators, associated with an accelerated decline in FEV_1, include moderate to severe chronic disease at presentation, active cigarette smoking, and BHR but not atopy (27). There is no evidence that any of the current asthma therapies can modify the natural history of asthma.

In cases of occupational asthma, more than 50% of patients remain symptomatic or maintain lung function abnormalities despite cessation of exposure to the responsible agent. Interestingly, cessation of exposure is associated with resolution of airway inflammation and reversal of SBM thickening, both in patients who improve clinically and in those who do not (50).

IMMUNOPATHOLOGY

In vivo evidence for Th2-mediated airway inflammation in asthma has come from the investigation of BAL fluid and bronchial biopsies obtained using the fiberoptic bronchoscope. Compared with normal controls, BAL fluid from atopic asthmatic patients contains greater numbers of cells expressing mRNA for IL-3, IL-4, IL-5, and GM-CSF, and these cells are predominantly T-lymphocytes (246). Similarly, bronchial biopsies from atopic asthmatics exhibit increased cellular expression of mRNA for IL-4, IL-5 (247), and IL-13 (34). Once again, these cells are predominantly $CD4^+$ T cells (Th2-like) but also include $CD8^+$ (cytotoxic) T cells (Tc2-like) (35). Unlike the expression of IL-5, that of IL-4 is also increased in individuals who are atopic and nonasthmatic (33,247), suggesting that IL-5 may be associated more closely with the asthma phenotype. As mentioned previously, chronic airway inflammation associated with a Th2/Tc2-deviated cytokine profile is also a feature of nonatopic asthma (33,34 and 35), including the nonatopic occupational form of the disease (248).

Airway Mucosal $CD4^+$ T-cell Activation

Activation of a $CD4^+$ T cell within the airway mucosa requires cognate interaction with a professional APC (249). The principal APC is considered the dendritic cell, which differentiates from a $CD34^+$ bone marrow precursor in the presence of GM-CSF and TNF-a, losing its monocyte marker (CD14) while gaining the dendritic cell marker (CD1a) (250). In the airway mucosa of patients with asthma, dendritic cell numbers are increased but fall in response to glucocorticoid therapy (251). Dendritic cells reside primarily within the airway epithelium, optimally positioned for making contact with inhaled antigen. If antigen does make contact, it is internalized, processed into short peptide sequences, and expressed on the APC surface in conjunction with human leukocyte antigen (HLA) class II (Fig. 65.7). Recognition of this HLA class II-peptide complex by the antigen-specific T-cell receptor (TCR) of a $CD4^+$ T cell provides the first of three signals necessary for maximal activation of the lymphocyte (249). The second, costimulatory signal is not antigen-specific and is generated when CD28, constitutively expressed on the $CD4^+$ T cell, binds to its ligands, CD80 (B7-1) and CD86 (B7-2), expressed on the APC (252). The third signal results from the secretion of IL-1b by the APC, enhancing T-cell responsiveness. Consequently, the activated T-lymphocyte expresses the IL-2 receptor (IL-2R, CD25), proliferates under the autocrine influence of IL-2 (clonal expansion), and produces Th1- or Th2-like cytokines. $CD4^+$ T cells that are activated by dendritic cells may be naive ($CD45RA^+$) or Th-memory cells ($CD45RO^+$). The latter arise from clonal expansion and provide long-term, antigen-specific immunologic memory, generating a more rapid and intense T-helper cell response on subsequent antigen exposure. Evidence suggests that when a naive $CD4^+$ T cell is activated, the Th1- or Th2-like phenotype becomes programmed into the daughter Th-memory cells (21). Although such programming of Th1- or Th2-like responses into long-term immunologic memory appears to be irreversible in mice, this may not be the case in humans (79). The mechanism directing the differentiation of activated airway mucosal $CD4^+$ T cells to a Th2-like phenotype remains a fundamental question in asthma

research, and attempts to answer this question have implicated all three signaling steps in the T-cell activation process.

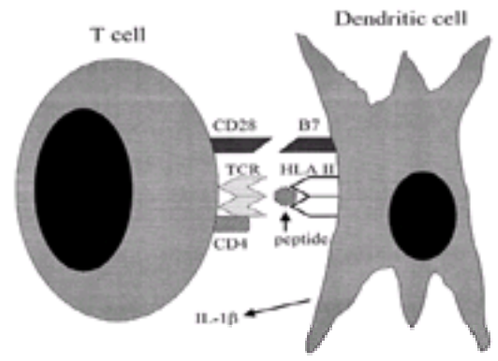

Figure 65.7. Dendritic cell–T cell interactions. Dendritic cells capture allergen and present processed peptide sequences, in conjunction with human leukocyte antigen (HLA) class II, to the T-cell receptor (TCR) of allergen-specific $CD4^+$ T cells. Costimulatory signals required for maximal T-cell activation include CD28/B7 and interleukin-1 (IL-1b).

Both HLA class II and the TCR have been proposed as candidate susceptibility genes for atopy and asthma, although most reported associations between HLA class II or TCR genotypes and the asthmatic phenotype have been controversial (71). Relatively strong associations with HLA class II genes have been observed with occupational asthma and AIA (71).

The role of CD28/B7 costimulation has been investigated with the human bronchial explant model, in which bronchial biopsy specimens from persons with mild atopic asthma and from normal controls were challenged with allergen *in vitro* (253). In biopsies from persons with asthma, transcription and release of IL-5 and IL-13 were augmented by allergen exposure. In contrast, these Th2 cytokines were not detected in normal biopsies. CTLA-4-Ig, a fusion protein that binds to CD80 and CD86, thereby blocking CD28/B7 signaling, almost totally inhibited the allergen-induced secretion of IL-5 and IL-13 in asthmatic biopsies (253). This finding suggests that CD28/B7 costimulation plays an important role in the activation of Th2-like cells by APCs and that this process occurs within the asthmatic bronchial mucosa. Additional studies with blocking antibodies to CD80 or CD86 demonstrated that Th2-like cell activation requires signals from both these costimulatory molecules (254). This finding is consistent with observations in the murine model of asthma, suggesting that CD28/CD86 costimulation is required for the induction of a Th2 phenotype (255), whereas CD28/CD80 costimulation is necessary for T-cell activation (256).

Interest also focused on the influence of the local cytokine milieu on the phenotype of activated $CD4^+$ T cells (79). As well as releasing IL-1b, normal mature dendritic cells also secrete IL-12, a cytokine that promotes the differentiation of naive $CD4^+$ T cells to a Th-1 like phenotype when activated *in vitro* (167). In addition, $CD4^+$ T-cell differentiation also appears to be dependent on the relative balance between Th1 (IFN-g) and Th2 (IL-4 and IL-10) cytokines present in the microenvironment at the time of T-cell activation. Whereas T cells are unable to differentiate into Th2-like cells in the absence of IL-4, IFN-g tends to prevent the development of this phenotype (167). Evidence suggests that IFN-g exerts its effect by inhibiting IL-4-induced downregulation of the IL-12 receptor b-chain, thereby reducing the sensitivity of the $CD4^+$ T cell to the Th1-promoting influence of IL-12 (257). IL-10 facilitates $CD4^+$ T-cell differentiation to a Th2-like phenotype by suppressing the production of IL-12 by APCs (258). The finding that IL-12 can induce the production of IFN-g in human Th2-like-memory cells *in vitro* (259) suggests that this cytokine might also be capable of changing a Th2-like cell to either a Th1-like or a Th0 cell (79), the latter characterized by a mixed cytokine profile that remains associated with the allergic response (260). Thus, programming of Th1- and Th2-like immune responses into long-term immunologic memory may be potentially reversible in humans, being strongly influenced by the local cytokine milieu at the time of $CD4^+$ T-cell activation (Fig. 65.8). Evidence therefore suggests that Th2-like immune responses are more likely to prevail in a microenvironment relatively deficient in IL-12 and IFN-g. This is consistent with the finding that the cellular expression of IL-12 mRNA (227) is decreased in bronchial biopsies from persons with asthma compared with normal controls; IFN-g mRNA is reduced in both groups (247). Finally, the ability of dendritic cells to direct the cytokine response of T cells led to the suggestion that there are two types of dendritic cell, designated DC_1 and DC_2 (261).

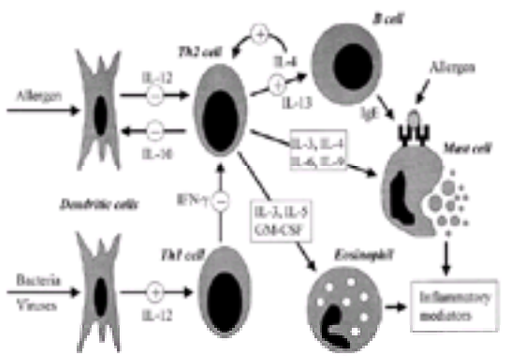

Figure 65.8. Proposed cellular and molecular mechanisms of allergy. Both soluble mediator and cognate interactions between dendritic cells and naive $CD4^+$ T cells direct their differentiation to the T helper cell type 2 (Th2) phenotype. Th2 polarization is effectively suppressed if T cells are driven along the Th1 pathway by interleukin-12 (IL-12) to produce increased interferon-g (IFN-g). It is postulated that exposure to bacteria, and possibly viruses, at a critical time during immune development in early childhood enhances Th1 at the expense of Th2 responses, thereby protecting against allergy. (Redrawn from Holgate ST. *Nature* 402(Suppl): B2–B4, with permission.)

Early Life Origins of Atopy and Asthma

The programming of cytokine responses into immunologic memory is greatly influenced by the maturity of the adaptive immune system at the time of antigen exposure (87). Studies suggest that exposure of the fetus to environmental allergens *in utero* is a normal phenomenon, probably involving the transfer of allergen across the placenta, and that fetal $CD4^+$ T-cell activation with allergen-specific Th-memory cell formation commonly occurs as early as the second trimester of pregnancy (262). Furthermore, neonatal cord blood mononuclear cells (CBMCs) universally exhibit a Th2-like response when challenged with allergen *in vitro* (263). The reason for this is not clear, but it is postulated that a Th1-like response is detrimental to pregnancy, reducing immunologic tolerance between mother and fetus, and that the Th2-like phenotype is the default response of the fetal immune system (99). This, at least in part, may reflect the influence of IL-10 on fetal APCs because IL-10 is produced by the placenta throughout pregnancy and is detectable in amniotic fluid (264).

At birth, the *in vitro* responses of CBMCs from neonates who become atopic in later life differ from those of CBMCs from neonates who do not become atopic. CBMCs in the atopic group not only exhibit a reduced IFN-g response to mitogen (PHA) but also show a weaker Th2-like response to allergen, suggesting that atopic persons have a generalized maturational defect in adaptive immune function at birth (263). During the first year of life, peripheral blood mononuclear cells (PBMCs) in the nonatopic group rapidly downregulate their Th2-like response to allergen but maintain a relatively weak IFN-g response. In contrast, PBMCs in the atopic group exhibit an age-related upregulation of both Th2-like and IFN-g responses, thereby adopting a Th0 phenotype. Between 2 and 5 years of age, further maturation of the Th1-like response occurs in the nonatopic group, corresponding to the upregulation of IFN-g, whereas downregulation of this cytokine occurs in the atopic group (263). Thus, the programming of cytokine responses into long-term immunologic memory appears to depend on the process of immune maturation during early life. To what extent this is influenced by maternal and infant allergen exposure is unclear. It is postulated that by increasing maternal Th2-like cytokine production, maternal atopy has the potential to cause a maturational defect in the adaptive immune system of the fetus, and continued allergen exposure during infancy reinforces a Th2/Th0 response in children so affected (262). This could explain why the inherited risk of atopy is greatest if the mother rather than the father is atopic (265). Furthermore, it is consistent with the finding that levels of IL-10 in amniotic fluid are higher in atopic mothers than in nonatopic controls (262). Studies are under way to assess whether the risk of developing atopy can be reduced through allergen avoidance strategies during the second trimester of pregnancy and the first year of life (266). Studies already showed that allergen avoidance measures taken during the first year of life in young children from atopic parents reduce the prevalence of wheezy illness, but only up to the age of 4 years (267), but these studies did not include allergen avoidance during pregnancy.

Although CBMCs exhibit a universal Th2-like phenotype in response to allergen, the ability of mitogen to induce IFN-g synthesis led to the suggestion and

demonstration that fetal Th2-like responses are, at least in part, the consequence of APC immaturity, characterized by reduced IL-12 production (268). Factors that facilitate postnatal APC maturation and IL-12 production might therefore promote Th1-type responses during infancy, thus reducing the risk of allergen-specific Th2-memory consolidation. The principal environmental stimulus for normal postnatal APC maturation is bacterial exposure (99). Both LPS (269) and components of the cell wall *of M. tuberculosis* (270) activate dendritic cells via the CD14 receptor, thereby inducing the production of IL-12. This mechanism might, at least in part, explain the inverse correlation between childhood infection rates and the prevalence of allergic disease (97,98), providing a mechanistic model in support of the hygiene hypothesis (99). A polymorphism of the CD14 gene, which resides on chromosome 5q31-33, is associated with total serum IgE (271). As mentioned previously, however, the hygiene hypothesis is more closely associated with atopy than with asthma (107), suggesting that other gene–environment interactions are likely to be responsible for localizing Th2-like responses to the airway mucosa. Based on epidemiologic evidence, candidate environmental factors would include aeroallergens, certain viruses, and air pollutants. For example, Martinez postulated that, instead of consolidating airway mucosal Th1-like responses, RSV bronchiolitis in early childhood might promote a Th2-like response in genetically susceptible persons, involving natural killer type 2 (NK2) and Tc2 cells (111).

Isotype Switching to IgE

Antigen-specific IgE production requires cognate interaction between Th2-like memory and B cells (272). The B cell acts as an antigen-specific APC, internalizing antigen recognized by its surface immunoglobulin. Subsequent Th2-like memory cell activation results from HLA class II–peptide complex recognition by the TCR in association with CD28/B7 costimulation. The switching of antibody synthesis from the IgM to IgE class then requires two signals. The first is generated when the costimulatory molecule, CD40, expressed by the B cell on antigen recognition, binds to its ligand, CD40L (CD154), expressed on the Th2-like cell (Fig. 65.9). The second signal results from the secretion of IL-4 and its homolog, IL-13, by the activated Th2-like cell (272). The IL-4 receptor (IL-4R) shares its a-chain (IL-4Ra) with the IL-13 receptor (IL-13R) (273), and activation of either of these receptors on the B cell leads to downstream activation of signal transducer and activator of transcription-6 (STAT-6) (274), initiating the isotype switch to IgE. IL-4 and IL-13, however, also activate different intracellular pathways, explaining why IL-13 cannot support differentiation of T cells to the Th2-like phenotype (275). Candidate genes for asthma and atopy include IL-4 and IL-13 on 5q31-33, and the IL-4Ra on chromosome 16p12.1 (71). Polymorphisms of the IL-4 gene promoter, causing increased IL-4 gene transcription, have been associated with elevated serum IgE levels and asthma, although this finding has not been confirmed by other studies (71). Polymorphisms of the IL-13 gene have been associated with elevated serum IgE levels (276), and polymorphisms of the IL-4Ra gene, giving rise to enhanced activation of STAT-6, have been associated with increased risk of atopy (71).

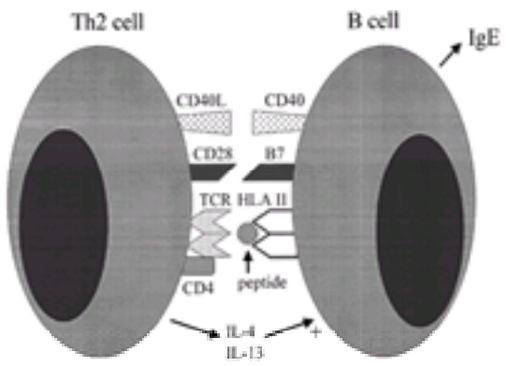

Figure 65.9. T-helper cell type 2 (Th2) cell–B-cell interactions involved in isotype switching to immunoglobulin E (IgE). Allergen-specific B cells can capture, process, and present allergen to Th2-memory cells. Costimulatory signals required by B cells for isotype switching to IgE include CD40/CD40L and interleukin-4 (IL-4)/IL-13.

In vitro studies show that isotype switching to IgE can also occur in the absence of Th2-like cells. Both mast cells and basophils can provide the necessary signals, expressing CD40L and releasing IL-4 in response to the activation of FceRI by IgE–allergen complexes (272). The mast cell–mediated induction of IgE synthesis by B cells requires additional, exogenous IL-4, however (272). In the absence of cognate interaction with allergen-specific Th2-like cells, B cells produce nonspecific, polyclonal IgE in the presence of basophils or mast cells *in vitro*.

When stimulated by IL-4 and IL-13, expression of the low-affinity IgE receptor (FceRII, CD23) is induced in the B cell (277). Activation of CD23 by IgE–allergen complex suppresses IgE production by the B cell, thereby acting as a feedback inhibition mechanism. Not only is Der p 1 an allergen, it also has cysteine protease activity (278) capable of cleaving CD23. Resulting disruption of feedback inhibition might explain, at least in part, why house dust mite allergen is relatively potent at inducing IgE responses in atopic persons (277). Inhibition of IgE production also has been shown to occur with IFN-g through a direct action on B cells (167).

Allergen Challenge Model

As a consequence of the anaphylactic theory of asthma, a great deal of research has been done with the allergen challenge model to elucidate the cellular and molecular mechanisms underlying the airway inflammatory response to aeroallergen exposure. Mast cell activation by allergen–IgE antibody complex already has been discussed, and this section focuses on mechanisms of inflammatory cell recruitment into the airway, characterizing the LAR. Because of associated eosinophilic inflammation and BHR, the LAR has been considered a better model of chronic asthma than the EAR. Although less pronounced, increased numbers of other inflammatory cells are also observed in BAL fluid during the LAR, including activated $CD4^+$ T cells (279), macrophages (280), neutrophils (281), and basophils (163).

The mechanism of inflammatory cell recruitment into the airways involves signals from cell adhesion molecules and chemotactic factors. During the EAR, histamine and the cysteinyl leukotrienes induce the expression of the P-selectin on vascular endothelial cells. The ligands for this cell adhesion molecule are specific oligosaccharides (lectins) expressed by all leukocytes, and interactions between these and P-selectin give rise to rolling and margination of leukocytes along the vascular endothelial wall (282). Activated mast cells also release a number of cytokines, including IL-3, IL-4, IL-5, IL-16, GM-CSF, and TNF-a (134,167). Within 6 hours of acute allergen challenge, TNF-a induces the expression of additional cell adhesion molecules on vascular endothelial cells, including E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (283,284), the last of which is also upregulated by IL-4 (167). The ligands for E-selectin are lectins, and those for ICAM-1 include the integrins, leukocyte functional antigen-1 (LFA-1), and Mac-1 (CD11b/CD18 integrin). Although these ligands are expressed on all leukocytes, IL-5 induced up-regulation of LFA-1 and membrane attack complex-1 (MAC-1) on eosinophils facilitates the selective recruitment of this inflammatory cell (285). In addition, VCAM-1 promotes eosinophilic inflammation because its ligand, very late antigen-4 (VLA-4), is expressed by eosinophils and basophils, but not by neutrophils (286). Furthermore, whereas E-selectin and ICAM-1 are downregulated at 24 hours after allergen challenge, VCAM-1 remains upregulated (287).

Chemotactic factors cause leukocytes adherent to the vascular endothelium to migrate across the vessel wall (*diapedesis*) and bronchial mucosa. The cysteinyl leukotrienes are potent, selective chemoattractants for eosinophils, and they increase eosinophil recruitment into the airway when inhaled by persons who have asthma (288). *In vitro* studies show LTB_4 and PAF to be chemoattractants for eosinophils and neutrophils (289); however, inhaled PAF fails to induce recruitment of eosinophils into the airways of atopic persons, casting doubt on its chemotactic function *in vivo* (290). IL-3, IL-5, and GM-CSF are selectively chemotactic for eosinophils (167). In addition, these cytokines prime eosinophils for activation and prolong their survival (167). IL-16 (lymphocyte chemoattractant factor) is chemotactic for $CD4^+$ T cells and eosinophils, and TNF-a is chemotactic for neutrophils and monocytes (167).

In addition to inhibiting the EAR, disodium cromoglycate also prevents the LAR (131), suggesting that mast cell activation at least initiates the recruitment of inflammatory cells into the airways. The continued recruitment of inflammatory cells probably occurs under the influence of activated Th2-like cells and eosinophils (143). Like mast cells, activated Th2-like cells and eosinophils contribute to the cytokine milieu by releasing IL-3, IL-4, IL-5, GM-CSF, and TNF-a (145,167,246,291). In addition, activated eosinophils release the cysteinyl leukotrienes (145) and IL-16 (167), whereas Th2-like cells are a source of IL-13, capable of upregulating VCAM-1 on vascular endothelial cells, like IL-4 (292). Activated Th2-like cells and eosinophils also release members of the CC chemokine (chemotactic cytokine) family, including RANTES (regulated on activation normal T cell expressed and secreted) and eotaxin (167). Eotaxin is chemotactic for eosinophils and basophils. RANTES is chemotactic for eosinophils and Th-memory cells. Because the receptor for RANTES, CCR3, is expressed by Th2-like but not Th1-like cells, it is postulated that this CC chemokine selectively recruits Th2-memory cells and therefore may play an important role in the target-organ localization of allergic responses (167,293). Allergen challenge is associated with increased BAL fluid levels of IL-4, IL-5, IL-13, IL-16, GM-CSF, RANTES, and eotaxin in atopic persons with asthma (279,294,295 and 296). Inhibition of the eosinophil chemotactic activity of BAL fluid by monoclonal antibodies against RANTES and IL-5 suggests that these two cytokines are the major eosinophil chemoattractants in asthmatic airways (297).

Doubts about the Allergen Challenge Model for Chronic Asthma

In the late 1960s, reported correlation between the inhibitory action of disodium cromoglycate on the EAR (131) and the clinical efficacy of the drug in asthma (298) helped to reinforce the mast cell theory of anaphylaxis and asthma. Consequently, the allergen challenge model became used as a screening test in the development

of novel asthma therapies. During the 1980s, it became increasingly apparent that the ability of a drug to suppress the EAR and LAR did not predict its clinical efficacy (299). For example, although nedocromil sodium inhibits the EAR and LAR, clinical trials have shown it to be no better than placebo in the treatment of chronic asthma (300). In addition, cellular and molecular discrepancies are observed between the LAR and clinical disease. Whereas BAL fluid cell counts in the LAR typically reveal an eosinophilia of 30% to 70%, with associated neutrophils and basophils, BAL fluids from patients with chronic asthma exhibit eosinophil counts of 5% to 8%, with neutrophils an inconsistent feature and basophils rarely seen (299). Furthermore, upregulation of E-selectin, ICAM-1, and VCAM-1 expression on vascular endothelial cells is not observed in chronic stable disease (301). Thus, doubt has been cast on the validity of the allergen challenge model and mast cell hypothesis with respect to chronic asthma.

T-cell Hypothesis

Over the past decade, after the discovery of the Th1/Th2 paradigm, several observations led to, and supported, the T-cell hypothesis of asthma (302). This hypothesis postulates that chronically activated Th2-memory cells, resident in the airways, play a pivotal role in the disease, orchestrating eosinophil recruitment and activation and associated IgE synthesis through the release of Th2-like cytokines. Supporting evidence includes the predominance of chronically activated Th2-memory cells as the major source of IL-4 and IL-5 in bronchial biopsies (193,247) and BAL fluid (303) from atopic and nonatopic (35) asthmatic persons. In addition, positive correlations have been demonstrated between the number of activated Th2-memory cells and (a) the number of eosinophils in bronchial biopsies (304), (b) the number of eosinophils in BAL fluid (303), and (c) the clinical severity of the disease (305). Furthermore, clinical response to glucocorticoid therapy is associated with a reduction in T-cell numbers in the bronchial mucosa (225).

Why Th2-memory cells should become chronically active remains unclear. Persistent low-grade exposure to aeroallergens may be one explanation in cases of atopic asthma. Studies have shown that airway dendritic cells express the a-subunit of the FceRI (306), and IgE-mediated capture and internalization of allergen increase the effectiveness of allergen presentation by 100- to 500-fold (307). b-chain polymorphisms of the FceRI (FceRI-b), which resides on chromosome 11q13, have been associated with atopy, BHR, and asthma; and it has been postulated that certain polymorphisms might enhance FceRI function and mast cell activation (71). No *in vitro* evidence in support of this hypothesis has been shown, however, and other population-based studies have failed to confirm the associations (71).

In nonatopic asthma, IgE-facilitated antigen presentation also may play a role in the induction and maintenance of chronic Th2-memory cell activation, perhaps involving unknown allergens, self-antigens, or persistent low-grade viral infection (308). In clinical trials, however, a humanized anti-IgE monoclonal antibody (rhuMAb-E25), which inhibits both EAR and LAR to acute allergen challenge (309), had only limited efficacy when administered for 12 weeks to patients with moderate-to-severe atopic asthma (310), despite causing marked reductions in serum levels of total and allergen-specific IgE. Thus, although IgE-dependent mechanisms may contribute to chronic Th2-mediated airway inflammation, non-IgE-dependent mechanisms are likely to be operating in parallel. This reiterates the conclusion drawn from epidemiologic data that factors other than atopy are likely to play an important role in asthma pathogenesis.

At 48 hours postactivation, the $CD4^+$ T cell normally expresses CTLA-4 (CD152), which binds to B7 (CD80 and CD86) on the APC. In contrast to the CD28/B7 signal, however, binding of CTLA-4 to B7 generates a negative signal, and it is postulated that this serves to downregulate the T-cell response (293). Studies showed that if the CTLA-4/B7 signal is blocked, Th2 cytokine production is increased, and the period of cytokine production is prolonged (293). Furthermore, some evidence suggests that CTLA-4 expression on T cells is both reduced and delayed in atopic persons compared with normal controls, and this may predispose to prolonged Th2-memory cell activation (293). Other studies suggest that CTLA-4 is also capable of generating a positive signal (311).

T helper-like cells are not the only source of proallergic cytokines (167). The contribution of Tc2-like cells, mast cells, eosinophils, and basophils to the cytokine network already has been mentioned. In addition, the structural cells of the airways are also potential sources of proinflammatory mediators, cytokines, chemokines, adhesion molecules, and growth factors (Table 65.4) (167). Furthermore, as mentioned, it is postulated that the damaged and stressed airway epithelium functions as a key regulator of inflammatory cell recruitment, airway wall remodeling, and local T-cell responses, driving sustained Th2-immune deviation and disease chronicity (209). Thus, the pivotal role of T cells in asthma remains controversial.

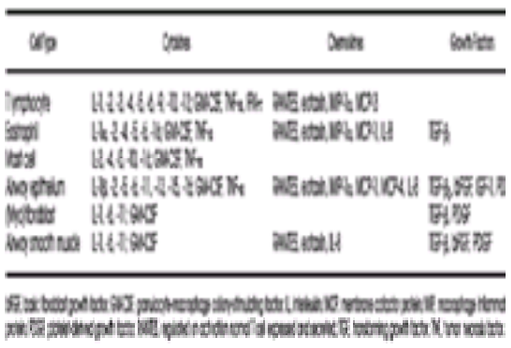

TABLE 65.4. Cell Sources of Cytokines, Chemokines, and Growth Factors

Role of the Airway Epithelium

The airway epithelium can respond to the inhalation of potentially damaging environmental agents by defensively adopting a stress phenotype, which normally functions to initiate the local recruitment of inflammatory cells and prepare the airway to clear the insult and respond to injury. Bronchial biopsies from asthmatic individuals provide evidence of widespread epithelial stress, characterized by the upregulated expression of proinflammatory transcription factors, including NF-kB (312), AP-1 (313), and STAT-1 (314). Target genes for NF-kB include proinflammatory cytokines (IL-1b, IL-6, GM-CSF, TNF-a), CC chemokines [RANTES, eotaxin, membrane cofactor protein (MCP-1), macrophage inflammatory protein (MIP-1)a], CXC chemokines [IL-8, growth-related oncogene (GRO)-a], cell adhesion molecules (ICAM-1, VCAM-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) (315). Target genes for STAT-1 include ICAM-1 (314), and many of those for NF-kB are also shared by AP-1 (316).

Normally, STAT-1 is activated by IFN-g, but levels of this Th1-like cytokine are not increased in asthma (314); however, STAT-1 is also downstream of the epidermal growth factor receptor (EGFR) (317), the expression of which is upregulated throughout the airway epithelium in relation to the severity of chronic disease (206). Because ligand-independent EGFR activation can occur in response to oxidant stress from air pollutants or inflammatory cells, or through transactivation by G-protein-coupled receptors, including protease-activated receptors, it is suggested that the EGFR is likely to play a key role in the induction of epithelial stress responses in asthma (209).

Known activators of NF-kB, many of which also activate AP-1, include Der p 1 (318), rhinovirus (319), proinflammatory cytokines (IL-1k, IL-6, TNF-a), PAF, and oxidant stress (315,316). Although there is potential for positive feedback to occur between epithelial stress responses and inflammatory cell recruitment, these events are normally self-limiting. Epithelial stress and airway inflammation are sustained in asthma, however, and it is postulated that this results, at least in part, from an airway epithelium that is intrinsically more susceptible to stress when exposed to potentially damaging environmental or inflammatory agents (209). This hypothesis is consistent with the *in vitro* observation that airway epithelial cells from persons with asthma produce greater quantities of GM-CSF, RANTES, and IL-8 both at baseline and in response to diesel exhaust particle exposure than cells from normal controls (320).

In addition to promoting the local recruitment of inflammatory cells, the airway epithelium has the potential to modulate T-cell responses, driving Th2-immune deviation. COX-2 catalyzes the production of PGE_2, which inhibits IL-12 production by dendritic cells *in vitro* (321). Airway epithelial cells may directly influence the phenotype of local T cells through the release of IL-5 (322), IL-13 (323), and IL-15 (167).

Airway Wall Remodeling

Although the precise mechanisms that underlie the remodeling process in asthma have yet to be elucidated, several observations support the hypothesis that airway wall remodeling is the consequence of chronic Th2-mediated airway inflammation. *In vitro* studies suggest that inflammatory cells have the potential to induce features of remodeling through the release of inflammatory mediators, cytokines, and proteases. Histamine (324), the cysteinyl leukotrienes (325), IL-1b, TNF-a (167), and mast cell tryptase (326) all can induce ASM proliferation. IL-1b, TNF-a (167), and mast cell tryptase (327) are also mitogens for fibroblasts. In addition, IL-4 (167) and mast cell tryptase (328) act on fibroblasts and upregulate the expression of genes encoding extracellular matrix (ECM) proteins. IL-4 induces the expression of a-smooth muscle actin, thereby promoting the differentiation of fibroblasts into myofibroblasts (329). As a consequence of changes in the protein composition of the ECM, (myo)fibroblasts have the potential to modulate inflammatory and structural cell function through altered cell–matrix signaling (330). For example, fibronectin prolongs

eosinophil survival (331).

Proteases are also likely to play an important role in the remodeling process through their action on ECM proteins. Matrix volume and turnover are dependent on ECM protein deposition and degradation, the latter mediated primarily by matrix metalloproteinases (MMPs). Inflammatory cells are a major source of MMPs, and mast cell tryptase can cleave and activate MMP-3 (stromolysin), which can itself cleave and activate MMP-9 (gelatinase-B) (332). Tissue inhibitors of MMPs (TIMPs) also regulate MMP activity, however, and although levels of MMP-9 are increased in sputum samples from patients with asthma, the MMP-9/TIMP-1 ratio is reduced and correlates with airflow obstruction. This finding led to the suggestion that an imbalance between MMP-9 and TIMP-1 accounts, at least in part, for the increase in ECM volume in asthma (333).

Eosinophils are also a source of transforming growth factor-b (TGF-b), and one study reported a positive correlation between the degree of subepithelial fibrosis and numbers of activated (EG^+) eosinophils expressing TGF-b_1 mRNA in bronchial biopsies from asthmatic individuals (212). Like IL-4, TGF-b can induce (myo)fibroblast proliferation and collagen gene expression (167) and can promote the differentiation of fibroblasts into myofibroblasts (329). In common with a number of other growth factors, TGF-b in its latent form binds to ECM proteins and remains encrypted until its release and activation by proteases, including MMP-9, plasmin, and thrombospondin. Thus, airway inflammation may promote the remodeling process through increased protease activity.

Epithelial–Mesenchymal Trophic Unit

Evidence supports the hypothesis that airway wall remodeling is driven primarily by repeated epithelial injury and repair (209). A three-dimensional coculture system of bronchial epithelial cells and myofibroblasts demonstrated that myofibroblast proliferation and ECM protein deposition can occur in response to paracrine growth factor signals from the repair phenotype of the airway epithelium. The fibroproliferative and profibrogenic growth factors involved include TGF-b_2, basic fibroblast growth factor (bFGF, FGF-2), ET-1, insulin-like growth factor-1 (IGF-1), and platelet-derived growth factor (PDGF) (334). Other *in vitro* studies showed that activated myofibroblasts also release TGF-b and PDGF, in addition to proinflammatory cytokines, and that ASM proliferation can be induced by ET-1, FGF-2, IGF-1, and PDGF (335). This coculture system models the epithelial-mesenchymal trophic unit (EMTU), which consists of the airway epithelium and subepithelial attenuated (myo)fibroblast sheath, separated by the basement membrane zone, and so named because of its pivotal role in branching morphogenesis during fetal lung development (209). This anatomic and functional unit is also thought to play a fundamental role in orchestrating an integrated response toward environmental insults and airway wall injury, with the epithelium as key regulator and (myo)fibroblast as key effector, amplifying and propagating defensive and repair responses throughout the airway wall. In asthma, it is postulated that inappropriate and persistent reactivation of the EMTU drives both chronic airway inflammatory and airway wall remodeling (209).

The phenotype of the asthmatic epithelium appears to be altered, giving rise to unusual fragility and impaired repair responses. This fragility is evident by the tendency of columnar cells to shed more easily than normal (173), suggesting a disturbance of intercellular adhesion mechanisms. This may be the consequence of direct attack on junctional proteins by environmental and inflammatory agents, including Der p 1 (177), major basic protein, eosinophil cationic protein (172), mast cell tryptase, and MMPs (336). Alternatively, increased fragility may reflect the sensitivity of asthmatic epithelial cells to oxidant-induced apoptosis (337). Despite upregulated expression of the EGFR (206), the asthmatic epithelium fails to proliferate as part of the normal repair response in areas of structural damage (338), suggesting suppression of mitogenic signaling by the EGFR (209). These changes in epithelial behavior are thought to account for the extensive epithelial damage in asthma, which is not a feature in other chronic inflammatory disorders of the airway, such as chronic bronchitis or cystic fibrosis. It is therefore suggested that chronic airway inflammation and airway wall remodeling are both the consequence of the asthmatic epithelium being “locked” in a repair/stress phenotype, continually releasing cytokines and growth factors (209).

Because primary asthmatic epithelial cells appear to proliferate as well as normal controls under standard *in vitro* culture conditions, it is suggested that *in vivo* suppression of EGFR-mediated mitogenic signaling in asthma results from antagonistic signals from other factors in the microenvironment. In this respect, interest has focused on TGF-b, which is known to generate antiproliferative signals via the activation of SMAD transcription factors. This hypothesis is consistent with increased levels of TGF-b_1 observed in BAL fluid from patients with asthma, when compared with normal controls (339) and would reflect events that normally occur within the EMTU at branching points during embryonic lung development (209). In view of the apparent importance of TGF-b in models of asthma, promoter polymorphisms for this growth factor have been proposed as candidate susceptibility and disease-modifying genes (340). The role of growth factor signaling in airway wall remodeling remains controversial, however, largely because of inconsistencies in comparative levels between individuals with asthma and normal controls (167).

Summary

Evidence suggests that programming of Th2-like responses into long-term immunologic memory results from multiple gene–environment interactions early in life. Evidence also suggests that determinants within the lung drive chronic Th2-mediated airway inflammation and airway wall remodeling, both of which appear to be necessary for the development of chronic asthma. In recent years, models of the disease had to take into account the recognition that structural cells of the airway wall are potentially important sources of cytokines, chemokines, and growth factors. In particular, attention focused on the airway epithelium, the phenotype of which appears altered in asthma, with increased susceptibility to chronic injury and stress from environmental and inflammatory agents. Persistence of the epithelial stress/repair phenotype might therefore be a key determinant in asthma, driving both inflammatory and remodeling processes. The underlying disturbance of the asthmatic epithelium is also likely to result from multiple gene–environment interactions, probably occurring in parallel with those responsible for the development of Th2-like responses (Fig. 65.10).

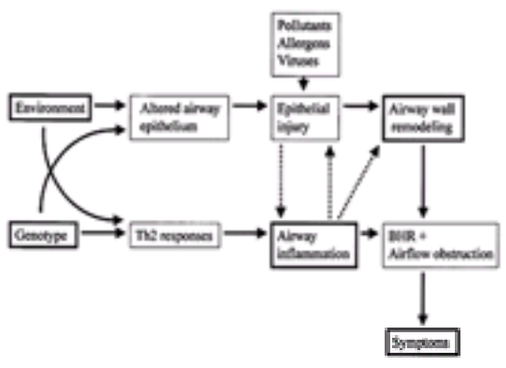

Figure 65.10. An integrated model of asthma. Multiple gene–environment interactions give rise to parallel and interacting processes of chronic T-helper cell type 2 (Th2)-mediated airway inflammation and airway wall remodeling, both of which contribute to BHR, airflow obstruction, and asthma symptoms.

ANIMAL MODELS

Asthma is a disease unique to humans, and animal models fail to simulate its complexity (341). Instead, animal models reproduce allergic airway inflammation, characterized by allergen-specific IgE production, IgE-mediated mast cell activation, eosinophil and T-cell recruitment to the airways, Th2 cytokine (IL-4 and IL-5) expression, airflow obstruction, and BHR. They lack a number of features characteristic of asthma, however, including the persistence of airway inflammation in the absence of allergen exposure and airway wall remodeling. Nevertheless, animal models have made an important contribution to our understanding of the cellular and molecular mechanisms underlying the allergic inflammatory response within the airway mucosa, and they have played an important role in the development of novel asthma therapies. The results of animal studies must be interpreted with caution, however, when attempting to apply them to the human disease. Of the animal models available, that of the mouse is most commonly used.

Although airway eosinophilia can be induced in the murine model after a single allergen provocation, induction of BHR requires repeated provocation (342). This finding suggests that factors other than airway eosinophilia alone are required for the expression of BHR. In the murine model, anti-IL-5 blocking antibodies can inhibit both eosinophilic inflammation and BHR (343), and this effect may last for up to 3 months after a single injection. In clinical trials, a single injection of a blocking anti-IL-5 humanized monoclonal antibody reduced eosinophil levels in blood and sputum for several weeks (344). Although this treatment prevented the recruitment of eosinophils into the airways in response to inhaled allergen challenge, it had no effect on the EAR, LAR, or BHR (344). Thus, the LAR and BHR may occur independently of recruited eosinophils, but eosinophils already resident in the airways at the time of allergen challenge may be involved.

Some murine models do behave in a manner similar to that of humans with asthma when treated with anti-IL-5 antibodies, with reduced eosinophil responses to allergen but no effect on BHR (345). In these mice, however, BHR can be reduced by anti-CD4 antibodies, which deplete $CD4^+$ T cells (345). This finding suggests that BHR is the consequence of mediator release, other than IL-5, from activated $CD4^+$ T cells. Indeed, the adoptive transfer of $CD4^+$ T cells from sensitized to naive mice

can induce BHR (346), supporting the concept of the T cell as key regulator of airway responses to allergen.

In the murine model, anti-IL-4R blocking antibodies inhibit allergen-induced eosinophil recruitment and BHR (347). Decoy soluble IL-4R provides an alternative means of competitively inhibiting IL-4 activity, and early clinical trials using once weekly nebulized soluble IL-4R in subjects with moderate atopic asthma appear promising, with significant improvements in symptom scores and lung function (348).

When administered to mice by nebulizer, IFN-g prevents allergen-induced eosinophil recruitment into the airways by inhibiting Th2 cells (349). Clinical trials that included patients with asthma have proved disappointing, however, with no significant reduction in eosinophilic airway inflammation demonstrated (350). Anti-IFN-g antibodies prevent allergen-induced BHR in the murine model, suggesting an important role played by IFN-g in this process (351). How this relates to clinical asthma is unclear, however.

When inoculated with BCG 14 days before allergen challenge sensitization, the murine model demonstrated reductions in allergen-specific IgE production and allergen-induced eosinophil recruitment and BHR, in association with increased levels of IFN-g (352). Similar results have been observed in mice treated with a single injection of heat-killed *Mycobacterium vaccae* (353). Thus, BCG and *M. vaccae* both appear to be capable of skewing T-cell responses toward the Th1 phenotype, consistent with the hygiene hypothesis. This raises the possibility of a vaccine that might be capable of promoting the maturation of Th1-like responses in early childhood, reducing the risk of atopy and allergic disease in later life. Although currently no published data are available that address the question of primary prevention in humans, there are promising signs that *M. vaccae* might be of use as tertiary prevention for patients with established asthma. When administered a single injection of heat-killed *M. vaccae*, allergen-challenged asthmatic patients demonstrated a significant reduction in the LAR, but not EAR (354). This reduction was associated with a fall in serum IgE level and decreased production of IL-5 by PBMCs in response to allergen, *in vitro* (354).

Other novel therapies have shown promise in the murine model but still must be assessed in humans with asthma. Anti-IL-9 (355) and anti-IL-13 (356) blocking antibodies both prevent allergen-induced eosinophilic inflammation and BHR. IL-10 therapy can dampen Th2 responses, inhibiting the release of IL-5, GM-CSF, TNF-a, and chemokines and reducing the eosinophil response to allergen challenge (357). By promoting Th1 responses, both IL-12 therapy (358) and the anti-CD86 blocking antibody (255) have been shown to prevent allergen-induced Th2 cytokine production, eosinophil recruitment, and BHR.

The likelihood of non-IgE-dependent mechanisms operating in parallel with IgE-dependent mechanisms to promote Th2-mediated airway inflammation already has been mentioned as an explanation for the partial response to anti-IgE monoclonal antibody treatment by patients with asthma. Of relevance is the observation in IgE-deficient mice that allergen provocation still can induce an eosinophil response and BHR (359). The mechanisms that are involved remain unclear, however.

FUTURE DIRECTIONS

Models of asthma have evolved over the past century, with focus shifting from the mast cell and the eosinophil to the T cell, and more recently to the structural cells of the airway wall. Although the past decade has seen advances in our knowledge of asthma pathogenesis, many fundamental questions remain to be answered. The challenge of the new century is to understand how environmental factors interact with the human genome to reveal the asthma phenotype. Elucidating the cellular and molecular pathways involved not only will aid the identification of novel therapeutic targets, but it also should lead to the development of preventive strategies so that the rising prevalence of the disease can be halted and reversed.

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66 URTICARIA AND ANGIOEDEMA: PATHOGENESIS, ASSESSMENT, AND TREATMENT

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Urticaria (hives) and angioedema (swelling) are common disorders that affect 20% of the population at some point during their lifetime. Urticarial lesions itch, have a central white wheal that is elevated, and typically are surrounded by an erythematous halo. The configuration of the lesions can vary; typically, they are rounded and circumscribed but also can be serpiginous or, rarely, more diffuse. Characteristically, hives blanch with pressure, and they generally resolve within 24 hours, leaving no residual change to the skin. Lesions that do not blanch, that cause pigmentation or scarring of the skin, or do not itch should be assessed for other dermatologic processes. The redness is due to dilated blood vessels in superficial layers of the skin, and the wheal is due to leakage of these vessels. Thus, fluid extravasates and compresses the vessels beneath it so that the central area appears clear.

Swelling of deeper layers of the skin, or *angioedema*, commonly accompanies urticaria. This swelling often results from the same inflammatory processes that cause hives. The redness that surrounds superficial lesions is not present, but the swelling can be readily appreciated. Angioedema generally occurs on the extremities and digits as well as on the head, neck, face, and, in men, genitalia. It is often described as painful or burning.

Urticaria alone or with angioedema is more common in women, whereas angioedema without urticaria is more common in men. Most urticarial outbreaks are acute and self-limiting. Fewer than 10% of urticarial eruptions become a chronic process. Acute urticaria is a common problem, and the cause is often elusive; however, its self-limited character limits morbidity. Chronic urticaria and angioedema tend to be recurring and recalcitrant problems, often causing significant disability and interfering with the patient's quality of life. Chronic urticaria is associated with angioedema in as many as 50% of cases. About 10% of cases have only angioedema in the absence of urticaria, and the remaining 40% have only urticaria.

CLASSIFICATION AND CAUSES OF URTICARIA

Although urticaria and angioedema can be classified by several characteristics, the most common classification is based on duration. Episodes that last for 6 weeks or less are considered *acute*, and those that persist beyond 6 weeks are designated *chronic*. This distinction is important because the causes and mechanisms of hive formation are different in each case, as are the prognoses and the approaches to treatment.

Acute Urticaria

Both children and adults commonly experience acute urticaria, which is often caused by an allergic [immunoglobulin E (IgE)-mediated] reaction. This type is a self-limited process that occurs when mast cells in the skin become activated, degranulate, and secrete histamine, leukotrienes, platelet-activating factor (PAF), enzymes such as tryptase and chymase, cytokines, and chemotactic cytokines (chemokines). When an allergen (for example, a food) to which the person is allergic is carried by the bloodstream to mast cells in the skin, it binds to the IgE; the mast cells become activated and degranulate. Allergens that can cause acute urticaria include foods; drugs, particularly antibiotics such as penicillin and sulfa; and venoms from bee, wasp, yellow jacket, hornet, and fire ants. Virtually any allergen that can be disseminated throughout the body, and to which there is an IgE response, has the potential to cause generalized urticaria. When a patient presents with an isolated event of urticaria, the physician must attempt to identify a specific cause or exposure. In a child, typical allergens that cause acute urticaria include medications such as antibiotics or food. The leading food allergens in children are derived from egg, milk, soy, peanut, and wheat. In adults, commonly encountered foods that result in allergic reactions include shellfish, peanut, and tree nuts (walnuts, hazelnuts, pecans). Historical evidence usually reveals that the initial outbreak of hives occurred shortly after ingestion of a particular food and repeated ingestion of that food results in repeated episodes of acute urticaria. One should be suspect of a patient's belief that he or she is allergic to a particular food if it has been ingested on other occasions without typical urticarial or allergic symptoms. When an allergen penetrates the skin locally, hives develop at the site of exposure. Contact urticaria, for example, might occur after exposure to latex from latex gloves if sufficient latex penetrates the skin (see Natural Rubber Latex Allergy, Chapter 73). In general, if an allergic reaction causes hives or swelling, it is usually ingested (food, oral drug) or injected (drugs, stings) and only rarely inhaled. Inhaled allergens typically cause allergic rhinitis and asthma and in children may contribute to atopic dermatitis.

Acute urticaria also can result from “nonspecific” stimulation of mast cells; in this case, IgE on the surface of mast cells usually is not directly involved. For example, mast cells can degranulate in such a fashion during exposure to certain radiocontrast media (RCM), which change the osmolality of the environment in which the mast cell resides. Complement also may be directly activated by these agents, and C5a can cause mast cell degranulation. Patients who develop acute urticarial eruptions can have other accompanying manifestations of allergy such as wheezing, laryngeal edema, cramps, diarrhea, and hypotension, that is, anaphylaxis. Newer low-ionic radiocontrast agents lessened the occurrence of this particular acute urticarial event. Acute viral illnesses in children can be associated with urticarial eruptions that last for a few weeks and then spontaneously subside. The children typically have symptoms of viral rhinitis, pharyngitis, or bronchitis. When such patients are also given an antibiotic, the cause of the hives becomes less clear because a drug reaction becomes an alternative possibility. If penicillin or related antibiotics have been given, it may be worthwhile to do skin testing for penicillin or cephalosporin allergy rather than simply assume that the child is “penicillin allergic.” Although viral infections can cause hives in children, they do not generally do so in adults; however, hives can be caused in any age group by early infection with hepatitis B (1) or infectious mononucleosis (Epstein–Barr virus) (2); and numerous helminthic parasites also are associated with hives. Codeine and other opiate-derived medications can cause mast cell degranulation by stimulation of opiate receptors rather than via IgE. This urticarial eruption is therefore not a specific allergic process, although it is indistinguishable from the urticarial eruption of allergy and is treated similarly. In certain persons, urticaria and angioedema can result from agents that alter the metabolism of arachidonic acid. Therapeutic agents in this category are aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs). These responses to NSAIDs have the potential to be fulminant and life-threatening because the associated angioedema can lead to prominent swelling of the tongue or throat or both. The widespread acceptance of NSAIDs for the treatment of musculoskeletal symptoms and their availability as over-the-counter medications have resulted in many episodes of urticaria and angioedema following their use. Furthermore, aspirin enjoys popularity because of its benefit in preventing heart disease and can cause repetitive urticarial eruptions that resemble chronic hives if taken daily. Over-the-counter preparations must be considered possible causes of urticaria in all patients. The use of angiotensin-converting enzyme (ACE) inhibitors, a class of drugs used to treat hypertension, can result in recurrent episodes of angioedema without urticaria. Examples include captopril, benazepril (Lotensin), enalapril (Vasotec), lisinopril (Zestril, Prinivil), and quinapril (Accupril). The swelling is thought to be due to elevated bradykinin levels because ACE inactivates bradykinin. Thus, the inhibition of ACE leads to the accumulation of bradykinin, which causes dilatation and leaking of vessels in deep layers of the skin.

Chronic Urticaria

Chronic urticaria and angioedema are diagnosed when hives and swelling are present for longer than 6 weeks and symptoms commonly exceed 12 weeks (Fig. 66.1). Moreover, it must be established that this protracted episode of urticaria is not caused by repeated exposure to an allergen or agent that results in recurrent acute urticaria. Although patients with chronic urticaria and angioedema often are assessed for IgE-mediated causes that result in recurring hives, IgE-mediated hypersensitivity is uncommonly found. Food-elimination diets and skin testing to foods, although generally negative, often help to convince the patient and the clinician alike that foods are not contributing to this process. A thorough review of the patient's medications will disclose whether any agents might be causing a chronic urticarial eruption, although this, too, is uncommon. Unnecessary medications should be eliminated, and alternative medications may be substituted for those that are necessary.

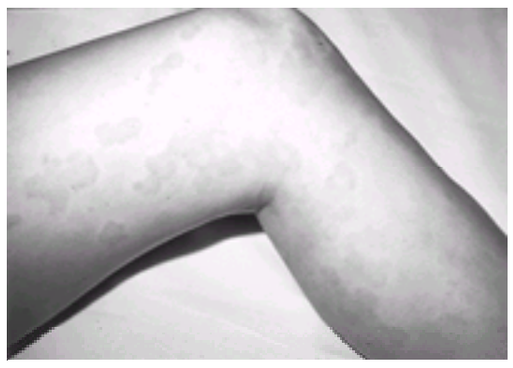

Figure 66.1. Typical lesions of chronic urticaria with serpiginous, elevated lesions borders variable degrees of central clearing. (See Color Figure 66.1.)

Once an evaluation has been completed and the chronic hives appear not to be associated with any other systemic disease process and are not due to one of the physically induced urtcarias (see later sections), the lesions are by exclusion deemed *idiopathic*, that is, of unknown origin. In the past, idiopathic urticaria accounted for more than 95% of all cases of chronic urticaria. New information has improved our understanding of chronic idiopathic urticaria, and the latest research suggests an autoimmune etiology in 35% to 45% of such patients (see later).

Other Modes of Classification

Urticaria also can be divided into two general types on the basis of the rate at which hive formation occurs and the length of time it is evident. One type of urticaria, with lesions that last 1 to 2 hours, typically is encountered with physically induced hives (see next section). The inciting stimulus is present only briefly, and there is prompt mast cell degranulation; biopsy of such lesions reveals little or no cellular infiltrate. The second type is accompanied by a prominent cellular infiltrate, and individual lesions can last from several hours up to 36 hours. This type is encountered with food or drug reactions, delayed pressure urticaria, chronic urticaria, and urticarial vasculitis.

An allergic mechanism, in the strictest sense, requires an interaction of IgE antibody with the allergen (e.g., food or drug) or other mechanisms for cross-linking either IgE or the IgE receptor (3), followed by degranulation of cutaneous mast cells. When an allergic person is skin-tested (e.g., intracutaneous administration of ragweed antigen to a person with ragweed-induced rhinitis), the immediate wheal-and-flare reaction lasts a few minutes and may be followed by swelling 4 to 6 hours later (the late-phase reaction) (4). The late-phase reaction is dependent on the preceding IgE reaction (5,6); it consists of a mixed cellular infiltrate containing mononuclear cells, neutrophils, eosinophils, and basophils (6,7) and is associated with a second wave of secretion of histamine (8) from infiltrating basophils. Cutaneous injection of 48/80, a polypeptide that causes mast cell degranulation, also can lead to late-phase reactions (9). It appears that the late-phase reaction in the skin requires something besides a single burst of mast cell degranulation, but no conclusive data are available. We can theorize that the persistence of mast cell degranulation over time or the persistence of antigenic stimulation over time is the critical difference. This situation would be absent in virtually all the physical urticarias except delayed-pressure urticaria. Perhaps the persistence of the hive in food or drug reactions is related to this phenomenon.

Serum sickness reactions (including the prodrome of hepatitis B infection) can be seen as a manifestation of drug reactions, and on biopsy there is evidence of a small-vessel cutaneous vasculitis (10). Urticaria in association with systemic lupus erythematosus (11,12) or other vasculitides appears similar. Atypical aspects of the gross appearance of the hives should heighten concern that the hives or swelling may be but one manifestation of such a more general disease process. Lesions that do not blanch or are associated with bleeding into the skin or black and blue marks suggest vasculitis (Fig. 66.2). In chronic urticaria, inflammation surrounds the blood vessels, but they are not damaged (Fig. 66.2A). Lesions that result in pigment changes, scarring, or blistering those that persist for longer than 36 hours require a more extensive evaluation, including a skin biopsy. The history, physical examination, and laboratory evaluation should seek evidence of organ involvement other than skin. With a true vasculitis, there is necrosis of the vessel wall (most prominent in small venules), infiltration with neutrophils, and deposition of immunoglobulins and complement. It is thought that these disorders are caused by the following sequential steps: (a) immune complex deposition in the dermal vasculature; (b) release of histamine (and other mediators) from perivenular mast cells as a result of local formation of the anaphylatoxins C3a, C5a, and C4a (13) (IgE antibody to the initiating antigen may also be contributory); (c) chemotactic attraction of cells, particularly neutrophils, to the vessel wall; (d) neutrophil activation and secretion of oxidants, serine proteases (elastase, cathepsins), and metalloproteinases (collagenases and proteoglycanases) to cause cell injury and digestion of connective tissue; and (e) local thrombus formation, activation of fibrinolysis, and formation of “fibrinoid” material by partial digestion of fibrin–fibronectin complex.

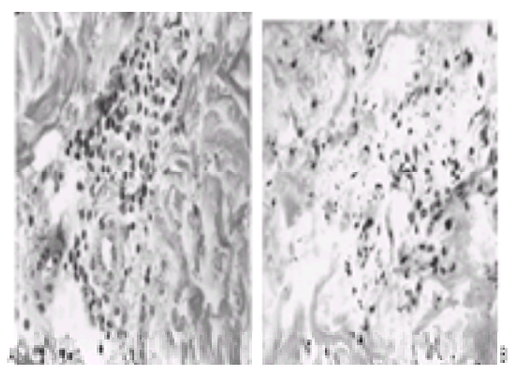

Figure 66.2. Comparison of biopsy specimens from a patient with chronic urticaria and a patient with cutaneous vasculitis. **A:** The vasculitis biopsy has a small venule just to the right of center that is destroyed. There is a predominantly neutrophilic infiltrate with fragmented cells (leucocytoclasis) throughout. **B:** In chronic urticaria, the integrity of the blood vessel wall is maintained, and a nonnecrotizing, predominantly mononuclear cell infiltrate is seen. (See Color Figure 66.2.)

PHYSICAL URTICARIAS

Physically induced hives and swelling share the property of being reproducibly induced by environmental factors such as a change in temperature or by direct stimulation of the skin by pressure, stroking, vibration, or light (14). These serve as investigative models (15) from which we have learned a great deal about the pathogenic mechanisms for hive formation and swelling. A classification of these disorders, which includes virtually all described types, is given in Table 66.1.

Cold-dependent disorders
idiopathic cold urticaria
Cold urticaria associated with abnormal serum proteins: cold agglutinins, cryoglobulin, cryofibrinogen, Donath-Landsteiner antibody
Systemic cold urticaria
Cold-induced cholinergic urticaria
Cold-dependent dermographism
Delayed cold urticaria
Localized cold urticaria
Localized cold reflex urticaria
Exercise-induced disorders
Exercise-induced anaphylaxis (idiopathic or food dependent)
Cholinergic urticaria (cold-dependent variant: cold-induced cholinergic urticaria)
Exercise-induced angioedema
Local heat urticaria
Familial variant
Dermographism
Urticaria pigmentosa–systemic mastocytosis
Cold-dependent variant (cold-dependent dermographism)
Delayed dermographism
Pressure-induced urticaria-angioedema (delayed)
Immediate pressure urticaria
Solar urticaria
Types I–VI
Aquagenic urticaria
Vibratory angioedema
Familial
Sporadic

TABLE 66.1. Classification of Physically Induced Urticaria and Angioedema

Cold-dependent Disorders

Idiopathic cold urticaria is characterized by the rapid onset of pruritus, erythema, and swelling after exposure to a cold stimulus. The location of the swelling is confined to those parts of the body that have been exposed; in this sense, it is a local, rather than a systemic, disorder. Total body exposure, such as that which occurs during swimming, can cause massive release of vasoactive mediators, resulting in hypotension; if the person “passes out,” death by drowning can result. The disease can begin at any age and has no apparent sex predilection. When the disease is suspected, an ice-cube test can be performed to confirm the diagnosis. The ice cube is placed on the patient's forearm for 4 to 5 minutes and then are removed. In a positive reaction, a hive the shape of the ice cube forms within 10 minutes after the cube is removed. The time course of this reaction (i.e., cold challenge followed by hive formation as the area returns to body temperature) demonstrates that a two-step reaction has occurred in which exposure to cold is a prerequisite, but hive formation actually occurs as the temperature increases.

The term *idiopathic* was used to indicate that the cause of cold urticaria is unknown and is unassociated with abnormal circulating plasma proteins such as cryoglobulins. There is evidence, however, that many of these cases are in fact caused by an immunologic reaction. Within this group, most have been shown by passive-transfer studies to be IgE dependent (16). The serum of the patient is injected intradermally into a normal recipient. After 48 hours, the injection site is challenged with an ice cube. Although the incidence of positive transfer has varied among studies, our experience suggests an incidence of about 10%. This figure is undoubtedly an underestimate because the passive transfer is far less sensitive than an ice-cube test in the propositus, and only those with sufficient pathogenic IgE in the circulation (rather than those with IgE bound to mast cells) are detected. In two cases, an IgM antibody was shown to mediate cold urticaria (17). In these cases, passive transfer was positive after a short interval of 3 to 6 hours but was negative at 48 hours, in contrast to an IgE-mediated reaction, which remains positive at 48 hours. We also reported a similar passive transfer (i.e., positive only after short-term sensitization) that seemed to be caused by an IgG antibody (18).

Studies of the pathogenesis of cold urticaria demonstrated release of mediators into the circulation on challenge by placing one hand of the patients in ice water for 5 minutes (Fig. 66.3) and obtaining serial blood samples for 20 minutes thereafter. As with the ice-cube test, swelling is usually not evident while the hand is being chilled; instead, swelling appears between 4 and 8 minutes thereafter and is associated with marked pruritus. In this case, chilling occurs in the deep dermis and subcutaneous tissue in addition to the more superficial skin layers; thus, the entire hand swells, and angioedema results. Studies documented the release of histamine (19), eosinophilotactic peptides (20), high-molecular-weight neutrophil chemotactic factor (NCF) (21), PAF (22), and prostaglandin D_2 (23,24) into the circulation with a time course that parallels the swelling. It is envisioned that chilling initiates a reaction mediated by IgE bound to mast cells and that, on warming, mediators are released into the circulation. When skin biopsy specimens were chilled and then warmed, histamine was released (25) (Fig. 66.4); however, chilling and warming the basophils of patients did not result in histamine release, even in those with documented IgE-mediated disease. Thus, it is unlikely that the disorder is caused by a circulating IgE cryoglobulin (unless the patient's basophils are desensitized). Rather, the presence of cutaneous mast cells seems essential.

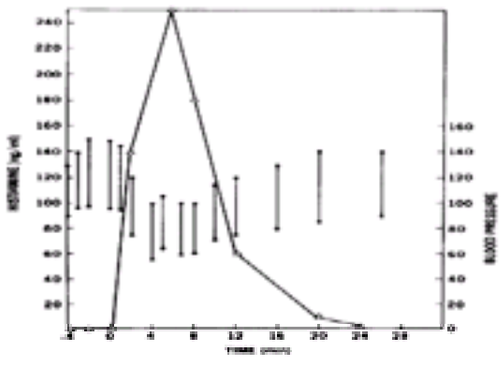

Figure 66.3. Time course of histamine release into the venous circulation of a patient with severe cold urticaria. The patient's hand was placed in ice water for 4 minutes, and arterial blood samples were obtained from the brachial vein draining that arm. Histamine was determined by radioeznyme assay. The vertical lines indicate systolic and diastolic blood pressure throughout the time course; a drop coincides with peak histamine levels.

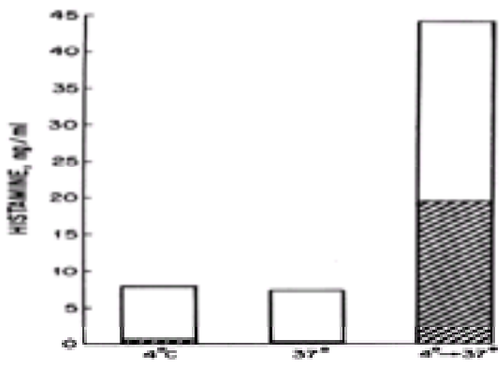

Figure 66.4. Histamine release from biopsy specimens taken from two patients with cold urticaria. Specimens were incubated at either 4°C for 30 minutes

One explanation for such a result is that patients have an IgE autoantibody to a cold-induced skin antigen. Thus, sensitization might occur in the cold, and mediators are released as the cells warm. Studies to test this hypothesis thus far have proved negative. We also found high levels of IgM and IgG antibodies directed against the Fc portion of IgE in patients with cold urticaria (18). Although the clinical significance of such autoantibodies is unclear (26), one such serum caused release of histamine when it was incubated with normal basophils. Because this reaction is demonstrable at 37°C and does not require chilling followed by warming, its relationship to the disease is not clear. Cyproheptadine (Periactin) in divided doses (4–8 mg four times daily) is the drug of choice for the treatment of cold urticaria. Histamine release is unaffected by doses that completely control symptoms (27); therefore, the drug to act as a classic antihistamine (i.e., by blockade of H_1 receptors). Other antihistamines of comparable potency are less effective; the reason is unclear. Although cyproheptadine has antiserotonin activity, serotonin does not appear to be released in cold urticaria. Some patients do not respond well to cyproheptadine, and symptoms do not always correlate well with histamine release (28). Thus, other vasoactive factors may make a significant contribution for some patients. Experimental cromolyn-like drugs, which inhibit mast cell degranulation, are effective in controlling symptoms and suppressing a positive response to the ice-cube test in patients who responded poorly to cyproheptadine (29). Ketotifen, in particular, inhibits histamine release and is effective for a variety of physically induced urticarias (30).

Localized cold urticaria, in which only certain areas of the body urticate with cold contact, has been reported after predisposing conditions such as cold injury as well as at sites of intracutaneous allergen injections, ragweed immunotherapy, and insect bites. We described a patient with cold urticaria confined to the head and face who had no identifiable antecedent or associated event (31). Such cases argue against the presence of a circulating factor and speak in favor of a local abnormality of mast cells.

Cold urticaria also has been described in association with cryoproteins such as cold agglutinins, cryoglobulins, cryofibrinogen, and the Donath–Landsteiner antibody of secondary syphilis (paroxysmal cold hemoglobinuria). The only reported studies that address mechanisms of hive formation are those performed in patients with associated cryoglobulins. The isolated proteins appear to transfer cold sensitivity and activate the complement cascade on incubation *in vitro* with normal plasma (32,33). It is possible that hive formation in these persons is due to cold-dependent anaphylatoxin release. Therapy is directed toward the underlying disease plus the administration of antihistamines. Cryoglobulinemia can be associated with cutaneous vasculitis as well as with cold urticaria, and other associations between these two entities have been reported. Eady and Greaves (34) reported that frequent and repeated cooling of the skin in patients with idiopathic cold urticaria can cause vasculitic lesions. In one patient, immune reactants (IgM and C3) were deposited in the vessels of such lesions (35). In two other patients, leukocytoclastic vasculitis occurred in association with cold urticaria, and circulating immune complexes were evident (36,37). It appeared that the mediator release caused by cold challenge could localize immune complexes to cutaneous sites where they caused vasculitis (36). Sites of typical urticarial vasculitis independent of temperature change were also evident (37).

Other cold-dependent syndromes have been reported, but their incidence is unknown. A delayed form of cold urticaria was described (38) in which swelling appeared 9 to 18 hours after cold exposure. Studies of mediator release were unrevealing, the cold sensitivity could not be passively transferred, and biopsy of a lesion revealed edema and a mononuclear cell infiltrate. Family studies suggested a dominant mode of inheritance. Four patients have been described in whom exercise in a cold

environment induced hives similar to those in cholinergic urticaria; however, hive formation did not occur if exercise was performed in a heated environment. In this disorder, the cold exposure is systemic rather than local, and the disorder should be suspected in any patient whose symptoms suggest either cold urticaria or cholinergic urticaria and in whom standard tests for each disorder are negative (39). Exercise in a cold room or running on a winter day will lead to generalized urticaria and confirm the diagnosis. Because of the visual resemblance of the lesions to those of typical cholinergic urticaria, the disorder has been called *cold-induced cholinergic urticaria*. In a study of 13 patients with symptoms suggestive of cold urticaria and cholinergic urticaria, two patients did not have both disorders but had the cold-induced cholinergic-type hives (40).

In another related disorder, called *systemic cold urticaria*, severe generalized hive formation occurs over covered or uncovered parts of the body after systemic cold challenge. Symptoms are unrelated to exercise or other activities (41), and the ice-cube test is negative. Histamine release on cold challenge (with or without exercise as appropriate) has been seen in cold-induced cholinergic urticaria as well as in systemic cold urticaria. Treatment regimens of hydroxyzine plus cyproheptadine in high doses (42) or of doxepin (43) have been used successfully.

Cold-dependent dermatographism is a disorder in which prominent hive formation occurs if the skin is scratched and then chilled (41). The ice-cube test and systemic cold challenge yield no hives. Scratching the skin yields a weakly positive dermatographic response that is dramatically accentuated when the scratched area is chilled. Treatment is the administration of high-dose antihistamines, for example, 200 mg of diphenhydramine per day or a combination of hydroxyzine (100–200 mg per day) and cyproheptadine (8–16 mg per day).

Finally, a disorder called *localized cold reflex urticaria* has been reported in which the ice-cube test is positive, but hives form in the vicinity of the contact site and not where the cube is applied (44,45). The hives resemble the punctate lesions of cholinergic urticaria, and there is no confluent hive where the ice cube is applied. A methacholine or acetylcholine skin test for cholinergic urticaria is negative, although the symptoms of one such patient resembled cold-induced cholinergic urticaria in that exercise-induced hives appeared in a cold environment (44).

Exercise-induced Disorders

Cholinergic or generalized heat urticaria is characterized by the onset of small punctate wheals surrounded by a prominent erythematous flare associated with exercise, hot showers, sweating, and anxiety (46). Typically, lesions first appear about the neck and upper thorax; when viewed from a distance, hives may not be perceived and the patient appears flushed. Pruritus is a prominent feature of the reaction; on close inspection, it is possible to discern small punctate wheals, sometimes no larger than 1 mm in diameter, which are surrounded by a prominent flare. Gradually, the lesions spread distally to involve the face, back, and extremities, and the wheals increase in size. In some patients, the hives become confluent and resemble angioedema (47). Symptoms of more generalized cholinergic stimulation, such as lacrimation, salivation, and diarrhea, occur occasionally. These stimuli have the common feature of being mediated by cholinergic nerve fibers, which innervate the musculature via parasympathetic neurons and innervate the sweat glands by cholinergic fibers that travel with the sympathetic nerves (48). The characteristic lesion of cholinergic urticaria can be reproduced by intradermal injection of 100 µg of methacholine (Mecholyl) in 0.1 mL of saline. When positive, the resultant localized hive surrounded by satellite lesions is indistinguishable from the patient's spontaneously induced lesions and confirms the diagnosis. We found that only about one third of patients give a clearly positive skin test, and these are generally the most severely affected. Challenge by exercise (e.g., running in a room warmed to 30°C (85°F) or using a bicycle ergometer for 10 to 15 minutes) is a far more sensitive test. Thus, the skin test can be used to confirm the diagnosis but cannot be used as a diagnostic test (19,49). Those patients who have a positive methacholine skin test demonstrate a “hypersensitivity ” to cholinergic mediators, but they have no evidence of an immunoglobulin-mediated allergy to acetylcholine. It is possible that the disorder is due to an intrinsic cellular abnormality that results in abnormal mediator release in the presence of cholinergic agents. One study addressing this issue demonstrated an increased number of muscarinic receptors in urticarial sites. These receptors were further augmented when exercise followed patch testing to copper-containing materials (50). The increased number of acetylcholine binding sites may be an important key to understanding the pathogenesis of cholinergic urticaria. The importance of copper is unclear, but it may affect ligand–receptor affinity.

There is evidence that a reflex consisting of afferent humoral and efferent neurogenic components is involved in this urticarial disorder. If a patient's hand is placed in warm water with a tourniquet tied proximal to that hand, there is no urticaria until the tourniquet is released. A generalized eruption then ensues. Thus, a central perception of a temperature change transmitted via the circulation appears to be followed by an efferent reflex leading to urticaria. Such a reflex could also account for the association of hives with anxiety (51), although the emotional reaction may be completely appropriate. Cholinergic urticaria is the only form of hives in which emotional stimuli can initiate an urticarial reaction in some patients.

Studies of mediator release during attacks of cholinergic urticaria demonstrated that in most cases elevated plasma histamine levels parallel the onset of pruritus and urticaria (19). Subsequent studies confirmed the presence of histaminemia in association with cholinergic urticaria (19,52), and the release of eosinophilotactic peptides and neutrophil chemotactic factor also has been observed (53). When patients were challenged while wearing a plastic occlusive suit to produce maximal changes in cutaneous and core body temperature, significant falls occurred in the 1-second forced-expiratory volumes, maximal midexpiratory flow rate, and specific conductance in association with a rise in residual volume. In four of seven patients, wheezing was detected by auscultation. Thus, under such conditions, an abnormality in pulmonary function can be detected, reflecting either primary pulmonary involvement or altered pulmonary mechanics secondary to circulating mediators. A clinically significant alteration in pulmonary function is unusual in cholinergic urticaria and has no known association with exercise-induced asthma. Kaplan et al. (54) also described two patients with typical cholinergic urticaria in whom lesions became confluent and were associated with prominent elevations of plasma histamine as well as with recurrent episodes of hypotension. Thus, some extreme cases of cholinergic urticaria can resemble the exercise-induced anaphylactic syndrome. An important distinguishing feature is that an increase in core body temperature of greater than 0.7°C with the use of hyperthermic blankets or submersion in warmed water causes hives, histamine release, and anaphylactic symptoms in patients with cholinergic urticaria and anaphylaxis-like symptoms but not in patients with the exercise-induced anaphylactic syndrome (55). Combinations of “physical urticarias” can occur in the same patient, for example, cold urticaria (52) or dermatographism (53) in association with cholinergic urticaria. Furthermore, combined cold and cholinergic urticaria, fulfilling separate criteria for each disorder (40,52), is distinguished from cold-induced cholinergic urticaria (39).

Generally, cholinergic urticaria is treated with hydroxyzine (100–200 mg daily) in divided doses (51). Many, but not all, patients respond to this regimen. Anticholinergic agents such as atropine or propantheline bromide (Pro-Banthine) have little effect, perhaps due to an inability to attain a sufficient systemic level; however, injected atropine can reverse the methacholine skin test (52).

Exercise-induced anaphylaxis was first described in a series of patients who experienced various combinations of pruritus, urticaria, angioedema, wheezing, and hypotension exercise. Symptoms did not occur with each exercise experience, and most of the patients were accomplished athletes (56). The disorder is distinguished from cholinergic urticaria by the following criteria. First, although exercise is the precipitating stimulus of each disorder, hot showers, sweating in the absence of exercise, and anxiety do not trigger attacks of exercise-induced anaphylaxis as they do with cholinergic urticaria (46). Second, the hives in exercise-induced anaphylaxis are large (10–15 mm), in contrast to the punctate lesions characteristic of cholinergic urticaria. Finally, when patients with exercise-induced anaphylaxis were challenged in an occlusive suit, pulmonary function was unchanged, although histamine release was documented (57). Optimal therapy for the exercise-induced anaphylactic syndrome is uncertain, and attempts at prophylaxis with H_1 and H_2 antagonists have not generally been effective (58). In contrast, classic cholinergic urticaria usually responds to prophylactic use of hydroxyzine.

Subtypes of food-related exercise-induced anaphylaxis also have been described. One patient experienced exercise-induced anaphylaxis if the exercise took place 5 to 24 hours after eating shellfish, whereas exercise alone or eating shellfish alone did not produce symptoms (59). In five other patients, two had symptoms if exercise followed the ingestion of any food within 2 hours (59,60); and three had symptoms if exercise followed the specific ingestion of celery within 2 hours (61). The latter patients also had positive skin tests to celery. Treatment of the various forms of food-dependent, exercise-induced anaphylaxis requires avoiding specific foodstuffs before exercising or avoiding exercise within certain time intervals after eating. Kivity et al. (62) performed skin tests to compound 48/80 (a cutaneous mast cell degranulating agent) and to histamine and demonstrated augmented wheal responses to 48/80, but not to histamine, in persons with food-induced (skin-test-positive), exercise-induced anaphylaxis. Food or exercise alone did not affect the response to 48/80, suggesting increased mast cell releasability was caused by the combination of food plus exercise.

Other Physically Induced Forms of Urticaria or Angioedema

The other forms of physically induced hives or swelling are, with the exception of dermographism, relatively rare disorders. They include local heat urticaria, pressure-induced urticaria/angioedema, solar urticaria, aquagenic urticaria, and hereditary vibratory angioedema.

Local Heat Urticaria

A local form of heat urticaria is a rare disorder in which urticaria develops within minutes after exposure to applied heat (63). Sixteen patients with local heat urticaria have been reported, most of whom are female. When suspected, the disorder can be tested for by the application of a test tube of warm water at 44°C to the arm for 4 to 5 minutes. A hive that appears a few minutes after the test tube is removed indicates a positive diagnosis. Mediator release in patients with local heat urticaria resembles that reported for cold urticaria; plasma histamine levels peak 5 to 10 minutes after heat exposure (64,65), although passive transfer studies have been negative. The release of neutrophil chemotactic factor was also identified in one study (64); thus, mast cell degranulation in response to heat challenge seems likely.

Complement abnormalities have been reported in the absence of histamine release (66). Association with other forms of physical allergy sometimes occurs, for example, combined cold urticaria and local heat urticaria (67). Therapy has been problematic, because antihistamines such as hydroxyzine or cyproheptadine as well as oral disodium cromoglycate have been ineffective. One patient was desensitized successfully by repeated daily immersion in hot baths, but caution is advised because systemic reactions are possible (66).

A familial variant of this disorder has been described in which urticaria occurred 1.5 to 2 hours after application of a warm stimulus (68) and persisted for 6 to 10 hours. Sunbathing with pronounced heating of the skin could produce wheals as a result of the temperature effect, whereas sunlight itself could be tolerated. An area could be desensitized with repeated challenge every few days. Skin biopsy specimens showed a pronounced inflammatory cell infiltrate in the upper dermis and around hair follicles. The pathogenesis of this form of local heat urticaria is unknown. Partial control can be achieved with oral antihistamines.

Dermographism

The ability to write on skin with a sharp object, termed *dermographism*, can occur as an isolated disorder that often presents as traumatically induced urticaria. It can be diagnosed by observing the skin after stroking it with a tongue depressor or fingernail. In patients who have this disorder, a white line secondary to reflex vasoconstriction appears, followed by pruritus, erythema, and a linear wheal, as seen in a classic wheal-and-flare reaction. Dermographism is said to be present in 2% to 5% of the population (15,69); however, only a small fraction of these individuals have cases of sufficient severity to warrant treatment. Biopsy of the skin reveals few changes, but most occur in the epidermis and consist of (a) vacuolation of keratinocytes and basal epidermal cell pseudopodia and (b) the appearance of typical mast cell granules (70). In about 50% of cases, passive transfer studies demonstrated an IgE-dependent mechanism (71,72). Thus, many patients have an abnormal circulating IgE that confers a particular form of pressure sensitivity to dermal mast cells. Such observations further suggest that histamine is one of the mediators of dermographism, although the demonstration of such release has been difficult because of the localized nature of the reaction. Early studies suggested the following: that (a) histamine is released into whole blood (73); (b) induced blisters over lesions contain elevated histamine levels (74); (c) 24-hour urine histamine levels are elevated (75); and (d) histamine is increased in the perfusate, as shown by *in vivo* subcutaneous perfusion studies (76). In a single patient with an unusually severe case of IgE-mediated dermographism, plasma histamine levels became elevated within 1 minute of stroking the skin, and the baseline histamine level was abnormal in multiple determinations, suggesting that “leakage” of histamine is continuous (77).

Although a formal study has not been performed, dermographism has been described anecdotally as a consequence of drug reactions (69); in one case, dermatographism could be observed only on challenge with the offending agent—in this instance, penicillin (78). Dermographism is treated with antihistamines (79,80); high doses may be needed for severe symptoms. The initial objective of therapy is to decrease pruritus so that scratching is diminished. Many patients complain of a sensation of itching or “skin crawling” that is readily relieved by antihistamines. At higher doses, the wheal-and-flare reaction to stroking is also markedly diminished.

Dermographism occurs in association with other disorders; for example, a mild form may be seen in some patients with chronic urticaria. Severe dermographism is associated with disorders in which the number of dermal mast cells is markedly increased (e.g., urticaria pigmentosa or systemic mastocytosis). Histamine is released in these disorders (75). It is also possible that other vasoactive agents released from cutaneous mast cells can be implicated in dermatographism and, in fact, in all forms of physically induced urticaria. An example is the increased level of prostaglandin D^2 (PGD^2) metabolites in the urine of patients with systemic mastocytosis, which may contribute to the hypotension associated with this disorder (81).

Pressure-induced Urticaria and Angioedema

Pressure-induced urticaria differs from most of the previously described types of hives or angioedema because symptoms typically occur 4 to 6 hours after pressure has been applied (82). The disorder is clinically heterogeneous. Some patients may complain of swelling secondary to pressure with normal-appearing skin (i.e., no erythema or superficial infiltrating hive); so the term *angioedema* is more appropriate; in others, the manifestation is predominantly urticarial and may or may not be associated with significant swelling. When urticaria is present, an infiltrative lesion characterized by a perivascular mononuclear cell infiltrate and dermal edema similar to those of chronic idiopathic urticaria is seen (83). Immediate dermographism is not present, but delayed dermographism is present and may represent the same disorder (42). Symptoms occur around tight clothing; the hands may swell with activity such as hammering; foot swelling is common after walking; and buttock swelling may be prominent after sitting for a few hours. A diagnostic test can be performed by placing a sling with a 5- to 15-pound weight attached over the forearm or shoulder for 10 to 20 minutes. Devices that apply gradual pressure in grams per square millimeter also can be used (83). Few studies are available regarding pathogenesis; mediators that cause pain rather than pruritus (e.g., kinins) have been considered because the lesions typically are described as burning or painful. Nevertheless, blisters induced over the lesions revealed histamine release following the time course of hive formation (74). Antihistamines, however, have little effect on the disorder, and patients with severe disease often have to be treated with glucocorticoids.

Although pressure urticaria and angioedema can occur as an isolated disorder, it occurs most often in association with chronic urticaria. Therapy usually is directed toward the chronic urticaria. Data suggest an increased incidence of food allergy in patients with chronic urticaria in whom pressure-induced symptoms are also prominent (84).

Immediate-pressure urticaria has been described in patients with the hypereosinophilia syndrome characterized by an acute wheal-and-flare reaction within 1 to 2 minutes of applied pressure (e.g., pressing on the back with one's thumb). Those patients also had dermographism, although those with dermatographism typically do not have immediate-pressure urticaria and require a stroking motion to produce a hive (85).

Solar Urticaria

Solar urticaria is a rare disorder in which brief exposure to light causes the development of urticaria within 1 to 3 minutes. Typically, pruritus occurs first, within about 30 seconds, followed by edema confined to the light-exposed area and surrounded by a prominent erythematous zone caused by an axon reflex. The lesions usually disappear within 1 to 3 hours. When large areas of the body are exposed, systemic symptoms may occur, including hypotension and asthma. Although most reported patients have been in their third and fourth decades of life, the disorder can occur in any age group and has no association with other allergic disorders.

Solar urticaria has been classified into six types, depending on the wavelength of light that induces lesions and the ability or inability to transfer the disorder passively with serum (86,87 and 88). Types I and IV can be passively transferred and may therefore be immunologically (IgE) mediated; they are associated with wavelengths of 280 to 320 nm and 400 to 500 nm, respectively. An antigen has not been identified. Histamine release, mast cell degranulation (89), and formation of chemotactic factors for eosinophils and neutrophil (90) occurred coincident with the induction of lesions when ultraviolet light (type I) was applied to skin. Type VI, indicated by a wavelength of 400 nm, is clearly an inherited metabolic disorder in which protoporphyrin IX acts as a photosensitizer; it is synonymous with erythropoietic protoporphyria. Type VI is caused by ferrochetalase deficiency (91,92). In contrast to other forms of porphyria, the urinary porphyrin excretion is normal; however, red-blood-cell protoporphyrin, fecal protoporphyrin, and coproporphyrin levels are elevated. The irradiation of serum samples from affected persons resulted in activation of the classical complement pathway and generation of C5a chemotactic activity (93); this activity was proportional to the serum level of protoporphyrin (94). Irradiation of the forearms of two such patients also resulted in *in vivo* complement activation as assessed by a diminution of titers of C3 and C5 and generation of C5a (95). Consistent with these observations is the deposition of C3 and the accumulation of neutrophils in the dermis (96,97) and the detection of complement fragments in the serum and suction blister fluid of irradiated skin (96). Type VI responds to oral b-carotene, which absorbs light at the same wavelengths as protoporphyrin IX (98). The mechanism by which urticaria is produced in types II, III, and V is unknown, but these types are induced by inciting wavelengths of 320 to 400, 400 to 500, and 280 to 500 nm, respectively. As a simple screen, fluorescent tubes that emit a broad, continuous spectrum can be used to test the patient, and filters then can be used to define the spectrum that causes urticaria. Therapy of this disease requires avoidance of sunlight, protective garments to cover the skin, and the use of topical preparations to absorb or reflect light. A 5% solution of paraaminobenzoic acid in ethanol, as in sunscreen lotions, can be helpful in the wavelength range of 280 to 320nm; however, it is more difficult to screen out the visible spectrum. The most effective agents for this purpose contain titanium oxide or zinc oxide. The efficacy of antihistamines, antimalarials, and glucocorticoids in these disorders is not clear and needs to be evaluated in each case.

Aquagenic Urticaria

Thirteen patients have been described who developed small wheals after contact with water, regardless of its temperature, and who could be distinguished from patients with cold urticaria or cholinergic urticaria. This disorder has been termed *aquagenic urticaria* (99), and its presence can be tested for by direct application of a compress of tap water or distilled water to the skin. The diagnosis should be reserved for those rare patients who test positively for water but test negatively for all other forms of physical urticaria. Combined cholinergic and aquagenic urticaria has been reported, and histamine release into the circulation has been documented on challenge with water (100).

Hereditary Vibratory Angioedema

Hereditary vibratory angioedema has been described in a single family in whom it was inherited in an autosomal dominant pattern. It is properly viewed as a physically induced angioedema because patients complain of intense pruritus and swelling within minutes after vibratory stimuli (101). The patients do not have dermographism or pressure-induced urticaria. Lesions can be reproduced by gently stimulating the patient's forearm with a laboratory vortex for 4 minutes. Rapid swelling of the entire forearm and a portion of the upper arm ensues, and histamine is released (102). With care, patients can avoid vibratory stimuli, and their symptoms can otherwise be

partially relieved with diphenhydramine (Benadryl). Nonfamilial, sporadic cases also have been described.

CHRONIC IDIOPATHIC URTICARIA AND IDIOPATHIC ANGIOEDEMA

This common disorder of unknown origin occurs in persons who do not have an increased incidence of atopic dermatitis, allergic rhinitis, or asthma compared with the incidence of these disorders in persons without chronic urticaria. Their IgE level, as a group, is within normal limits. Some patients have dermographism, although it is usually of milder degree than occurs with the IgE-dependent dermographism described earlier. The dermographism may wax and wane, just as the urticaria may vary from severe to mild or may intermittently subside. These patients have a normal white blood cell count and erythrocyte sedimentation rate (ESR) and have no evidence of systemic disease. Thus, they do not have evidence of any of the causes of urticaria or angioedema discussed earlier (i.e., foods, drugs, additives, infection, systemic disease, association with other allergic phenomena, or triggering by physical agents). Chronic urticaria does not appear to be an allergic reaction in the classic sense, because IgE antibody is not involved and no external allergen is needed to initiate or perpetuate the process. It differs from allergen-induced skin reactions or from physically induced urticaria (e.g., dermatographism or cold urticaria) in that histologic studies reveal a cellular infiltrate predominantly about small venules (1). External examination reveals infiltrative hives with palpably elevated borders, sometimes varying greatly in size and shape but generally rounded.

The typical lesion of chronic urticaria consists of a nonnecrotizing perivascular mononuclear cell infiltrate (Fig. 66.2A); however, many types of histopathologic processes can occur in the skin and manifest as hives. For example, patients with hypocomplementemia and cutaneous vasculitis can have urticaria (or angioedema); and biopsy specimens from patients with urticaria, arthralgias, myalgias, and an elevated ESR as manifestations of necrotizing venulitis reveal fibrinoid necrosis with a predominant neutrophilic infiltrate (103,104). The incidence of vasculitis in patients with urticaria varies widely (105,106,107 and 108). Mathison et al. (105) found that 10 of 78 patients had hypocomplementemia, and many showed evidence of activation of the classical complement pathway. Six of the ten had elevated levels of circulating immune complexes. If hypocomplementemia and vasculitis are equated, the incidence of presumed vasculitis was 14%. Monroe et al. (106) found neutrophilic leukocytoclastic angiitis in 20% of patients; the other 80% had a perivascular infiltrate of mononuclear cells that was classified as “dense” or “sparse.” The “vasculitis,” “dense,” and “sparse” groups had circulating immune complex levels of 33%, 29%, and 13%, respectively, as measured by multiple assays. Phanuphak et al. (108) used the criterion of significant cellular infiltrate within vessel walls to define vasculitis (rather than that of endothelial damage, nuclear dust, fibrin deposition, or red blood cell extravasation).They found a 52% incidence of vasculitis (various types of predominant cells) and a 48% incidence of a perivascular mononuclear-cell infiltrate. Deposits of immune complexes were found in the skin of 18% of the persons with vasculitis, almost exclusively in those with an abundance of neutrophils.

Our group studied the histopathology of chronic idiopathic urticaria in 43 consecutive patients (107). All but one patient had a nonnecrotizing perivascular infiltrate consisting primarily of lymphocytes. We found necrotizing vasculitis to be a rare cause of urticaria and believe that our group of patients is comparable to the group without vasculitis in the study of Mathison et al. (105), to the “sparse” or “dense” infiltrate groups of Monroe et al. (106), and to the “perivasculitis” group of Phanuphak et al. (108).

We noted a tenfold increase in the number of mast cells and a fourfold increase in the number of mononuclear cells in skin biopsy specimens from the patients with chronic urticaria compared with specimens from unaffected control subjects. The number of basophils was not increased; however, circulating basophils of patients with chronic urticaria are reported to be less responsive to anti-IgE than are circulating basophils of unaffected individuals, suggesting *in vivo* desensitization (109). Although eosinophils were not prominent in the skin biopsies of our series of patients when considered as a group, an occasional patient had a prominent eosinophil accumulation.

In another study, deposits of eosinophil major basic protein were identified in skin specimens of 50% of patients with chronic urticaria, although only a fraction of the specimens had obvious eosinophil infiltration (110). Thus, degranulated eosinophils may be present, perhaps more commonly than previously recognized. The increase in the number of mast cells may account for (a) the increased amount of histamine found in suction blister fluid obtained from patients with chronic urticaria compared with the amount from unaffected control subjects (74) and (b) the increased level of total skin histamine in such patients (111). Immunoperoxidase staining of cell-surface antigens with monoclonal antibodies specific for monocytes, T cells, B cells, and natural killer (NK) cells and histochemical staining of mast cells revealed the infiltrate in chronic urticaria to consist of 50% T-lymphocytes, 20% monocytes, 11% mast cells, and 19% unidentified cells (112). Perhaps a lymphokine or monokine is released that causes mast cell proliferation (or accumulation) and degranulation. A factor [called histamine-releasing factor (HRF)] that can cause basophil degranulation is released from lymphocytes (113,114), including T cells, B cells, monocytes (115), and alveolar macrophages (116) as well as from neutrophils (117) and platelets (118).

We concluded that most chronic urticaria is characterized by a nonnecrotizing perivascular mononuclear cell infiltrate with an accumulation of mast cells. Patients with vasculitis and urticaria are a separate subpopulation in whom the cause and pathogenesis of hive formation probably involves immune complexes, complement activation, anaphylatoxin formation, histamine release, and neutrophil accumulation, activation, and degranulation.

Association with Autoimmune Thyroid Disease

The first studies of autoimmunity in patients with chronic urticaria were by Leznoff et al. (119,120), who reported that patients with chronic urticaria have an increased frequency of Hashimoto thyroiditis. The association was with the presence of antibodies to thyroglobulin or a microsomal-derived antigen (peroxidase), even if the patients were euthyroid. In these initial reports, the incidence of thyroid autoantibodies in patients with chronic urticaria was about 12%. We now routinely check thyroid function and thyroid antibodies in patients with chronic urticaria. The incidence of abnormal thyroid function, either increased or decreased thyroxine (T_4) or increased or decreased thyroid-stimulating hormone (TSH) or both, is 19%. The incidence of finding an elevated level of antithyroglobulin antibody is 8%, an antimicrosomal antibody is 5%, and the coincidence of elevated titers of both antibodies is 14%, for a total incidence of 27%. There are no data to suggest that either of these antibodies is pathogenic in terms of hive formation, and we believe these are associated, parallel, autoimmune events. A report (121) suggested that there is a positive effect on the severity of the urticaria if the dose of thyroid hormone is raised somewhat (i.e., to high normal levels but not overtly hyperthyroid). We do not recommend thyroid supplementation beyond that required to achieve the euthyroid state. A significantly elevated T_4 or depressed TSH need not be indicative of the presence of Graves disease or hyperfunctioning nodules but rather may reflect the early stage of Hashimoto thyroiditis with liberation of thyroxine before thyroglobulin stores are depleted. Eventually, all such patients are at risk of becoming hypothyroid, with low T_4 and elevated TSH levels.

Antibodies to IgE or IgE Receptor

Gruber et al. (122) first reported an increased incidence of IgG antibodies to IgE in patients with chronic urticaria. The incidence was 10%, and about half of these were shown to be functional by basophil degranulation. We were able to stimulate basophil degranulation and histamine release by cross-linking cell-surface IgE with a patient's serum. Although an occasional patient with cold urticaria had demonstrable IgM or IgG anti-IgE antibody, those with other forms of urticaria did not. Such antibodies were not specific for chronic urticaria, however, and were detected in patients with atopic dermatitis. The next important observation was made by Grattan et al. (123), who found that a substantial number of patients with chronic urticaria have a positive autologous skin test (123); that is, if serum of the patient is used as a skin-testing reagent in that patient, a significant wheal and flare reaction occurs. Most members of subpopulation of positive patients studied in much greater detail were found to have an IgG antibody directed to the a subunit of the IgE receptor (124,125) (Fig. 66.5). By using rat basophil leukemia cells transfected with the human a subunit, the release of histamine or the enzyme b-hexosaminidase could be demonstrated (124,126). Thus, it appeared that the antibody could cross-link the IgE receptor (a subunit) and degranulate the cells. The incidence of such antibodies is about 35%, and an additional 5% to 10% of patients have anti-IgE antibodies rather than anti-IgE receptor antibody. Activation of basophils *in vivo* may relate to the basopenia reported in patients with chronic urticaria (127,128,129 and 130).

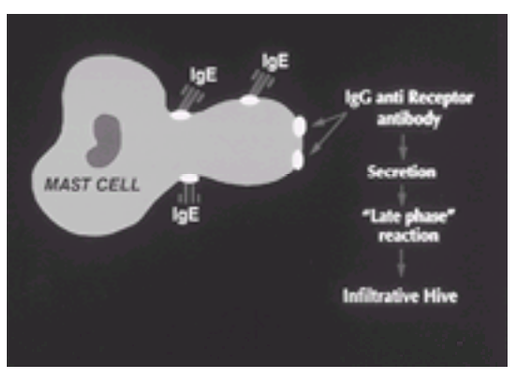

Figure 66.5. Diagrammatic representation of cutaneous mast cell activation by immunoglobulin G (IgG) antibody to the IgE receptor. (See Color Figure 66.5.)

Immunoblot Analysis

Fiebiger et al. (131) and Tong et al. (126) first used immunoblot analysis to demonstrate anti-IgE receptor antibodies, which were shown to bind to the 34-kd cloned a subunit. A Western blot depicting the presence of antibody in a serum at 1:500 and 1:1,000 dilutions is shown in Fig. 66.6. We found that normal sera tested at a 1:50 dilution or less are often positive; thus, it is common to have a low titer of this antibody present. Nevertheless, at a 1:500 dilution, normal sera are positive only rarely, whereas the sera of patients with chronic urticaria have an incidence of 45% positive (132). A second concern is the observation that positives can be obtained in patients with connective tissue disorders, particularly dermatomyositis, bullous pemphigus, systemic lupus erythematosus, and bullous pemphigoid (133); however, none of these sera released histamine from human basophils. This fact was attributed, in part, to a skewing of the antibody population to the IgG_1 and IgG_3 subclasses in chronic urticaria and to the IgG_2 and IgG_4 subclasses in the other disorders. These data also suggested that activation of basophils might require complement (133), C5a in particular, and that cross-linking of the IgE receptor may not explain cell activation.

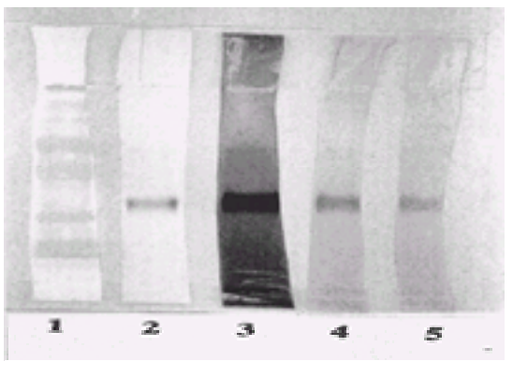

Figure 66.6. Immunoblot analysis to ascertain the presence of immunoglobulin G (IgG) antibody to the a subunit of the IgE receptor Fc_eRIa. Soluble recombinant Fc_eRIa was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto nitrocellulose. Membranes were inoculated with monoclonal antibody or whole sera from patients with chronic urticaria or unaffected control subjects. Antibody binding was detected with alkaline phosphatase-conjugated goat antimouse IgG antibody or goat antihuman IgG fragment-specific antibody, respectively. Lane 1, molecular marker; lane 2, mouse monoclonal antibody (positive control); lanes 3–5, serum at dilutions of 1:50 (lane 3), 1:500 (lane 4), and 1:1,000 (lane 5).

Another important variable in these assays is the donor basophils used for functional analysis. Basophils saturated with IgE (using an IgE myeloma protein) are best for the demonstration of activation by anti-IgE, whereas basophils stripped of IgE by treatment with lactate buffer are used to assay antireceptor antibodies. With unaltered basophils, the level of responsiveness to antireceptor antibodies may depend on the percentage of receptors occupied by IgE. Zweiman et al. (134) showed that the addition of exogenous IgE inhibits the detection of antireceptor antibodies, and we have abolished the responsiveness of blood basophils and cutaneous mast cells by saturating IgE receptors with an IgE myeloma protein (unpublished observations).

Activation of Mast Cells: A Role for Complement

Nimi et al. (135) were first to demonstrate that antibodies to the IgE receptor activate cutaneous mast cells. This finding is important because the mast cell is a critical effector cell in chronic urticaria, whereas the role of the infiltrating blood basophil, if any, is unclear. We observed that the portion of cutaneous mast cells activated by sera from patients with chronic urticaria approaches 50% and correlates strongly with the activation of basophils and the results of immunoblot analysis, although there are occasional sera for which the functional assay is positive and the immunoblot is negative or vice versa. When we compared whole sera with IgG isolated from those sera, the purified IgG was incapable of activating cutaneous mast cells. When IgG isolated from patient sera was added to pooled normal sera, however, the normal serum was capable of activating mast cells; the addition of the IgG to either C2-deficient plasma or C5-deficient plasma was ineffective (Fig. 66.7). These data indicate a role for the classical complement pathway in cutaneous mast cell activation, and C5a is the likely effector. Attempts to inhibit mast cell activation with commercially available peptides that compete with C5a for binding to the C5a receptor were equivocal, in part because the competitors had agonist activity. On a molar basis, C5a is superior to C3a as an activator of cutaneous mast cells, and C3a is rapidly degraded to an inactive peptide (C3a des-arg) by a plasma carboxypeptidase; the analogous peptide, C5a des-arg, retains activity that is augmented by vitamin D–binding protein (C5a cochemotaxin) (136,137). Although C5a is a potent chemotactic factor, cellular infiltration secondary to injected peptide is largely mast cell dependent (138).

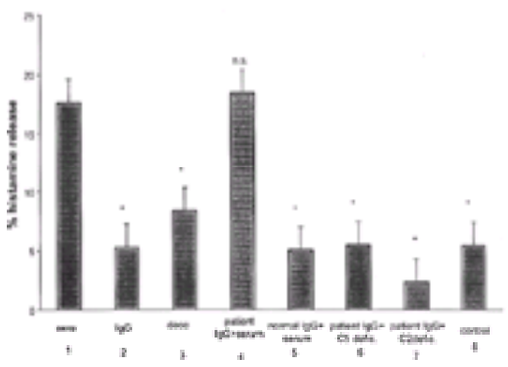

Figure 66.7. Complement dependence of histamine release; 75-µL samples of a cell suspension containing 2×10^5 mast cells were incubated for 20 minutes at 37°C with one of the following: 75 µL of patient serum (1), 1 to 10 mg/mL of protein G-eluted IgG (2), 75 µL of decomplemented patient serum (for 1 hour at 56°C) (3), 15 µL of purified patient IgG plus 60 µL of pooled normal serum (4), 15 µL of purified patient IgG plus 60 µL of pooled normal serum (5), 15 µL of purified patient IgG plus 60 µL of C5-deficient serum (6) or C2-deficient serum (7), or 75 µL of buffer (8). Results are expressed as a percentage of histamine release from two cell suspension replicates. Cells were boiled to determine total histamine content. Significant differences ($p < 0.05$, as determined by the Student *t*test) for paired samples, each of which was compared with the histamine release for patient sera (far left bar), are indicated by asterisks. Each bar represents an average of histamine release by using five different patient sera, each of which was assayed five times with duplicates ($n = 50$)

The cellular infiltration with T cells and monocytes described above appears to be critical because chronic urticaria responds rapidly to glucocorticoids and steroids have little effect on the degranulation of cutaneous mast cells. Thus, like late-phase allergic reactions, the perpetuation of the inflammatory response by cellular infiltration is critical to disease manifestations, and degranulation of mast cells alone, regardless of the mechanism, is insufficient to explain the clinical course. Thus, antihistamines, although of value in the treatment of chronic urticaria, are often not fully effective. About 40% to 50% of patients with chronic urticaria often have associated angioedema, commonly facial. Thus, when maximal $H_{1/>}$ or H_2 receptor blockade has been achieved, we advocate alternate day steroids as the most effective treatment (139). In general we administer 20 mg of prednisone in a single morning dose on alternate days and decrease it by 2.5 to 5.0 mg every 3 weeks, depending on the patient's response. The goal is a slow, gradual decline in dosage toward zero. Antileuleotriene agents may also be helpful. Experimental approaches that have a rationale, given the pathogenic mechanism described here, include intravenous gamma globulin, plasmapheresis, and immunosuppression with cyclosporine. Some preliminary reports using these approaches have appeared (140,141 and 142), but I believe these approaches should be used in research protocols or reserved for the small percentage of patients who do not respond to glucocorticoids or who require prohibitive dosages to achieve control. In general, NSAIDS, hydroxychloroquine sulfate (Plaquenil), sulfasalazine, colchicine, and dapsone have been ineffective. One exception is the hypocomplementemic urticarial vasculitis syndrome (143) associated with autoantibodies to C1q (144), which responds to Plaquenil (145).

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67 Atopic Dermatitis

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Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease commonly associated with respiratory allergy (1,2). It is the most common chronic skin disease of young children, affecting 5% to 20% of children in most countries (3). Similar to the trends seen with asthma, the prevalence of AD has increased, especially in Western countries. Poorly controlled AD is often associated with sleep deprivation, school absenteeism, occupational disability, and emotional stress and has a significant impact on the quality of life of patients and their families. AD also may be associated with significant morbidity, especially when complicated by erythroderma or concomitant infection.

The term *atopic dermatitis* was first introduced in the 1930s to describe both the weeping eczema of infancy and childhood and the chronic xerosis and lichenified lesions more typical of older patients (4). This term also recognized the close relationship between AD, asthma, and allergic rhinitis. Acute AD, like asthma and allergic rhinitis, is associated with the local infiltration of T cells with a T helper 2 (Th2) cytokine profile (5). In addition, the eosinophil is a common effector cell in chronic AD and the allergic respiratory diseases. Furthermore, patients with AD can have nonspecific, as well as specific triggers, similar to patients with asthma and allergic rhinitis. A recent review suggested that the immune mechanisms underlying AD and asthma have more similarities than differences (6). In more than 50% of patients with AD, asthma and allergic rhinitis develop (7), and thus AD is an important early marker of the atopic diathesis.

EPIDEMIOLOGY

Genetics

Although genetic susceptibility to respiratory allergy has been suggested by localization of a locus for atopy on chromosome 11q13 (8), linkage to this gene had previously not been demonstrated in patients with AD (9). More recently, however, linkage of both AD and asthma to polymorphisms within the gene for the b subunit of the high-affinity immunoglobulin E (IgE) receptor on chromosome 11q12-13 has been reported but must be substantiated (10). By contrast, there has been particular interest in the role of chromosome 5q31-33 in AD, as it contains a clustered family of cytokine genes [interleukin (IL)-3, IL-4, IL-5, IL-13, and granulocyte/macrophage colony-stimulating factor (GM-CSF)] expressed by Th2 cells (11). Chan et al. (12) found that abnormal IL-4 gene expression in AD may be linked to alterations in nuclear protein interactions with IL-4 promoter elements. A study examining linkage between markers at and near the IL-4 gene and AD in 88 Japanese nuclear families found a genotypic association between the T allele of the –590 C/T polymorphism of the IL-4 gene and AD (13). Because the T allele was reported to be associated with increased IL-4 gene promoter activity compared with the C allele, the data suggest that genetic differences in transcriptional activity of the IL-4 gene may influence predisposition for AD, particularly in this population, because of the high frequency of the T allele. In addition, an association of atopy with a gain-of-function mutation in the a subunit of the IL-4 receptor has been reported in a small group of patients with AD (14). The authors speculated that the R576 allele may predispose persons to allergic diseases by altering the signaling function of the receptor. Finally, Mao et al. (15) demonstrated a significant association between a specific polymorphism in the mast cell chymase gene and AD, but no association with asthma or allergic rhinitis. This finding may reflect the population studied and requires confirmation; but it suggests that a genetic variant of mast cell chymase, which is a serine protease secreted by skin mast cells, may have an organ-specific effect and contribute to the genetic risk for AD.

Although the mode of transmission remains uncertain, limited studies support an autosomal dominant inheritance pattern. Uehara and Kimura (16) found that 60% of adults with AD had children with AD. The prevalence of AD in children was 81% when both parents had AD, 59% when one parent had AD and the other had respiratory allergy, and 56% when one parent had AD and the other had neither AD nor respiratory allergy. Diepgen and Fartasch (17) showed that 42% of first-degree relatives of patients with AD also had AD, whereas 28% had respiratory allergy. In contrast, only 12% of first-degree relatives of persons with respiratory allergy without skin disease had AD, whereas 43% had respiratory allergy. In a follow-up study of the familial aggregation of AD, allergic rhinitis, and allergic asthma in the relatives of 426 patients with AD and 628 persons with no history of AD, the odds ratio of familial aggregation for AD was 2.16 if no distinction was made between the degree of relationship, 3.86 among siblings, and 1.90 between parents and siblings (18). For AD, the odds ratio differed between mother/sibling pairs (2.66) and father/sibling pairs (1.29), suggesting environmental events affecting the fetus *in utero* or shared environmental exposures. The fact that all of the atopic diseases demonstrated a stronger correlation between siblings than between siblings and parents supports the hypothesis that exposure to environmental factors during childhood is responsible for the recently observed increased prevalence of atopic diseases.

Environmental Factors

Studies indicate an increasing prevalence of AD. Schultz Larsen (19) demonstrated a cumulative incidence rate up to 7 years of 12% for twins born between 1975 and 1979 compared with a rate of 3% for twins born between 1960 and 1964. A cross-sectional questionnaire study conducted in 1992 confirmed this increased prevalence (20). In this study of 3,000 7-year-olds from Denmark, Germany, and Sweden, the frequency of AD was 15.6%. Similarly, questionnaire studies of Swedish schoolchildren showed an increase in prevalence of AD from 7% in 1979 to 18% in 1991 (21), and the point prevalence of AD in schoolchildren living in northern Norway has been reported to be 23% (22).

In a study in Japan, the authors performed skin examinations rather than relying on questionnaires to ascertain the prevalence of childhood and adolescent AD (23). More than 7,000 patients were examined, with AD observed in 24% of the 5- to 6-year-old group, 19% of the 7- to 9-year-old group, 15% of the 10- to 12-year-old group, 14% of the 13- to 15-year-old group, and 11% of the 16- to 18-year-old group. The prevalence of AD in 9- to 12-year-old children was two times, and in 18-year-old adolescents, five times as high as that in similar age groups examined 20 years ago.

Increased exposure to pollutants and indoor allergens, especially house-dust mites, and a decline in breast-feeding, along with increased awareness of AD, have been suggested as reasons for the increased frequency of AD (24). In a prospective study, Zieger et al. (25) found that restricting the mother's diet during the third trimester of pregnancy and lactation as well as the child's diet during the first 2 years of life resulted in decreased prevalence in AD of the prophylactic group as compared with a control group that followed standard feeding practices at age 12 months, but not at 24 months. Follow-up through age 7 years showed no difference between the prophylactic and control groups for AD or respiratory allergy (26). A large study of an ethnically and socially diverse group of children in suburban Birmingham, England, also found that breast-feeding did not affect the lifetime prevalence of 20% (27). A study of prevalence of childhood eczema found a correlation with increased socioeconomic class that was not due to heightened parental awareness (28). Finally, the effects of genetic and environmental factors on allergic diseases were studied in two Japanese cities with differing climates (29). The prevalence of AD in the city with a temperate climate was significantly higher than in the one with a subtropical climate, even after controlling for genetic and environmental factors. In both cities, children from atopic families had a significantly higher risk of contracting respiratory allergies and AD.

Atopic Diathesis

AD, like asthma and allergic rhinitis, is classified as an atopic disease occurring in individuals with a genetic predisposition to develop an IgE response to common environmental allergens. Abnormal IgE responses are associated with cellular abnormalities, resulting in overproduction of Th2 cytokines, which also contribute to the eosinophilia in these diseases. Genetic linkage of the atopic diseases has been suggested, as discussed earlier.

Early onset of AD is associated with an increased risk for respiratory allergy. The highest incidence of asthma at a given age has been observed in children with onset of AD before age 3 months, in those with severe AD, and in those with a family history of asthma (30). A more recent study confirmed the association of increased risk for respiratory allergy (asthma and/or rhinoconjunctivitis) with early onset of AD (31). Respiratory allergy occurred in 50% of children with onset of AD in the first 3 months of life and with two or more atopic family members compared with 12% of those with onset of AD after age 3 months and with no atopic family members.

Like patients with asthma and allergic rhinitis, patients with AD can react to both allergic and nonspecific triggers. Skin reactivity to irritants such as sodium lauryl sulfate (SLS) has been shown in patients with both active and inactive AD, as well as in patients with allergic respiratory disease with no dermatitis, compared with nonatopic individuals (32). The authors hypothesized that an abnormal intrinsic hyperreactivity in inflammatory cells in atopic individuals predisposes to a lowered threshold of irritant responsiveness. A more recent study confirmed and extended these observations by showing that stratum corneum abnormalities in noninvolved AD skin were associated with increased diffusional water loss even 7 days after application of SLS (33). Furthermore, SLS induced a similar eosinophilic infiltrate in patients with allergic rhinitis. In patients with AD, no constitutionally impaired stratum corneum barrier has been definitively proven (34), whereas atopy has been transferred through bone marrow transplantation (35), suggesting that the cutaneous abnormality results from a complex interaction of resident and infiltrating cells. Finally, in a study of bronchial and cutaneous reactivity in asthmatic patients with or without AD (36), the authors found a latent predisposition for bronchial asthma in patients with AD and implicated activated eosinophils as the common effector cells.

A global survey of the prevalence of asthma, allergic rhinoconjunctivitis, and AD reported on 463,801 children aged 13 to 14 years in 155 collaborating centers in 56 countries (37). The highest prevalence rates for AD were reported from scattered centers, including Scandinavia and Africa, that were not among centers with the highest asthma prevalence rates; however, the lowest prevalence rates for AD were in centers located similarly to those with the lowest prevalence rates of asthma and allergic rhinoconjunctivitis. Thus the ultimate presentation of an atopic disease may depend on a complex interaction of environmental exposures with end-organ response in a genetically predisposed individual.

CLINICAL PRESENTATION

Natural History

AD typically is seen in early childhood with onset before age 5 years in approximately 90% of patients (1). Although a 20-year follow-up study found that approximately 84% of children outgrew their AD by adolescence, these results may be flawed, as patients with seborrheic dermatitis may have been included in the study population (38). Other studies show less optimistic outcomes. AD resolved in only 18% of children followed up from infancy until age 11 to 13 years, although it had become less severe in 65% (7). In another study, 72% of patients diagnosed in the first 2 years of life continued to have AD 20 years later (39). Finally, in a prospective study from Finland, between 77% and 91% of adolescent patients treated for moderate or severe AD had persistent or frequently relapsing dermatitis as adults, although only 6% had severe disease (40). More than half of those adolescents with mild disease experienced a relapse as adults.

Clinical Features

AD has no pathognomonic skin lesion(s) or unique laboratory parameters; thus diagnosis is based on the presence of major and associated clinical features (Table 67.1). The principal features include severe pruritus, a chronically relapsing course, typical morphology and distribution of the skin lesions, and a history of atopic disease (1). The presence of pruritus is critical to the diagnosis of AD, and patients with AD have been shown to have a reduced threshold for pruritus.

Major features
Pruritus
Chronicity of skin disease
Typical distribution of dermatitis
Facial and extensor involvement in children <2 yr old
Flexural involvement in children >2 yrs old or adults
Other features
Age of onset <5 yrs old
Course influenced by environmental/emotional factors
Itch when early sweating
Intolerance to wool and lipid solvents
Xerosis (dry skin)
White dermatographism
Recurrent conjunctivitis
Infraorbital darkening
Facial pallor or erythema
Hand/foot dermatitis
Hyperlinear palms
Frequent cutaneous infections, especially by *Staphylococcus aureus*

TABLE 67.1. Clinical Features of Atopic Dermatitis

Acute AD is characterized by intensely pruritic, erythematous papules associated with excoriations, vesiculations, and serous exudate. Subacute AD is characterized by erythematous, excoriated, scaling papules, whereas chronic AD is characterized by thickened skin with accentuated markings (lichenification) and fibrotic papules. Patients with chronic AD may have all three types of lesions present concurrently. In addition, patients usually have dry skin. Significant differences exist between the pH, capacitance, and transepidermal water loss of AD lesions and those measurements in uninvolved skin in the same patients and in the skin of normal control subjects (41).

During infancy, AD involves primarily the face, scalp, and extensor surfaces of the extremities; the diaper area is typically spared. When involved, it may be secondarily infected with *Candida*, in which case, the dermatitis does not spare the inguinal folds. In contrast, infragluteal involvement is a common distribution in children. In older patients with long-standing disease, the flexural folds of the extremities are the predominant location of lesions. Localization of AD to the eyelids may be an isolated manifestation but should be differentiated from an allergic contact dermatitis. Chronic traumatization of the skin due to pruritus can result in prurigo nodules.

Complicating Features

Patients with AD often have a nonspecific hand dermatitis. This condition is frequently irritant in nature and aggravated by repeated wetting, especially in the occupational setting. A recent study suggested that a history of AD at least doubles the effects of irritant exposure and, thus, doubles the risk in occupations in which hand eczema is a common problem (42).

Ocular complications associated with AD can lead to significant morbidity. Increased numbers of IgE-bearing Langerhans cells have been found in the conjunctival epithelium in patients with AD (43). These cells may capture aeroallergens and present them to infiltrating T cells, thus contributing to ocular inflammation. Atopic keratoconjunctivitis is always bilateral, and symptoms include itching, burning, tearing, and copious mucoid discharge. It is frequently associated with eyelid dermatitis and chronic blepharitis and may result in visual impairment from corneal scarring. Vernal conjunctivitis is a severe bilateral recurrent chronic inflammatory process of the upper eyelid conjunctiva, usually occurring in younger patients. It has a marked seasonal incidence, often in the spring. The associated intense pruritus is exacerbated by exposure to irritants, light, or sweating. Examination of the eye reveals a papillary hypertrophy or “cobblestoning” of the upper inner eyelid surface. Keratoconus is a conical deformity of the cornea believed to result from persistent rubbing of the eyes in patients with AD and allergic rhinitis. Anterior subcapsular cataracts may develop during adolescence or early adult life.

Patients with AD have an increased susceptibility to infection or colonization with a variety of organisms including *Herpes simplex* and *Molluscum contagiosum*. Raychaudhuri and Raychaudhuri (44) demonstrated a direct relationship between interferon g (IFN-g) concentrations and the cytopathic effect of *Herpes simplex* and an inverse relationship between IL-4 and the cytopathic effect of *Herpes simplex*. This finding suggests that the T cell–associated cytokine abnormalities in AD may enhance viral infections. Superimposed dermatophytosis including *Trichophyton rubrum* can also cause AD to flare (45). *Pityrosporum ovale* has been associated with a predominantly head and neck distribution of AD (46).

A number of studies have elucidated the importance of *Staphylococcus aureus* in AD. *S. aureus* can be cultured from the skin of more than 90% of patients with AD

compared with only 5% of unaffected individuals (47). A recent study confirmed the higher rate of *S. aureus* colonization in AD lesions compared with that in lesions from other skin disorders and showed that this finding may be associated with colonization of the nares (48). In addition, this study pointed to the importance of *S. aureus* carriage on the hands, suggesting that this may be the vector for transmitting these bacteria from the nasal reservoir to lesional skin and to close contacts of these patients. In another study looking at reservoirs, the prevalence of *S. aureus* in the anterior nares of patients with AD was more than 5 times higher than that in the anterior nares of patients with other skin diseases or in healthy adult control subjects, and the prevalence of *S. aureus* in the subungual spaces was 10 times higher in the patients with AD compared with that in the other groups studied (49). The phage type of *S. aureus* strains isolated from the anterior nares was similar to that of the strains isolated from the subungual spaces. Although recurrent staphylococcal pustulosis can be a significant problem in AD, invasive *S. aureus* infections occur rarely and should raise the possibility of an immunodeficiency such as hyper-IgE syndrome.

Patients with AD have high levels of anxiety and lower life quality compared with unaffected individuals (50). Patients often respond to stress or frustration with itching and scratching. Stimulation of the central nervous system may intensify cutaneous vasomotor and sweat responses and contribute to the itch/scratch cycle. In some instances, scratching is associated with significant secondary gain or with a strong component of habit. Severe disease may lead to problems with social interactions and self-esteem.

Differential Diagnosis

A contactant should be considered in those patients whose AD does not respond to appropriate therapy. Typical distribution for a suspected contactant may be suggestive, although allergic contact dermatitis complicating AD may appear as an acute flare of the underlying disease. Proper diagnosis depends on confirmation of a suspected allergen with patch testing. Scabies is an intensely pruritic skin disease. However, distribution in the genital and axillary areas, presence of linear lesions, and skin scrapings should distinguish scabies from AD. In young infants, immunodeficiencies or metabolic disorders can present as an eczematoid eruption recalcitrant to treatment. In adults with an eczematous dermatitis and no history of childhood eczema or other atopic features, cutaneous T-cell lymphoma must be ruled out. Eczematous rash suggestive of AD can be seen in human immunodeficiency virus (HIV) disease.

THERAPEUTIC MANAGEMENT

Conventional Therapy

Conventional therapy includes skin hydration, antiinflammatory therapy, and elimination of exacerbating factors and clinically relevant allergens (Table 67.2). In children who have undergone blinded, controlled challenges, milk, egg, peanut, soy, wheat, and fish account for approximately 90% of the food allergens found to exacerbate AD (51). Avoidance of foods implicated in controlled challenges results in clinical improvement (51,52). More recently, the *in vitro* CAP test has been shown to measure specific IgE to egg, milk, peanut, and fish allergens with clinically relevant predictive values (53). In dust-mite–allergic individuals, environmental control measures aimed at reducing dust-mite load result in clinical improvement of AD in patients with specific IgE to dust-mite allergen (54). Counseling, relaxation, behavioral modification, or biofeedback may be of benefit, especially in those patients with habitual scratching. Patients and their families should be counseled about the natural history and prognosis of the disease, with appropriate vocational counseling.

Recover skin barrier function with skin hydration/emollients
Reduce chronic inflammatory response
Remove allergens and microbes which aggravate skin disease and can alter response to steroids
Importance of patient education
New role for topical macrolide immunomodulators

TABLE 67.2. Principles for Management of Atopic Dermatitis

Atopic skin shows enhanced transepidermal water loss and reduced water-binding capacity. Use of an effective emollient, usually in ointment or cream form, in combination with hydration therapy, helps to restore and preserve the stratum corneum barrier and can result in a decreased need for topical glucocorticoids.

Glucocorticoids reduce inflammation and pruritus in AD. In addition, topical glucocorticoids can decrease *S. aureus* colonization. In a double-blind, randomized, 1-week trial of a low-potency topical glucocorticoid compared with vehicle in children with AD, clinical scores improved and *S. aureus* density significantly decreased in the glucocorticoid-treated group, but not in the vehicle-treated patients (55). Although topical corticosteroids have been used to treat visibly abnormal skin, studies have shown that the uninvolved skin of patients with AD has an inflammatory cell infiltrate and manifests cutaneous hyperreactivity (32,56). To define a potential role for intermittent treatment with topical glucocorticoids as part of maintenance therapy in adult patients with recurring AD, Van Der Meer et al. (57) treated patients with moderate to severe AD for 2 weeks with topical fluticasone propionate. Patients whose AD had completely healed were entered into a second phase with topical fluticasone propionate or placebo ointment applied to healed skin lesions twice per week for 16 weeks. Patients treated with intermittent fluticasone propionate were more successful in maintaining the skin improvements achieved after the initial treatment phase with significantly reduced risk of relapse. Side effects include thinning of the skin with telangiectasias, bruising, hypopigmentation, acne, striae, and secondary infections, which may occur especially with more potent glucocorticoid preparations. Contact allergy to topical glucocorticoids can complicate treatment of inflammatory skin diseases, often in an insidious manner, and may be difficult to identify through patch testing (58). Systemic corticosteroids should be avoided in the management of chronic AD, as improvement may be followed by dramatic flaring of AD after discontinuation.

Systemic antibiotic therapy may be necessary to treat AD secondarily infected with *S. aureus*. Maintenance antibiotic therapy is rarely indicated and may result in colonization by methicillin-resistant organisms. Patients with disseminated eczema herpeticum usually require treatment with systemic acyclovir. Recurrent cutaneous herpetic infections can be controlled with daily prophylactic oral acyclovir. Superficial dermatophytosis and *P. ovale* can be treated with topical or, rarely, systemic antifungal drugs.

Systemic antihistamines and anxiolytics may be most useful through their tranquilizing and sedative effects and can be used primarily in the evening to avoid daytime drowsiness. Nonsedating antihistamines are less effective in treating the pruritus associated with AD. However, a beneficial effect has been reported in a double-blind study in children with AD treated with cetirizine, 5 to 10 mg daily for 8 weeks (59). Cetirizine, 10 mg twice daily, also has been shown to reduce both eosinophils and neutrophils in early- and late-phase reactions in a placebo-controlled, double-blind study in allergen-specific challenge, suggesting antiinflammatory along with antihistamine effects (60).

Ultraviolet (UV) light therapy can decrease the expression of activation markers such as human leukocyte antigen (HLA)-DR, IL-2 receptor, and CD30 on cutaneous lymphocyte antigen (CLA)-positive T cells (61). UVB has been shown to be effective in the treatment of AD, although narrow-band UVB may be a safer alternative (62). Alternatively, high-dose UVA1 is a fast-acting and effective phototherapeutic approach in patients with acute exacerbations of AD. Unlike traditional UVA/UVB phototherapy, which appears less effective for acute exacerbations and acts primarily in the epidermis, high-dose UVA1 therapy significantly decreases dermal IgE-binding cells, including mast cells and dendritic cells (63). UVA1 may exert antiinflammatory effects indirectly by downregulating proinflammatory cytokines or directly by inducing apoptosis in skin-infiltrating $CD4^+$ T cells (64).

Photochemotherapy with oral methoxypsoralen therapy followed by UVA (PUVA) may be indicated in patients with severe AD, especially with failure of topical therapy in patients with significant glucocorticoid side effects. Short-term adverse effects may include erythema, pruritus, and pigmentation, whereas long-term adverse effects include premature skin aging and cutaneous malignancies (65). Topical psoralens combined with UVA may be equally effective. Topical PUVA has no risk of systemic side effects and may be especially useful for patients with chronic hand eczema who are resistant to other topical medications (66). PUVA therapy in children with severe AD and growth suppression has resulted in significant clinical improvement, with a significant number of patients experiencing sustained remission as well as accelerated growth (67). However, long-term risk of cutaneous malignancies has precluded general use of this treatment modality in children.

Patients with AD who are erythrodermic or who appear toxic may need to be hospitalized. Hospitalization also may be appropriate for patients with severe disseminated disease resistant to first-line therapy. Removing the patient from environmental allergens or stressors, together with intensive education and assurance of compliance with therapy, usually results in marked clinical improvement. In this setting, the patient also can undergo appropriately controlled provocative challenges to

help identify potential triggering factors.

Immunomodulatory Therapy

INTERFERON-g

IFN-g suppresses IgE synthesis (68) and inhibits Th2-cell function (69). Treatment with subcutaneous recombinant human (rh)IFN-g reduces the clinical severity and the total circulating eosinophil counts in patients with AD (70,71). In addition, treatment with rhIFN-g significantly decreases the number of circulating activated lymphocytes (72). Clinical improvement also correlates with reduction in white blood cell (WBC), eosinophil, and lymphocyte counts and normalization of the CD4/CD8 ratio among large lymphocytes (73). Some patients may show persistent improvement several months after discontinuing therapy. Thus, the *in vivo* effects of rhIFN-g therapy are likely to be complex, with modulation of Th2 cell–directed allergic inflammation the primary action. Recently, two open long-term studies have shown clinical efficacy in patients with AD treated for a minimum of 22 months with 50 μg/m^2 rhIFN-g given daily or every other day (74,75). These studies demonstrate that patients with AD can be treated on a long-term basis with rhIFN-g without deterioration of their disease or significant adverse effects. This is noteworthy because IFN-g has been shown to have proinflammatory effects in some clinical settings. Importantly, effective dosing with rhIFN-g is associated with a decrease in eosinophil counts, suggesting that rhIFN-g acts primarily on the allergic inflammatory response, as opposed to IgE synthesis. Thus it is possible that a subset of patients treated with rhIFN-g would respond to individualized titration of their treatment dose (76).

CALCINEURIN INHIBITORS

Cyclosporin A (CsA) is an immunosuppressive agent that acts primarily on T cells, interfering with cytokine transcription (77). The drug binds to an intracellular protein, cyclophilin, and this complex in turn inhibits calcineurin, a Ca^{++} calmodulin-dependent protein phosphatase involved in signal transduction (78). Activation of calcineurin is necessary for cytokine gene transcription to be initiated. Maintenance of chronic inflammation in AD appears to be associated with increased IL-5 gene expression and eosinophil infiltration (5), and preliminary *in vitro* data with mononuclear cells from atopic patients demonstrate suppression of IL-5 production by CsA (79). A significant decrease in the number of circulating eosinophils and in soluble CD30 and soluble E-selectin levels also has been observed with CsA therapy (80,81). A number of studies of oral CsA in severe AD have documented both clinical benefits (82,83) and improvement in quality of life (84). These data have been substantiated in long-term trials, although patients can relapse even after 48 months of therapy (85). Open studies have demonstrated that children with AD treated with CsA tolerated the treatment and showed significant improvement in both clinical signs and quality of life (86,87). Discontinuation of treatment resulted in relapse, although the rate of relapse was variable. Short-term oral CsA therapy can result in increased serum urea, creatinine, and bilirubin concentrations, but these normalize after treatment is discontinued (83). Because of the concern for progressive or irreversible nephrotoxicity with extended treatment, few patients have been evaluated on maintenance therapy. Patients with severe AD treated with oral CsA, 5 mg/kg per day for 6 weeks, were followed up until relapse, and then those who relapsed were treated with a second 6-week course (88). After both treatment periods, approximately half the patients relapsed after 2 weeks, and after 6 weeks, the relapse rate was 71% and 90%, respectively. However, a few patients did not relapse after the first or second treatment courses. All of these patients were still in remission after 1 year, suggesting that although this treatment regimen did not result in lasting remission for the majority of patients, a subset of patients may have derived extended clinical benefit. Because of concerns for systemic toxicity, topical administration of CsA has been tried. However, in a 3-week double-blind vehicle-controlled study with 10% CsA gel and 10% CsA ointment, patients failed to show significant improvement (89).

Tacrolimus (FK506), a macrolide isolated from *Streptomyces tsukabaensis*, is an immunosuppressive agent with a spectrum of activity similar to that of CsA, although it is structurally unrelated to CsA (90). Tacrolimus also acts by binding to a cytoplasmic protein, FK506-binding protein, and this complex in turn inhibits calcineurin, interfering with gene transcription (78). Of note, other cells important in allergic skin inflammation including mast cells, basophils, eosinophils, keratinocytes, and Langerhans cells have tacrolimus (FK506)-binding proteins and downregulate their mediator or cytokine expression after treatment with tacrolimus (91,92). Preliminary data with tacrolimus in ointment form suggested clinical benefit in AD with markedly diminished pruritus within 3 days of initiating therapy, and biopsies done on days 3 and 7 of treatment showed diminished T-cell and eosinophilic infiltrates (93). Randomized, double-blind vehicle-controlled studies in adults and children with moderate to severe AD showed that three concentrations of tacrolimus ointment were significantly better than vehicle alone (94,95). In these 3-week trials, burning at the application site was the only significant adverse event seen with the active drug, and mean blood tacrolimus levels were not elevated during these studies. Because skin inflammation in AD is associated with increased permeability, an aspect of therapy with tacrolimus ointment that possibly contributes to its safety profile is its self-regulatory activity (i.e., as it heals inflamed skin, tacrolimus absorption is decreased) (96). In addition, unlike topical glucocorticoids, tacrolimus ointment does not cause cutaneous atrophy (97). Multicentered, blinded, vehicle-controlled phase 3 trials with tacrolimus 0.03% and 0.1% ointment have been completed in both adults and children with moderate to severe AD; because AD is a chronic disease, long-term studies with tacrolimus ointment applied on up to 100% body surface area are in progress. The optimal treatment regimen with this agent remains to be defined. However, because recent studies suggest that the T-cell activation in AD is biphasic, with predominance of the Th2 cytokines, IL-4, IL-5, and IL-13, during the acute phase and increased expression of the Th1 cytokines, IFN-g and IL-12, in chronic lesions (5,98,99 and 100), the capacity of tacrolimus to inhibit the activation of multiple cell types and cytokines may account for its efficacy in AD.

Topical 1% SDZ ASM 981 has been studied in a randomized, double-blind, vehicle-controlled, right-and-left comparison trial in adult patients with moderate AD (101). Twice-daily application of 1% SDZ ASM 981 cream was significantly more effective than use of the corresponding vehicle and more effective than once-daily treatment over a 21-day period. No clinically relevant drug-related adverse effects were noted. Phase 3 trials in children are in progress.

MYCOPHENOLATE MOFETIL

Mycophenolate mofetil, a purine biosynthesis inhibitor, has been used as an immunosuppressant in organ transplantation and more recently for inflammatory skin disorders (102). Oral mycophenolate mofetil, 2 g daily as monotherapy, resulted in clearing of skin lesions in two adults with AD resistant to other treatment including topical and oral glucocorticoids and PUVA (103). Improvement was evident 2 to 4 weeks after initiation of therapy. Both patients continued on mycophenolate mofetil for an additional 4 weeks, and no side effects were noted. Neither patient experienced relapse of their AD during a 12-week follow-up period. Because the side-effect profile is favorable compared with that of other systemic therapies for AD, further studies with this agent may be warranted.

INTRAVENOUS IMMUNOGLOBULIN

Because chronic inflammation and T-cell activation appear to play a critical role in the pathogenesis of AD, intravenous immunoglobulin (IVIG) could have immunomodulatory effects in this disease. IVIG also could interact directly with antigens, such as infectious agents or toxins involved in the pathogenesis of AD. IVIG contains high concentrations of staphylococcal toxin-specific antibodies that inhibit the *in vitro* activation of T cells by staphylococcal toxins (104). In this study, two different pooled IVIG preparations both contained high titers of antibodies to eight staphylococcal toxins, and binding by the pooled IVGG was toxin specific. The mechanism of inhibition by IVIG was shown to be direct blocking of toxin binding to, or presentation by, antigen-presenting cells. Further rationale for use of IVIG in AD includes the recent observation that IVIG reduces IL-4 protein expression in AD (105). Treatment of severe refractory AD with IVIG has yielded conflicting results. Studies have not been controlled and have involved small numbers of patients. Kimata (106) reported dramatic improvement of AD with this treatment modality. However, in another open label study, nine patients with severe AD were treated with high-dose (2 g/kg) IVIG (Venoglobulin I; Alpha Therapeutic Corporation, Los Angeles, CA) monthly for seven infusions (107). Skin disease lessened slightly in six patients, whereas their average daily prednisone dosage did not change significantly. Mean serum IgE levels did not decrease significantly during IVIG therapy, and *in vitro* IgE production by peripheral blood mononuclear cells (PBMCs) IL-4 and anti-CD40 stimulation was not significantly reduced. The authors concluded that IVIG was of no clear clinical benefit in this small group of patients. However, controlled studies are needed to answer the question of efficacy in a more definitive manner.

PHOSPHODIESTERASE INHIBITORS

Leukocytes from patients with AD have increased cyclic adenosine monophosphate (AMP)-phosphodiesterase (PDE) enzyme activity, with monocytes having a unique, highly active PDE isoenzyme. Monocytes from patients with AD produce elevated levels of prostaglandin (PG) E_2 and IL-10, both of which inhibit IFN-g production. PDE inhibitors such as Ro 20-1724 decrease IgE synthesis (108) and basophil histamine release *in vitro* (109). Culture of AD monocytes with Ro 20-1724 also resulted in significant reduction of abnormal levels of IL-4, IL-10, and PGE_2 (110). Patients with AD treated with CP80,633, a potent inhibitor of PDE type IV, applied topically in a blinded, placebo-controlled paired-lesion study, showed significant clinical improvement with the active drug (110). In a recent study, the selective PDE type IV inhibitor rolipram inhibited *S. aureus* enterotoxin B–mediated generation of cutaneous lymphocyte-associated antigen positive $CD3^+$ cells from PBMCs by reducing IL-12 production in a concentration-dependent manner (111).

Experimental and Unproven Therapies

ALLERGEN DESENSITIZATION

Although uncontrolled trials have suggested that desensitization to specific allergens may ameliorate AD, such results have not been verified in controlled trials (112).

Controlled trials of sufficient duration with standardized extracts of allergens relevant to AD are needed before this form of therapy can be recommended.

TRADITIONAL CHINESE HERBAL THERAPY

Traditional Chinese herbal therapy comprising herbs with antimicrobial, sedative, antiinflammatory, and glucocorticoid-like activities has been shown to inhibit the low-affinity receptor for IgE on peripheral blood monocytes in a dose-dependent manner not due to a toxic effect (113). It has been used in the form of decoctions to treat AD in both children (114) and adults (115). A controlled trial of this therapy failed to show any benefit in the treatment of recalcitrant AD (116). In addition, the possibility of toxicity associated with long-term use, contamination with glucocorticoids, or idiosyncratic reactions remains a concern (117,118).

ESSENTIAL FATTY ACIDS

A number of disturbances in the metabolism of essential fatty acid have been reported in patients with AD (119). A double-blind, placebo-controlled, parallel-group randomized study that avoided the methodologic and analytic problems of a number of previous studies found no clinical benefit of either evening primrose oil or fish oil in AD (120).

LEUKOTRIENE MODIFIERS

The role of cysteinyl leukotrienes has not been well defined in AD, although a recent study with skin chambers demonstrated antigen-specific release of LTB_4 *in vivo* in nonlesional skin from patients with AD (121). Case reports with the LTD_4 receptor antagonist zafirlukast suggest benefit in some patients with AD (122), although controlled studies are needed to evaluate the role of leukotriene modifiers in the treatment of AD.

IMMUNOPATHOLOGY

Immune Responses in AD

The finding of elevated serum IgE levels and the occurrence of eczematous lesions indistinguishable from AD in patients with primary T-cell immunodeficiency disorders suggest an immunologic basis for AD (123). In Wiskott-Aldrich syndrome, bone marrow transplantation results in correction of the immunologic defect and resolution of the dermatitis. In addition, nonatopic recipients of bone marrow transplants from atopic donors have been shown to develop atopic symptoms and positive skin tests after successful engraftment (35). These data suggest that AD results from a bone marrow–derived cell dysfunction rather than from a constitutive skin defect.

Immunoregulatory Dysfunction

A number of immunoregulatory abnormalities have been described in AD (Table 67.3). B cells from patients with AD synthesize high levels of IgE (124). Lymphocytes from these patients produce increased amounts of IL-4 and express abnormally high levels of IL-4 receptor (124,125). In addition, the spontaneous production of IgE can be inhibited *in vitro* by addition of anti–IL-4 (126). PBMCs isolated from patients with AD have a decreased capacity to make IFN-g, and this characteristic is inversely correlated with serum IgE levels (127).

Increased serum IgE
Eosinophilia
Increased basophil spontaneous histamine release
Increased expression of CD23 on mononuclear cells
Chronic macrophage activation with increased secretion of GM-CSF, PGE_2 and IL-10
Expansion of IL-4- and IL-5-secreting Th2-type cells
Decreased numbers of IFN-γ-secreting Th1-type cells
Increased serum soluble IL-2 receptor levels
Increased serum levels of eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil major basic protein

GM-CSF, granulocyte–macrophage colony-stimulating factor; $IFN_γ$, interferon-γ; IgE, immunoglobulin E; IL, interleukin; PGE_2, prostaglandin E; Th1, T-helper cell type 1; Th2, T-helper cell type 2.

TABLE 67.3. Peripheral Blood Immunologic Findings in Atopic Dermatitis

Studies also have shown an increased frequency of both circulating and cutaneous allergen-specific IL-4 and IL-5 secreting Th cells in patients with AD (98,128). Recently, Nakazawa et al. (129) used flow cytometry to demonstrate at the single-cell level that the frequency of Th2 cytokine–secreting cells was significantly higher in patients with AD compared with that in normal controls (129). In addition, CD30, an activation marker of T-cell clones with a Th2 cytokine profile, is significantly elevated in soluble form in children with AD (130).

In addition to acting as an IgE isotype-specific switch, IL-4 also inhibits production of IFN-g and downregulates the differentiation of Th1 cells (131). IFN-g production also is inhibited by PGE_2 and IL-10, both of which are secreted in increased amounts by monocytes from patients with AD (132,133). A recent study did not find any defect in the capacity of cells from AD patients to produce IL-12, an important inducer of IFN-g (134). However, neutralization of IL-10 and IL-4 was able to correct production IFN-g. Thus the activation of Th2 cells and monocytes may be central to the immune dysregulation in AD.

A role for the costimulatory molecules CD80/CD86 has been investigated in AD. With immunohistochemical analysis, Ohki et al. (135) showed predominantly CD86 on Langerhans cells in both the epidermis and the dermis in AD. They also demonstrated almost complete inhibition of antigen-specific T-cell proliferation with an anti-CD86 monoclonal antibody. Studies also have suggested that these accessory molecules differ in their capacity to generate Th1 versus Th2 T-cell responses. A recent study found that the expression of CD86 on B cells of AD patients was significantly higher than that on B cells from normal patients and psoriasis patients, whereas there was no significant difference in CD80 expression among the three subject groups (136). Interestingly, total serum IgE from AD patients and normal subjects correlated significantly with CD86 expression on B cells, suggesting a role for $CD86^+$ B cells in IgE synthesis. Purified $CD86^+$ B cells produced significantly more IgE than did $CD86^-$ B cells *in vitro*, and anti-CD86, but not CD80, monoclonal antibody (mAb) significantly decreased IgE production by PBMCs stimulated with IL-4 and anti-CD40 mAb. Furthermore, $CD86^+$ B cells had a significantly higher level of IL-4R and CD23 expression than did $CD80^+$ B cells. These data demonstrate the predominant expression of CD86 in AD and suggest a role in IgE synthesis.

Immunohistology

Clinically *normal*-appearing skin of patients with AD contains a sparse perivascular T-cell infiltrate (56). Acute papular skin lesions are characterized by marked intercellular edema (spongiosis) of the epidermis. Dendritic antigen-presenting cells, including Langerhans cells and macrophages in lesional and, to a lesser extent, in nonlesional skin of AD, exhibit surface-bound IgE molecules (137). A sparse epidermal infiltrate consisting primarily of T lymphocytes also is frequently observed. In the dermis of the acute lesion, there is a marked perivenular T-cell infiltrate with occasional monocyte/macrophages. The lymphocytic infiltrate consists predominantly of activated memory T cells bearing CD3, CD4, and CD45 RO, suggesting previous encounter with antigen. Eosinophils, basophils, and neutrophils are rarely present in acute AD, whereas mast cells are present in various stages of degranulation.

Chronic lichenified lesions are characterized by a hyperplastic epidermis with elongation of the rete ridges, prominent hyperkeratosis, and minimal spongiosis. There are increased numbers of IgE-bearing Langerhans cells in the epidermis, and macrophages dominate the dermal mononuclear cell infiltrate. The number of mast cells and eosinophils is increased, and eosinophils undergo cytolysis with release of granule protein contents into the upper dermis of lesional skin (138). Eosinophil-derived extracellular major basic protein can be detected in a fibrillar pattern associated with the distribution of elastic fibers throughout the upper dermis. Eosinophils are thought to contribute to allergic inflammation by the secretion of cytokines and mediators that augment allergic inflammation and induce tissue injury in AD through the production of reactive oxygen intermediates and release of toxic granule proteins (139). Eosinophil major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin are elevated in AD sera and correlate with disease severity (140).

Cytokine Expression in Skin Lesions

Both Th2 and Th1 cytokines contribute to the pathogenesis of skin inflammation in AD, with the relative contribution of each cytokine dependent on the acute nature of the skin lesion (Fig. 67.1). As compared with the skin of normal control subjects, uninvolved skin of patients with AD has an increased number of cells expressing IL-4 and IL-13 messenger RNA (mRNA), but not IL-5, IL-12, or IFN-g mRNA (5,100). Acute and chronic skin lesions, when compared with normal skin or with uninvolved skin of patients with AD, have significantly greater numbers of cells that are positive for IL-4, IL-5, and IL-13 mRNA. However, acute AD skin lesions do not contain significant numbers of IFN-g or IL-12 mRNA-expressing cells.

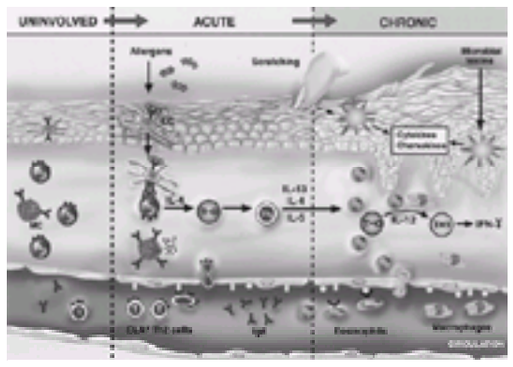

Figure 67.1. Cellular interactions in atopic dermatitis. The acute skin lesion is associated with overexpression of Th2 cytokines, whereas the chronic skin lesions show a shift to Th1 cytokine expression. Two potential reasons for the shift to Th1 cytokine expression include interleukin (IL)-4–induced eosinophil secretion of IL-12 or superantigen-induced stimulation of macrophage, or dendritic cell secretion of IL-12. *MC*, mast cell. (See Color Figure 67.1.)

Chronic AD skin lesions have significantly fewer IL-4 and IL-13 mRNA-expressing cells, but increased numbers of IL-5, GM-CSF, IL-12, and IFN-g mRNA-expressing cells than do acute lesions. Thus acute T-cell infiltration in AD is associated with a predominance of IL-4 and IL-13 expression, whereas maintenance of chronic inflammation is associated with increased IL-5, GM-CSF, IL-12, and IFN-g expression accompanied by the infiltration of eosinophils and macrophages. The increased expression of IL-12 in chronic AD skin lesions is noteworthy because IL-12 plays a key role in Th1 cell development, and its expression in eosinophils and/or macrophages is thought to initiate the switch to Th1 cell development in chronic AD (141).

The importance of Th1 and Th2 cytokines in the development of allergic skin inflammation was demonstrated by a study with a murine model of AD in mice with targeted deletions of the IL-4, IL-5, and IFN-g cytokine genes to assess the role of these cytokines. The data suggested that both the Th2 cytokines IL-4 and IL-5 as well as the Th1 cytokine IFN-g play important roles in the skin inflammatory response of AD. Allergen-sensitized skin from IFN-g knockout mice had reduced dermal thickening; IL-5 knockout mice had no detectable eosinophils and exhibited decreased epidermal and dermal thickening; and IL-4 knockout mice displayed normal thickening of the skin layers but had a drastic reduction in eosinophils (142).

The biphasic pattern of T-cell activation in AD also has been demonstrated in studies with the atopy patch test (reviewed in ref. 141). Twenty-four hours after allergen application to the skin, the expression of IL-4 mRNA and protein is increased, after which IL-4 expression declines to baseline levels. In contrast, IFN-g mRNA expression is not detected in 24-hour patch-test lesions, but is strongly overexpressed at the 48- to 72-hour times. This finding is consistent with studies demonstrating that T-cell clones obtained from early times of evolving allergen patch-test sites secrete Th2-type cytokines, whereas most of the allergen-specific T-cell clones derived from later patch-test sites (>48 hours) exhibit a Th1- or Th0-type cytokine profile. The increased expression of IFN-g mRNA in atopic patch test lesions is preceded by a peak of IL-12 expression coinciding with the infiltration of macrophages and eosinophils.

Identification of mechanisms controlling infiltration of inflammatory cells into AD skin is an area of active investigation (143). Studies have demonstrated that IL-16, a chemoattractant for $CD4^+$ T cells, is more highly expressed in acute than in chronic AD skin lesions (144). Levels of C-C chemokines, RANTES (regulated on activation normal T cell expressed and secreted), monocyte chemotactic protein (MCP)-4, and eotaxin also are increased in AD skin lesions and likely contribute to the chemotaxis of eosinophils and Th2 lymphocytes into the skin (145, 146). A role has been suggested for cutaneous T cell–attracting chemokine (CTACK/CCL27) in the preferential attraction of CLA^+ T cells into the skin (147). The chemokine receptor CCR3, which is found on eosinophils and Th2 lymphocytes and can mediate the action of eotaxin, RANTES, and MCP-4, has been reported to be increased in nonlesional and lesional skin of patients with AD (146). LTB^4 also is released on exposure of AD skin to allergens and may act as a chemoattractant for the initial influx of inflammatory cells (121).

Persistence of Cutaneous Inflammation

Chronic AD is linked to the prolonged survival of eosinophils and monocyte/macrophages in atopic skin. The increase in IL-5 expression during the transition from acute to chronic AD likely plays a role in the prolongation of eosinophil survival and function. In chronic AD, there also is a marked increase in GM-CSF expression (148). GM-CSF plays an important role in maintaining the survival and function of monocytes, Langerhans cells, and eosinophils. Enhanced production of GM-CSF by epidermal keratinocytes and infiltrating macrophages has been shown in AD lesions (148,149). Supernatants from cultured keratinocytes of patients with AD induced a significantly higher proliferative response in PBMCs than did supernatants from control keratinocytes of nonatopic individuals, and this proliferative response could be totally abrogated by an anti–GM-CSF monoclonal antibody. Supernatants from PMA-stimulated keratinocytes of patients with AD, together with exogenous IL-4, also supported the maturation of monocytes into dendritic cells. Epidermal keratinocytes stimulated simultaneously with IFN-g and tumor necrosis factor (TNF)-a produced increased levels of RANTES, which enhanced the chemotaxis of eosinophils (150,151). This may serve as one mechanism by which the increase in IFN-g during chronic AD enhances the chronicity and severity of eczema. Mechanical trauma also can induce the release of TNF-a and many other proinflammatory cytokines from epidermal keratinocytes (152). Thus prolonged scratching plays a role in the perpetuation and elicitation of skin inflammation in AD.

The environmental triggers that contribute to the chronicity of AD are unknown. However, chronic AD is frequently associated with colonization by superantigen-producing *S. aureus* (153). To examine a potential role for microbial superantigens in the prolongation of monocyte/macrophage survival, Bratton et al. (154) incubated PBMs from patients with AD with various concentrations of toxic shock syndrome toxin-1, a prototypic superantigen, and examined the incidence of apoptosis. This superantigen, in a concentration-dependent manner, significantly inhibited monocyte apoptosis and stimulated production of the prosurvival cytokines GM-CSF, IL-1b, and TNF-a. Their data also showed that GM-CSF production was the primary cytokine responsible for inhibition of apoptosis. More recently, staphylococcal enterotoxin B (SEB)-stimulated CLA^+ $CD4^+$ and $CD8^+$ T cells were shown to increase the life span of eosinophils, with the $CD8^+$ subset significantly more effective than the $CD4^+$ subset (155).

Finally, recent studies on mononuclear cells from patients with atopic asthma indicated that allergen-induced immune activation can alter T-cell response to glucocorticoids by inducing cytokine-dependent abnormalities in glucocorticoid receptor binding affinity (156). PBMCs from patients with chronic AD also have reduced glucocorticoid receptor binding affinity, which can be sustained with the combination of IL-2 and IL-4 (157). Endogenous cortisol levels control the magnitude of cutaneous allergic inflammatory responses, suggesting that impaired response to steroids could contribute to chronic AD (158).

Immunologic Triggers

FOOD ALLERGENS

Based on double-blinded, placebo-controlled food challenges, approximately 40% of infants and young children with moderate to severe AD have food allergy (159). Guillet and Guillet (160) evaluated 250 children with AD and found the increased severity of AD and younger age of patients was directly correlated with the presence of food allergy. Removal of the food allergen from the patient's diet can lead to significant clinical improvement but requires a great deal of education, because most of the common causal allergens (e.g., egg, milk, wheat, soy, and peanut) contaminate many foods and are therefore difficult to avoid.

Laboratory studies also support a role for food allergy in AD. Infants and young children with moderate to severe AD frequently have positive immediate skin tests or serum IgE directed to various foods. Positive food challenges are accompanied by significant increases in plasma histamine concentrations and eosinophil activation (159,161). Furthermore, children with AD who are ingesting foods to which they are allergic have increased spontaneous basophil histamine release compared with children without food allergy. Immediate skin tests to specific allergens, however, do not always indicate clinical sensitivity, and patients who outgrow AD frequently

continue to have positive skin tests. Thus the relationship between IgE and clinical AD is not exclusively dependent on IgE-mediated mast cell degranulation.

IgE molecules also can participate in T cell–mediated reactions by binding to Langerhans cells through their high-affinity IgE receptors. Food allergen–specific T cells have been cloned from lesional skin and normal skin of patients with AD (162,163). In support of a role for food allergen–specific T cells in AD, patients with food-induced AD have been studied to analyze the relationship between eczematoid reactions to food allergens and the expression of skin-homing receptors on T cells activated *in vitro* by the relevant allergen. In these studies, T cells from children with casein-induced AD were assessed for their CLA expression after stimulation *in vitro* with casein and compared with T cells collected from patients with milk-induced gastroenteropathy or healthy controls (164). Casein-reactive T cells from patients with milk-induced eczema had significantly higher levels of CLA than did *Candida albicans*–reactive T cells from the same patients, and either casein- or *C. albicans*–reactive T cells from nonatopic controls. These studies provide strong scientific evidence that foods can play a role in the pathogenesis of AD.

AEROALLERGENS

As atopic children grow older, inhalant allergens play a more important role in the pathogenesis of their AD (reviewed in ref. 165). Walker (166) first reported, in 1918, that several of his patients had exacerbation of their AD after exposure to horse dander, timothy grass, or ragweed pollen. In the 1950s, Tuft and Heck (167) found that patients with AD developed pruritus and eczematoid skin lesions after intranasal inhalation challenge with either *Alternaria* or ragweed pollen, but not with placebo. More recently, Tupker et al. (168) reported a double-blind, placebo-controlled challenge study that demonstrated inhalation of house-dust mites by bronchial challenge resulted in both new AD lesions and exacerbation of existing skin lesions.

Most reports, however, have focused on the development of eczematoid eruptions after epicutaneous application of aeroallergens. In these studies, patch testing of uninvolved atopic skin with aeroallergens gave rise to eczematoid reactions in 30% to 50% of patients with AD (169,170). In contrast, patients with respiratory allergy and healthy volunteers rarely have positive allergen patch tests. Because there is considerable variation in the performance and interpretation of patch tests, however, their clinical usefulness is still questionable. Nonetheless, patch testing continues to be used as a research tool.

Several studies have examined whether avoidance of aeroallergens results in reduced skin symptoms due to AD. Most of these reports have involved uncontrolled trials in which patients were placed in mite-free environments, and their AD improved. One double-blind placebo-controlled study with a combination of effective mite-reduction measures in the home has been reported (171). Although both active and control groups decreased *Dermatophagoides pteronyssinus* (Der p 1) concentrations in carpeting, the reduction in the amount of dust was greater on the mattresses in the active group, and their AD symptoms decreased significantly more.

These clinical studies suggest that inhalation or contact with aeroallergen may exacerbate AD. Laboratory data supporting a role for inhalants include the finding of IgE antibody to specific inhalant allergens in most patients with AD. One study found that sera from 95% of patients with AD had IgE to house-dust mites compared with sera from 42% of individuals with asthma (172). The degree of sensitization to aeroallergens was directly associated with the skin severity of AD. The isolation from AD skin lesions and allergen patch test sites of T cells that recognize Der p 1 and other aeroallergens provides further evidence that the inflammatory response in AD can be elicited by aeroallergens (173).

MICROBES

Patients with AD have an increased tendency to develop bacterial and fungal skin infections. *S. aureus* colonizes more than 90% of AD skin lesions. In contrast, only 5% of normal subjects harbor this organism. The importance of *S. aureus* is supported by the observation that even AD patients without superinfection show a reduction in severity of skin disease when treated with a combination of antistaphylococcal antibiotics and topical glucocorticoids (174,175). The fact that a combination of antibiotics and topical glucocorticoids is much more effective than topical glucocorticoids alone in reducing the severity of AD suggested that *S. aureus* may secrete a toxin that alters the response to glucocorticoids.

Studies suggest that one strategy by which *S. aureus* exacerbates or maintains skin inflammation in AD is through the secretion of superantigens that stimulate marked activation of T cells and macrophages. Several lines of investigation support a role for superantigenic toxins in AD. First, more than half of patients with AD have *S. aureus* cultured from their skin that secretes superantigens such as enterotoxins A and B and toxic shock syndrome toxin-1 (TSST-1) (153,176,177). An analysis of the skin-homing CLA^+ T cells from these patients as well as their skin lesions reveals that they have undergone a T-cell receptor V-b expansion consistent with superantigenic stimulation (178,179). Second, most patients with AD make specific IgE antibodies directed against the staphylococcal toxins found on their skin (175,176 and 177). Basophils from patients with anti-toxin IgE release histamine on exposure to the relevant toxin but not in response to toxins to which they had no specific IgE. Third, a correlation has been found between the presence of IgE antisuperantigens, the density of superantigen-producing *S. aureus*, and the severity of AD. In a humanized murine model of skin inflammation, *S. aureus* superantigen plus allergen had an additive effect in inducing cutaneous inflammation (178). Superantigens also augment IgE synthesis by PBMCs to specific allergens and induce glucocorticoid resistance, suggesting that several mechanisms exist by which superantigens could aggravate the skin severity of AD (179,180). Fourth, the superantigen SEB, applied to the skin, can induce skin changes of erythema and induration, and the infiltrating T cells are selectively expanded in response to SEB (181,182). Furthermore, in a prospective study of patients recovering from toxic shock syndrome, it was found that in 14 of 68 patients, chronic eczematoid dermatitis developed, whereas in no patients recovering from gram-negative sepsis did eczema develop (183). Thus superantigens can induce an eczematoid process in the skin. Superantigens have been demonstrated to induce T-cell expression of the skin-homing receptor through stimulation of IL-12 production (184). In the case of AD, staphylococcal superantigens secreted at the skin surface may penetrate inflamed skin and stimulate epidermal macrophages or Langerhans cells to produce IL-1, TNF, and IL-12. Local production of IL-1 and TNF induces the expression of E-selectin on vascular endothelium, allowing an initial influx of CLA^+ memory/effector cells. Local secretion of IL-12 could increase CLA expression on those T cells activated by allergen or superantigen and thereby increase their efficiency of T-cell recirculation to the skin, perhaps including areas with only low levels of vascular E-selectin and minimal inflammatory activity. IL-12 secreted by toxin-stimulated Langerhans cells that migrate to skin-associated lymph nodes (and serve as antigen-presenting cells there) could upregulate the expression of CLA and influence the functional profile of virgin T cells activated by the toxins, thereby creating additional skin-homing memory-effector T cells. Together, these mechanisms would amplify the initial cutaneous inflammation in AD.

There also has been considerable interest in fungus, particularly *Malassezia furfur* (*P. ovale* or *P. orbiculare*), as a pathogen in AD. *M. furfur* is a lipophilic yeast commonly present in the seborrheic areas of the skin. IgE antibodies against *M. furfur* are frequently found in AD patients, most often in those with head and neck dermatitis (185). In contrast, IgE sensitization to *M. furfur* is rare in normal controls or in patients with asthma. The potential importance of *M. furfur* as well as other dermatophyte infections is further supported by the reduction of AD severity in the skin of such patients after treatment with antifungal agents (186,187).

AUTOALLERGENS

Valenta et al. (reviewed in ref. 188) has reported that the sera from most patients with severe AD contain IgE antibodies directed against human proteins. Such antibodies were not detected in patients with chronic urticaria, systemic lupus erythematosus, graft-versus-host disease, or healthy controls. Several of the autoallergens have been cloned and found to be mainly intracellular proteins. Nevertheless, they have been detected in IgE immune complexes of AD sera, suggesting that release of these autoallergens from damaged tissues could trigger IgE- or T cell–mediated responses. These data suggest that although IgE immune responses are initiated by environmental allergens, allergic inflammation can be maintained by human endogenous antigens particularly in severe AD.

FUTURE DIRECTIONS

The pathogenesis of AD is complex and incompletely understood. Most patients have a systemic Th2 response with high serum IgE levels, eosinophilia, and an expansion of T cells expressing IL-4, IL-5, and IL-13, a finding also in patients with AD with an increased tendency to develop respiratory allergy. Once the process of skin inflammation is elicited, there is an evolution from uninvolved skin, which has a sparse infiltrate of Th2 cells, to acute papular skin lesions with epidermal spongiotic changes and a marked influx of Th2 cells. The activation of Th2 cells by epicutaneous application of allergens is thought to involve antigen capture and presentation by IgE-bearing Langerhans cells. However, ingestion of foods or inhalation of aeroallergens also may involve mucosal antigen-presenting cells, which become armed with allergens and activate T cells that then home to the skin. Th2-type cytokines released from T cells and mast cells also enhance development of Th2 cells and induce the release of chemokines, such as eotaxin, from various cell types including keratinocytes, fibroblasts, and endothelial cells. Prolonged skin inflammation results in chronic AD associated with epidermal hyperplasia and lichenification. Unlike acute AD, the chronic inflammatory response is dominated by a Th1-type response driven by the subsequent infiltration of IL-12–expressing eosinophils, dendritic cells, and macrophages, which follows the initial Th2 response (Fig. 67.1). The mechanism for increased IL-12 expression during chronic AD is not known but may be related to the ability of IL-4 to induce IL-12 expression by eosinophils (189). In the case of dendritic cells and macrophages, microbial superantigens are known to be a potent inducer of IL-12 production (190). IFN-g, but not IL-4, has been correlated with the clinical severity of AD. This may be related to the capacity of IFN-g to enhance eosinophil viability and augment eosinophil activation and cytotoxic activity as well as activation of vascular endothelial molecules, which increase the infiltration of eosinophils and thereby contribute to chronic AD.

Atopic skin inflammation involves both allergen-specific and nonantigen responses that amplify inflammatory responses. This process is reflected in studies demonstrating AD is associated with a lower itch threshold, cutaneous hyperreactivity, and an overly active inflammatory response. Scratching plays a key role in the elicitation of eczematoid skin lesions. Once the itch/scratch cycle is triggered, mechanical trauma to the keratinocytes results in the release of a proinflammatory

cytokine cascade that contributes to the infiltration of cells into the skin.

Finally, studies indicate that AD is associated with defective skin-barrier function. Although these changes may be related to the constant scratching and immune activation that is ongoing, these patients also have decreased ceramide levels in their skin, which may lead to reduced water-binding capacity, higher transepidermal water loss, and decreased water content (191). These changes also contribute to increased antigen absorption by the skin and therefore create a vicious cycle leading to further immune activation and perpetuation of skin inflammation.

Targeting specific cytokines or their receptors represents a potentially unique and specific treatment modality for AD. Anti–IL-5 antibody completely blocks eosinophil infiltration in sensitized animals, whether administered before or after allergen challenge (192). Pilot studies are under way with a humanized monoclonal antibody against IL-5 in patients with asthma, and such an approach could also be considered in AD. Soluble IL-4 receptor molecules can effectively bind IL-4 and suppress IL-4–mediated T- and B-cell functions (193). Preliminary clinical trials with soluble IL-4 receptor in patients with moderate asthma have found it to be safe and effective (194). In patients with AD, soluble IL-4 receptor downregulates allergen-specific lymphocyte function *in vitro* and may prove to be an effective therapeutic agent (195). Development of specific chemokine antagonists may also lead to use in AD. Finally, novel therapies based on an improved understanding of the immunopathogenesis of AD would be expected to result in improved outcomes, especially in recalcitrant disease, and lead to specific, rather than symptomatic therapy. However, much remains to be learned about the complex interrelationship of genetic, environmental, immunologic, and pharmacologic factors in AD before the ultimate goal of prevention can become a reality.

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68 ALLERGIC REACTIONS TO FOODS

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Allergic reactions to foods result from heightened immunologic responses to glycoprotein components in these substances. Children and adults in whom such reactions develop are said to have a *food allergy*, a term synonymous with *food hypersensitivity* (1). *Food intolerance* is defined as an exaggerated or abnormal reaction to a food that has a pharmacologic, toxic, or metabolic basis. Conceptually, food allergy is divided into two subgroups: those reactions that depend on food antigen-specific immunoglobulin E (IgE), and those problems that result from non–IgE-dependent immunologic mechanisms. Most food allergies in adults depend on IgE-mediated mechanisms, whereas in infants and children, there is evidence that a substantial minority have food allergies because of non–IgE-dependent events.

The prevalence of adverse reactions to foods in children younger than 6 years has been stated to be approximately eight in 100 (2). One fourth to one half of these reactions seem to depend on immunologic mechanisms. A prospective study of Danish infants during the first 3 years of life found the prevalence of cow's milk allergy to be 2.4% (3). The prevalence of food allergy in adults may be somewhat less. In a study in the Netherlands based on questionnaires, clinical follow-up, and double-blind, placebo-controlled food challenge, the prevalence of food allergy and intolerance together was 2.4% (4). The prevalence of peanut and tree nut allergy in the United States as determined with a random-digit-dial telephone survey was found to be 1.1% of the general population (5).

It also is clear from general population surveys that a large number of individuals believe they have had allergic reactions to foods. For example, in a study in Great Britain commissioned by the Ministry of Agriculture, Fisheries, and Food, and with the electoral register used as a sampling frame, 15.6% of 18,582 respondents stated that they had allergies to foods, and 7.4% stated that they had allergies to food additives (6). Although the responses to the question about food allergy were not evaluated, the responses to the question about additive reactions were. Patients who believed themselves sensitive to additives were interviewed; and if the reaction was thought possibly to be due to food additives, they underwent placebo-controlled, double-blind challenges with the additives. After this evaluation, which excluded possible reactions to monosodium glutamate or sulfites, the investigators estimated a 0.01% to 0.23% prevalence of food additive intolerance.

FOOD ALLERGENS

The gastrointestinal barrier is exposed to a heterogeneous group of proteins that varies, in part, according to age. For example, infants ingest proteins from fewer sources than do older individuals; their food sources consist of mother's milk or commercial formulas, including those based on bovine milk or soybean proteins. The dietary proteins ingested by older children and adults are derived from meat, poultry, and fish (42.3%); dairy products (21.2%); flour and cereal products (18.6%); dry beans, peas, nuts, and soya products (5.4%); vegetables (6.2%); eggs (4.9%); and fruits and miscellaneous sources (1.4%) (7). Commercially processed proteins used as food ingredients include casein and whey from bovine milk; gelatin and collagen from animal tendons and hides; soy protein isolates from soybeans; and gluten from the flours of wheat, corn, and oats (8).

The sources of dietary proteins are changing in response to the nutritional requirements of progressively larger populations. Newer sources of proteins now include genetically modified foods. Changes in the processing of foods also are having their impact. Chemical modification of foods by ultrafiltration, high-temperature processing, or preservation by irradiation have the potential to create new antigenic sites or to decrease digestibility.

Despite the wide variety of foods ingested, only a relatively few cause allergic reactions. For example, in children, most allergic reactions are due to milk, peanuts, egg, and soybean. In adults, the most common food allergens are peanuts, tree nuts, crustaceans, fish, and eggs. Other foods not infrequently implicated include cereal grains and seeds.

Much has been done to isolate and characterize components of food that are responsible for immunologic reactions. Attempts to isolate relevant antigens have often focused on their ability to bind to an antigen-specific IgE. In early studies, water-soluble food components were evaluated for antigenicity by their ability to induce immediately positive skin tests in individuals with immediate reactions to foods. Later studies have characterized antigens with *in vitro* assay techniques that examine the ability of water-soluble food proteins to bind IgE in serum from these patients, followed by the isolation and determination of the antigenic molecular structure.

Food antigens are almost always proteins or glycoproteins. Molecular masses of these antigens tend to be between 10 and 40 kd. These antigens are often, but not always, resistant to denaturation by heat and to degradation by proteases.

Allergen M (Gad c 1) from codfish has been well studied. Codfish hypersensitivity is common in countries where consumption of this fish is high. Allergen M is a parvalbumin, a calcium-binding protein found in the muscle of fish and amphibians. It has a molecular mass of approximately 12 kd, is heat stable, is partially resistant to proteases, and exists as a single polypeptide chain (9,10). Allergen M consists of 113 amino acids and one glucose molecule. Linear peptides corresponding to regions 13 to 32, 49 to 64, and 88 to 103 have been synthesized and bind IgE from cod-allergic individuals.

A number of other food antigens have been purified (Table 68.1). The major allergen in shrimp is tropomyosin (Pen a 1, Pen i 1, Met e 1) with a molecular mass of 34 to 36 kd. Linear fragments bind tropomyosin-specific IgE (11,12). Tropomyosin is a common allergen also found in lobster, crab, and crawfish.

Allergen	Source	Mol Wt (Da)	Characteristics
Gad c 1	Codfish	12,328	A parvalbumin, chelates calcium
Pen a 1, Pen i 1, Met e 1	Shrimp	34,000–36,000	A tropomyosin
Ara h 1	Peanut	63,500	
Ara h 2	Peanut	17,000	
Ara h 3	Peanut	14,000	
Ory s 1	Rice	14,764	α-amylase inhibito
Gal d 1	Egg	28,000	Ovomucoid
Gal d 2	Egg	45,000	Ovalbumin
Gal d 3	Egg	77,700	Ovotransferrin
Gal d 4	Egg	14,300	Lysozyme

TABLE 68.1. Examples of Water-Soluble Food Antigens

Peanuts are highly allergenic. Three main peanut allergens (Ara h 1, Ara h 2, Ara h 3) account for much of this legume's allergenicity (13,14). Peanut products such as flours retain their allergenicity, although highly refined peanut oil appears generally safe (15). Soybeans are similarly a major crop of the legume family. At least in one instance, an allergic reaction to soybeans was traced to a reaction to Kunitz soybean trypsin inhibitor (16). Similarly, the rice major allergenic protein has been isolated from a complementary DNA (cDNA) library of maturing rice seeds (17). The cDNA had an open reading frame of 486 nucleotides that coded a 162-amino acid residue

polypeptide. The deduced amino acid sequence of the rice major allergenic protein was similar to those of barley trypsin inhibitor and wheat a-amylase inhibitor.

In addition to identifying and isolating specific proteins with allergenic activity, many studies have examined major proteins or protein fractions for their allergenic activity when available. Such is the case for milk. For milk, casein, a-lactalbumin, b-lactoglobulin, and bovine serum albumin produce the highest rates of skin-test reactivity in children with milk allergy (18). In oral challenge studies of 45 children, 62% reacted to b-lactoglobulin, 60% to casein, 53% to a-lactalbumin, and 52% to bovine serum albumin (19). Studies indicate that children who are allergic to cow's milk are also frequently allergic to goat's milk (20).

Chicken egg is a common food allergen, particularly in children. The yolk is often less allergenic than the white. Ovomucoid (Gal d 1) constitutes 10% of the egg-white protein and is the dominant allergen. Other major allergens are ovalbumin (Gal d 2; 50% of egg white), ovotransferrin (Gal d 3), and lysozyme (Gal d 4) (21,22,23,24 and 25).

IgE-MEDIATED REACTIONS

A number of reactions involving multiple target organs are caused by IgE-mediated immediate adverse reactions to food antigens. These reactions include hives, atopic dermatitis, angioedema, gastrointestinal reactions, asthma, the oral allergy syndrome (26) (see later) anaphylaxis, and allergic eosinophilic gastroenteritis. These patterns of food allergies share a common pathogenesis, are evaluated with similar testing procedures, and in general are managed with related treatment approaches.

Pathogenesis

Clinical and experimental observations support a role for mast cells, basophils, and IgE in immediate reactions to foods. The presence on mast cell surfaces of IgE that reacts with food antigens has been demonstrated by immediate wheal-and-erythema reactions in the skin after local injection of minute amounts of food antigen (27). Such reactions have been passively transferred by intracutaneous injection of serum from an allergic donor into the skin of a normal recipient, followed 24 hours later by local injection into the recipient of the food antigen to which the donor was sensitive (Prausnitz-Küstner test). This test, useful for demonstrating the passive transfer of a sensitizing agent in the serum, was discovered when the serum of Küstner, who was allergic to fish, was injected into Prausnitz, and a positive response was elicited on local challenge with fish antigen (28). Similar local reactions have been reported when the food antigen was administered orally to the normal recipient 24 hours after injection of allergic donor serum into the skin (29).

Food antigen–specific IgE has been demonstrated indirectly to lead to degranulation of mast cells in the gastrointestinal tract. Local mucosal reactions reminiscent of those that follow mast cell degranulation in human skin have been reported *in vitro* after passive sensitization of human ileum and colonic mucosa (30). Local reaction after passive sensitization could be induced by ingestion or by direct application of the antigen. Human mucosal mast cells have been reported to degranulate *in vitro* in IgE-dependent reactions (31).

Mast cells exert their effects on surrounding tissues during allergic reactions by the generation, synthesis, and release of potent chemical mediators (32). Mediator release, or degranulation, is triggered by a variety of stimuli. These diverse stimuli may be endogenous substances, such as neuropeptides; or they may be exogenous substances to which mast cells become exposed *in vivo*, such as antigens. Although food antigen–specific IgE appears to be responsible for the induction of immediate food reactions, other stimuli including anaphylatoxins may play a role in delayed reactions.

Mast cells in the mucosa of the gastrointestinal tract are uniquely positioned to affect reactions to substances presented orally. Small quantities of luminal proteins may gain access to the mucosa. If IgE antibody specific to such substances is on the surface of mucosal mast cells, mast cell activation and mediator production result. This local intestinal anaphylaxis increases mucosal permeability and the entry of a food antigen into the body, where it may be distributed to other target organs and initiate degranulation of mast cells at those sites. The gastrointestinal tract, by virtue of food processing, also is in a position to alter the timing of such reactions. Thus antigenic sites on food antigens may be unmasked or degraded as food is digested.

The ability of some individuals but not others to limit or prevent the synthesis of food-specific IgE appears to be largely genetically determined. There is some ingress of intact antigen in the normal state. Consequently, there is no requirement for an alteration in mucosal permeability as the underlying defect in the genesis of IgE-mediated food allergy. This conclusion is consistent with clinical data that have failed to demonstrate defects in IgA synthesis, to find gastrointestinal disease including achlorhydria, or to generate evidence of immune complex diseases in patients with immediate reactions to foods. The most reliable clinical correlates of immediate reactions to foods are a family and personal history of atopy and the presence of positive skin tests to foods and inhalants, strongly suggesting that the basis of the production of IgE in response to foods is related to inherited patterns of IgE synthesis and regulation. Food allergy is not necessarily associated with the overproduction of IgE. Patients with food allergy mediated by IgE may not give a family history of adverse reactions to specific foods, suggesting that multiple factors contribute to the development of IgE directed to food antigens.

Clinical Patterns of Target-Organ Responses

Foods are generally implicated as the etiologic agents of immediate reactions to foods because of a clear temporal relation between eating and induction of symptoms. However, in any discussion of food antigen–induced reactions, a number of alternative conditions and diseases must be considered (Table 68.2). These alternative diagnoses represent diseases exacerbated by the process of ingestion; those caused by intrinsic pharmacologic agents, additives, and contaminants in foods; and those due to infectious organisms and their products.

TABLE 68.2. Differential Diagnosis of Immunologically Mediated Reactions to Foods

The clinical expression of immediate reactions logically follows the sequence in which target tissues are exposed to the food antigen that is the etiologic agent. Oropharyngeal reactions are often observed first and are characterized by pruritus and urticaria in and around the mouth. Uvular edema also may be present. Rarely does the initial exposure to antigen result in obstructed airflow. Such reactions are transient and are not necessarily followed by other allergic manifestations.

This development of oropharyngeal symptoms with food ingestion is sometimes referred to as the oral allergy syndrome, especially when it occurs in the absence of systemic reactions. In such cases, it is most commonly due to fruits or vegetables. Symptoms include pruritus, tingling, and angioedema of the lips. Throat tightness, facial flushing, oral mucosal blebs, and hoarseness have been reported. As with other immediate reactions, symptoms occur within minutes after exposure to the provoking foods. The entry of the offending food into the stomach and subsequently into the intestine may result in nausea, cramping pain, abdominal distention, vomiting, flatulence, and diarrhea. In children, gastrointestinal manifestations of immediate food reactions are common and may be the only problem noted. In adults, isolated gastrointestinal complaints caused by immediate adverse reactions to foods are less common. In one study of 27 patients with irritable bowel syndrome who suspected foods as being the cause of their symptoms, only three patients had their complaints reproduced by food challenge (33).

Food allergy also may be expressed in one or more extraintestinal target tissues. The basis of the preferential reactivity of one system over another is unknown. The skin is a common target organ in food allergy. Skin reactions are evidenced by acute urticaria or angioedema and much less frequently by chronic urticaria. In one report of 554 patients with urticaria, food allergy was demonstrated in only 1.4% (34). When the acute urticaria is due to foods, in most situations, it is not a diagnostic problem, as the symptoms usually occur shortly after eating the food, and the patient is aware of which food caused the symptoms.

Food reactions that include asthma have been verified by oral challenge with suspected foods. The frequency of reactions that include asthma is unknown but seems to be low (35). Although hay fever and asthma are frequently suspected to be caused by foods, they appear less frequently than do gastrointestinal symptoms and

rarely occur without other organ involvement (36,37).

Systemic anaphylaxis as a result of allergy to ingested foods generally occurs within minutes, but occasionally has been reported to occur hours after ingestion of the offending food (38). The first anaphylactic episode may be unexpected or may have been preceded by minor symptoms such as abdominal discomfort or urticaria on previous exposure to the food. Systemic anaphylaxis involves multiple target organs, as evidenced by abdominal pain, nausea, emesis, diarrhea, dyspnea, cyanosis, chest pain, urticaria, angioedema, arrhythmias, hypotension, and shock. These severe, life-threatening reactions are most often associated with the ingestion of peanuts, tree nuts, fish, and shellfish. Fatal reactions may develop quickly or begin with mild symptoms and progress over a 1- to 3-hour period to cardiorespiratory arrest and shock. Factors that predispose to anaphylaxis include a personal history of atopy, a family history of atopy, and dietary exposure. Atopic patients have an increased risk of developing food allergies if they have asthma and allergic rhinitis (39). Food allergy is the single most common cause of anaphylaxis seen in hospital emergency departments (40).

In addition to foods, other factors such as exercise may be necessary to provoke the reaction. Anaphylaxis has been reported in persons exercising within 3 to 4 hours after the ingestion of certain foods such as shrimp, wheat, milk, fish, and celery (41,42 and 43). Attacks are precipitated by various types of strenuous exercise, including running, fast walking, tennis, and dancing (44). In some patients, attacks are associated with meals, but no foods specifically can be implicated. The general assumption is that anaphylaxis that occurs during exercise and after meals is associated with increased antigen absorption.

Atopic Dermatitis

Atopic dermatitis is an inflammatory condition or state of the skin, seen as a small, round patch as in nummular eczema or as a widespread eruption involving large areas of the body. Atopic dermatitis can be acute (with erythema, edema, and weeping) or chronic (with scaling, thickening, and hyperpigmentation). The chronicity of atopic dermatitis distinguishes it from immediate and self-limited immediate reactions as described earlier. Atopic dermatitis is frequently associated with hay fever and asthma. In fact, bronchial hyperreactivity with or without asthma is more frequent in patients with atopic dermatitis (45). The prevalence of atopic dermatitis is estimated to be 10% to 12% of the pediatric population (46). A complete review of atopic dermatitis appears in Chapter 67.

A number of studies have examined the role of foods in the pathogenesis of atopic dermatitis and have concluded that food hypersensitivity contributes to skin symptoms in children in at least one third of the cases (47); and that the more severe the dermatitis, the greater the possibility that food allergy is playing a role (48). In one classic study of 26 children, 16 had positive blind challenges (49). Positive responses included pruritus, erythematous macular or maculopapular rashes, nausea, abdominal pain, vomiting, diarrhea, nasal congestion, rhinorrhea, sneezing, and mild wheezing. All symptoms developed 10 minutes to 2 hours after challenge. Foods producing positive challenges were egg, milk, peanut, wheat, soy, chicken, fish, chocolate, potato, and rye. Complete removal of the incriminated foods resulted in significant improvement of the dermatitis.

Atopic dermatitis is associated with elevated serum IgE levels. In some instances in which challenge with food allergen provokes the disease, a concomitant increase in plasma histamine levels has been documented (50). These observations and positive skin tests to provocative foods strongly implicate IgE-mediated mast cell activation in the skin. The reaction is not self-limited, as in food-induced urticaria, but results in chronic dermatologic changes. The frequent findings of peripheral blood eosinophilia, elevated serum eosinophil cationic protein levels after food challenge (51), and the dermal deposition of eosinophil granule major basic protein (MBP) suggest that the recruitment of eosinophils to the skin may contribute to the pathologic changes (52). The involvement of eosinophils indicates that the pathogenic mechanism in the dermis in atopic eczema is similar to that for IgE-mediated late phase reactions. Surface-bound IgE also is found on Langerhans cells in biopsy specimens (53). In addition to IgE-dependent dermal mast cell activation, patients with atopic dermatitis exhibit a high “spontaneous basophil histamine release,” possibly due to release of a “histamine-releasing factor” from immune peripheral monocytes (54).

Eczematous lesions in patients with atopic dermatitis also are infiltrated with T cells, most of which synthesize interleukin (IL)-4 and IL-5. These infiltrating T cells are enriched for cutaneous lymphocyte antigen (CLA)-positive cells. In contrast, T cells from the lungs of patients with asthma are CLA negative (55).

An intestinal mucosal defect independent of food reactions is suggested by studies with polyethylene glycol. High-molecular-mass polyethylene glycol (4 kd) was absorbed in patients with eczema to a greater extent than in normal individuals. Low-molecular-mass polyethylene glycol (0.6 kd) was absorbed equally (56).

Allergic Eosinophilic Gastroenterocolitis

Eosinophilic gastroenterocolitis (57) is an uncommon chronic digestive disease characterized by peripheral eosinophilia and by edema and eosinophilic infiltration of the stomach or, to a lesser extent, the small and large intestines (58). It is seen most frequently during the third decade of life (59), although this disease develops also in children (60). Clinical symptoms are related to the extent of eosinophilic infiltration in the bowel wall. When the eosinophilic infiltration is in the mucosa, symptoms correspond to those of malabsorption. If the infiltration is predominantly in the muscular layer, the clinical picture is that of obstruction. Significant eosinophilic infiltration of the serosa is characterized by a clinical picture of ascites. These pathologic findings may occur singly or in combination. Thus when the eosinophilic infiltration is limited to the esophagus and disease manifestations reflect this pathology, the disease is referred to as allergic eosinophilic esophagitis. Similarly, when the disease is limited to the stomach, it may be referred to as allergic eosinophilic gastritis (57).

Many patients with the mucosal pattern of this disease complain of abdominal symptoms that are brought on or are aggravated by the ingestion of specific foods. These patients often are atopic, have an elevated serum IgE level, and have specific IgE antibodies to multiple food antigens (58,61). Taken together, these clinical and laboratory findings support the thesis that allergic mucosal diseases are due to severe food allergies, leading to repeated immediate and late phase immediate hypersensitivity reactions in the mucosa. This construct is supported by the finding that patients with this disease have T cells that produce abnormally high amounts of IL-4 and IL-5 after mitogenic stimulation (62). In fact, lymphocytes in some patients produced IL-5 in the absence of mitogen. In contrast, interferon-g (IFN-g) messenger RNA (mRNA) could not be detected by the reverse transcription (RT) polymerase chain reaction (PCR) in biopsy specimens from patients with allergic eosinophilic gastroenteritis but was present in those from normal individuals.

Diagnostic Tests

The diagnosis of an immediate IgE-mediated reaction to a food depends on the history, an appropriate exclusion diet, selective skin tests or antigen-specific IgE *in vitro* tests, and blinded provocation. There is no convincing evidence that measurement of food-specific IgG or IgG4 antibody levels or of food antigen/antibody complexes has diagnostic value in the evaluation of immediate reactions to foods.

IN VITRO *TESTS*

Laboratory procedures supporting the clinical diagnosis of an IgE-mediated food hypersensitivity depend on the identification of antigen-specific IgE in serum to water-soluble food allergen extracts. Examples of such tests are *in vitro* measurements of antigen-specific IgE, as in the radioallergosorbent test (RAST) or enzyme-linked immunosorbent assay (ELISA), and basophil histamine release. Laboratory tests such as total IgE determinations and eosinophil counts do not correlate with immediate hypersensitivity reactions to foods.

The RAST, an *in vitro* test, is generally considered somewhat less sensitive than skin testing (63). Although there have been modifications of the RAST, all involve antigens coupled to a solid phase (e.g., paper disk). Patient sera are incubated with the solid phase. After washing, the amount of bound IgE antibody is calculated by adding labeled antihuman IgE antibodies. The ELISA is a variation on this method. Depending on the available antibody sites, such tests may be more subject to inhibition by antigen-specific IgG.

Basophil degranulation tests also may be used to evaluate allergic reactions to foods. For this test, heparinized venous blood or separated blood leukocytes are incubated with extracts of suspected foods. Histamine is released into the supernatant fluid and is measured as an index of reactivity and the amount of antigen-specific IgE on the basophil surface that interacts with allergens within the food extract. The outcome of the basophil degranulation test is generally comparable to that of the RAST.

IN VIVO *TESTS*

Skin testing with food extracts consists of the application of extracts to the dermis. It is the most reliable method to demonstrate food allergen–specific IgE antibodies. The usual method is the puncture or prick skin test, in which a drop of food extract is placed on the skin. The skin is then punctured through the drop with a sterile needle. Extracts of testing are usually supplied as 1:20 (wt/vol) extract in 50% glycerine. Skin testing with 1:1,000 (wt/vol) extracts may be performed by the intradermal technique. Intradermal tests, however, are more likely to produce clinically irrelevant positive results (64) and are associated with a higher frequency of systemic reactions that, in rare instances, have been fatal. Allergic reactions to foods are unusual in the face of negative skin tests (false negatives). Food extracts, including peanuts, tree nuts, egg, milk, soy, and fish, induce reactions that correlate reliably with allergic manifestations.

Patients should never be advised that they are allergic to certain foods solely on the basis of positive skin tests, because skin tests may be positive to foods in the absence of symptomatic food allergy (false-positive tests). In cases of the oral allergy syndrome, the use of extracts of fresh fruits and vegetables is often necessary to exclude IgE-mediated food hypersensitivity. Patients with allergic eosinophilic gastroenterocolitis have as many as 15 to 25 positive skin tests to various foods. Skin testing is not feasible or recommended in some clinical situations. Thus, in patients with extensive skin disease or significant and prolonged dermographism, or in patients in whom exposure to minute quantities of a specific food resulted in a life-threatening reaction, *in vitro* diagnostics are used to demonstrate food allergen–specific IgE.

ORAL CHALLENGE

Double-blind, placebo-controlled food challenge is the diagnostic procedure by which other diagnostic approaches are measured (65). Such testing is used in difficult cases in children and adults (66). Before these challenges, suspected foods should be eliminated for 10 to 14 days. Antihistamines are discontinued long enough to establish a normal histamine skin test. Other medications are minimized to levels sufficient to prevent breakthrough of acute symptoms. The food challenge is administered in a fasting state, starting with a dose unlikely to provoke symptoms. The dose is then doubled every 30 to 60 minutes or more, depending on the type of reaction suspected and the length of time required to produce symptoms. If the patient has tolerated 10 g lyophilized food blinded in capsules or liquid, clinical reactivity is generally ruled out. If the blinded challenge is negative, the food must be given openly in usual quantities under observation to rule out the rare false-negative challenge. To control for a variety of confounding factors, an equal number of placebo and food-antigen challenges are necessary, and the order of administration should be randomized. Double-blind, placebo-controlled food challenges should be conducted in a clinic or hospital setting, especially if an IgE-mediated reaction is suspected; furthermore, they should be done only if trained personnel and equipment for treating systemic anaphylaxis are present, and only with informed consent.

Treatment

Strict elimination of offending foods is the one proven therapy for immediate reactions to foods. However, severe elimination diets may lead to malnutrition or eating disorders (or both) and must be instituted with nutritional guidance. Patients must be instructed on how to read and understand food labels to detect hidden food allergens. Clinical reactivity to food allergens is generally specific. Patients may react to more than one member of a botanic family or animal species. No appropriately designed trial has demonstrated clear efficacy for the use of prophylactic medications or injection immunotherapy for preventing allergic reactions to foods.

Patients sometimes inadvertently consume food to which they are sensitive. Treatment for a specific symptom that results from inadvertent exposure is the same as that used when other factors provoke symptoms. Laryngeal or pulmonary symptoms after an inadvertent food exposure should be treated immediately with epinephrine, bronchodilator therapy, or both. The treatment of food-induced anaphylaxis is essentially the same as that for anaphylaxis due to a medication or insect sting (67). A patient with potential anaphylactic reactivity must be taught how to self-administer epinephrine and must have an epinephrine-containing syringe and an antihistamine available at all times. For children, day-care centers and schools should have a plan of action including a list of emergency numbers with backups to be called. Patients may exhibit only mild symptoms during the first few minutes after ingesting a food to which they are allergic, but this reaction may be followed 10 to 60 minutes later by hypotension and other severe problems. After self-medication for systemic reactions, the patient should immediately seek medical attention. All patients with IgE-mediated food allergy should be warned about the possibility of developing a severe anaphylactic reaction and should be educated in the treatment measures to be instituted in case of an accidental ingestion.

For those with atopic dermatitis, when exacerbation has been documented to food allergens, those foods should be eliminated from the diet. Symptomatic control of pruritus may be attempted with antihistamines. In severe cases, a brief application of topical glucocorticoid to control a flare of the disease may be warranted. When an infection is suspected, an antistaphylococcal antibiotic may be prescribed. Other general measures include avoidance of irritants such as soaps, wools, and trauma (e.g., that due to scratching). Maintenance of skin hydration is an important component of therapy.

Treatment of allergic eosinophilic gastroenterocolitis is often unsatisfactory. History and skin testing or *in vitro* measurements of food antigen-specific IgE may identify food allergens for a trial of elimination. A trial diet that eliminates multiple food antigens from the diet may be performed when no clear evidence of IgE-mediated hypersensitivity is found. In patients with eosinophilic gastroenteritis and food sensitivity, the number of foods involved may preclude the long-term use of an elimination diet for symptom control. Patients who respond poorly to dietary restrictions and those without evidence of food hypersensitivity may require oral glucocorticoid therapy with prednisone. Although this measure usually results in clinical improvement, the need for long-term glucocorticoid therapy is variable. As evidenced by improvement of clinical symptoms and resolution of the histologic abnormalities, prednisone may be tapered to an every-other-day regimen (58).

NON–IgE-MEDIATED REACTIONS

Dietary Protein Enterocolitis

Dietary protein enterocolitis is a disease of infants and children (57). It is diagnosed by documenting changes in the structure of the intestinal mucosa that occur in response to the ingestion of particular foods. These changes are transient and resolve with time, presumably because of maturation of the gastrointestinal and immune systems. Transient enteropathies have been associated with cow's milk, soy protein, fish, rice, chicken, and egg, as summarized elsewhere (68,69).

The pathogenesis of food protein–induced enteropathy is unknown. Because it is a disease of infants and resolves with age, it is assumed to be due in part to the immaturity of the gastrointestinal and immune systems. In some instances, food protein–induced enteropathy follows episodes of acute gastroenteritis, a condition that facilitates uptake of intact antigen. It is possible that the increased uptake of food protein due to the immaturity of the mucosal barrier and exacerbated by infections is the initial pathogenic feature. Multiple immune responses are then provoked, including recruitment of T cells secreting tumor necrosis factor-a (TNF-a) (70), and damage to the intestinal mucosa results.

A role for antibodies in the pathogenesis of mild protein-induced enteropathy has not been clearly demonstrated. During the neonatal period, circulating antibodies to cow's milk protein are virtually absent. However, by age 2 years, 95% of formula-fed infants have specific antibodies, primarily IgA and IgG. The presence of such antibodies provides evidence only of milk consumption and is not diagnostic of milk allergy. The serum IgE level is often normal in patients with cow's milk gastroenteropathy (71).

The most common cause of food protein–induced enteropathy is cow's milk. Clinically, in patients with cow's milk–induced enteropathy, chronic diarrhea and malabsorption may develop, typically associated with mucosal changes. At least 30% of the affected infants have a family history of some form of allergy. The mean onset of the milk-induced malabsorption syndrome is approximately 1 month after the introduction of cow's milk into the diet (68). Symptoms, typically vomiting and diarrhea, occur with either acute or gradual onset. It may be difficult to distinguish this illness from acute gastroenteritis in cases of acute onset. The presence of blood in the stool is indicative of milk protein colitis (72).

The intestinal villous structure is virtually normal if gastrointestinal symptoms are mild. Patients who are diagnosed and treated early typically show normal jejunal histology of partial villous atrophy. With active disease, marked changes consisting of increased numbers of lymphocytes, plasma cells, eosinophils, and neutrophils are seen in the mucosa and between the epithelial cells. The distribution and severity of associated lesions can vary substantially. Some infants have severe damage to the jejunum without involving the colon; in other cases, damage occurs predominantly in the colon (72). When cow's milk in eliminated from the diet, the prognosis is good for patients with gastrointestinal symptoms. Most patients become milk tolerant by age 2 years (73).

The treatment of food-induced enteropathy depends on the removal of milk or other sensitizing protein from the infant's or child's diet. An elemental formula or formulation or even a period of total parenteral nutrition may be required to allow repair and return of normal gastrointestinal function. Because this disease often resolves with time, reintroduction of the offending protein may eventually be attempted.

Other Food-induced Reactions

Vasoactive substances in food (e.g., caffeine, histamine, ethanol, nitrites) have been implicated in the onset of migraine headaches. Most studies have failed to associate migraine headaches with immunologically mediated adverse reactions to foods. This area remains controversial. One double-blind, placebo-controlled study implicated specific food-induced symptoms in 15% of 104 adults with migraine headaches (74).

Allergic reactions to foods have been implicated in some cases of sudden infant death syndrome (SIDS). Elevated serum tryptase levels were found in 40% of 50 infants who had died of SIDS. Fifteen of 38 had b-lactoglobulin–specific IgE compared with one of nine normal subjects (75). Similarly, another study found elevated serum mast cell tryptase levels in infants dying of SIDS compared with others dying unexpectedly of causes later explained; this finding suggests anaphylaxis around the time of death (76).

NATURAL HISTORY OF FOOD ALLERGIES

Food allergies in general cannot be prevented. However, food hypersensitivity reactions in infants may be delayed and in some cases avoided by breast-feeding. Many infants eventually become tolerant to the foods provoking reactions. In prospective studies of adverse food reactions in infants, 80% to 87% of confirmed symptoms were no longer present by age 3 years (2). In addition to infants, older children (77,78) and even some adults (79) lose their sensitivity if the responsible food allergen is identified and completely eliminated from the diet; after 1 to 2 years of allergen avoidance, as many as one third of children and adults lose their clinical sensitivity (78,79 and 80). Loss of sensitivity correlates with allergen avoidance, but skin-prick test and RAST may remain positive and do not allow prediction of which individuals will lose clinical reactivity to specific foods. Patients with an allergy to peanut, tree nut, fish, or shellfish rarely lose their clinical reactivity (78,80,81).

GENETIC MODIFICATION OF FOODS

The identification of Brazil-nut allergen in transgenic soybean (82) and the subsequent decision not to market this product have paralleled efforts to develop rational approaches to prevent or limit the spread of food allergens. This effort is occurring when it has become technically possible to alter the genetic composition of foods derived from both plant and animal sources to increase yield, to provide pesticide or drought resistance, or to alter the composition of the food to decrease allergenicity or to improve its nutritional value.

At least four technical approaches may be used to determine if a modified food may be or is enhanced in its allergenic potential (82,83,84,85 and 86). First, the amino acid sequence coded for by a transferred gene may be determined. A search is then performed to identify similarity to known allergens from both plant- and animal-derived foods; inhalant allergens such as pollens and fungal spores; insect venoms; and allergens that provoke immediate contact reactions. Because the optimal peptide length for binding appears to be between eight and 12 amino acids for T-cell epitopes and even longer for B-cell epitopes, some have suggested that the sequence identity requires a match of at least eight contiguous identical amino acids. There are significant limitations to this molecular approach. For example, not all food allergens have been sequenced, and this strategy does not identify conformational or noncontiguous epitopes. As a second approach, when a gene is transferred from a known allergenic food or an allergenic food of concern, a determination may be made about whether serum from individuals sensitive to the food of origin contains allergen-specific IgE that will bind with the gene product. These assays are usually performed as solid-phase immunoassays, such as the RAST or RAST inhibition assays or the ELISA. If the gene transferred is derived from a source in which there is no general history of its use in the population and thus its allergenic potential cannot be assessed, a third approach, stability to digestion, may be used. As a general principle, food allergens appear to be relatively resistant to acids and proteases. It has thus been argued that stability of food allergens to digestion may be used to help identify the products of transferred genes as to their possible allergenicity (87). However, insufficient information is available on possible differences in susceptibility to acid denaturation and susceptibility to proteases among various allergenic food proteins and with proteins that possess weak or no allergenic potential to use this test in any way other than as a relative indication of allergenic potential (88). Finally, the use of animal models in the determination of food-protein allergenicity also may be considered. Such an approach would help identify whether a gene product is allergenic in the absence of a history of exposure to that protein in the general population. A screening test to determine the allergenic potential of a modified food with an animal model also would help to address the question as to whether the genetic manipulation led to the expression or upregulation of an allergen in the recipient organism. It is possible to make animals of various species allergic to specific proteins, usually by injecting them intraperitoneally with the protein in an adjuvant. The most commonly applied mouse model of asthma uses ovalbumin as its antigen, which is administered intraperitoneally in alum. The difficulty arises in using sensitization by this means as a test for allergenicity, because both allergenic and nonallergenic proteins applied in this manner may provoke antigen-specific IgE. Indeed, it may be too much to expect any one animal model to predict allergenicity in humans to a specific food. However, it may be reasonable to expect (with careful selection of the study animal, the route, and the frequency of administration of the antigen) that an animal model might be developed that provides some ranking of allergenicity.

SUMMARY AND CONCLUSIONS

Adverse reactions to foods can be either immediate or delayed. Immediate reactions to foods seem to be due to the production of food-specific IgE in some predisposed individuals. Interactions between food antigen and specific IgE leads to a pathologic process mediated by mast cells and basophils. The food antigens involved are those more likely to survive the process of digestion by virtue of their intrinsic properties. Delayed reactions to food are more diverse in their expression and in the biologic mechanisms involved.

It is perhaps surprising that adverse reactions to foods are not more frequent. Survival depends on food ingestion, which is a continual process. Mechanisms to regulate digestion and immune responses are complex and interrelated. For these reasons, the belief that many more diseases might be related to, or influenced by, adverse responses to foods has long been attractive. The search for such associations should continue, with sufficient scientific skepticism to ensure that valid observations result.

In all probability, the number of adverse reactions to foods will increase with time. Much of the increase will be directly related to two factors: the need to feed a growing population with protein from increasingly diverse sources, and the development of new biologic techniques to process, preserve, and alter foodstuffs. In the first case, potential reactors are simply exposed to more antigens to which they may respond. In the second instance, new antigens are created that are capable of eliciting adverse reactions. The potential for a real increase in the number of adverse reactions to foods is accompanied by an increasing public awareness of the reality that reactions to foods do occur. These factors will undoubtedly combine to heighten scientific interest in reactions to foods.

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69 INSECT VENOM ALLERGY

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Warm breeze aloft,
Bees in nectar buzz. Does this
foretell my next sting?
A Poet
Hot day.
Step on a bee, you may get stung.
You expected maybe honey?
A Cynic

A small proportion of individuals who are stung by members of the order Hymenoptera have not only local irritation but also varying degrees of the signs and symptoms of acute, generalized anaphylaxis. This chapter is devoted primarily to a review of the evolution of our understanding of the anaphylactic response to insect stings: its diagnosis, treatment, and prevention. Inhalant allergy to the detritus of other insects and insectlike arthropods (cockroach, midge, and house-dust mite) is covered in Chapter 61.

TAXONOMY

The phylum Arthropoda includes creatures with segmented bodies and jointed appendages. It is divided into a number of classes, among which is the class Insecta, which includes bugs, beetles, butterflies, and the order Hymenoptera. The only insects that have a true stinger are the Hymenoptera; and because the stinger is a modified ovipositor, only the female can sting.

The family Vespidae of the order Hymenoptera includes a variety of socially organized insects that make paper nests constructed from wood fiber, chewed until pulpy; they are referred to as hornets or wasps. There are three important genera of vespid insects. The genus *Vespula* includes the ground-nesting yellow jackets and the aerial New World hornets, which construct large globular nests found suspended from the branches of shrubs or trees. Wasps of the genus *Polistes* also construct suspended paper nests; but because they do not enclose the nest in a paper sheath, the hexagonal cells where eggs are laid and the young develop are in plain sight. The genus *Vespa* comprises the Old World hornets, which are common in North America.

The family Apidae includes the honeybee *Apis mellifera*, which was introduced from Europe and is represented by several races of a single species. The African “killer” bee represents an aggressive race introduced to Latin America in an attempt to breed bees with superior honey-producing qualities. Bumblebees (genus *Bombus*) are closely related to honeybees and have been reported to cause allergic sting reactions, as have their more distant relatives, the sweat bees (family Halictidae).

In North America, allergic reactions to Hymenoptera stings are usually due to the honeybee (family Apidae), yellow jacket, yellow hornet, bald-faced hornet (genus *Vespula*), and paper wasp (genus *Polistes*). In the South, such reactions are also caused by the imported fire ants (genus *Solenopsis*), which have true stingers in addition to the mandibles used to grasp and pivot on the victim.

Honeybees, if held individually, expel a droplet of venom when the abdomen is placed in apposition to a suitable surface. The solids that remain after evaporation of the volatile portion of the venom weigh, on average, 50 µg per droplet and appear to represent the entire contents of the venom apparatus of the honeybee, which, under more natural circumstances, stings only once in its life. The honeybee's barbed stinger resists easy removal and usually remains behind as the bee flies away, resulting in fatal exenteration for the bee. Vespids do not generally leave their stingers behind and may sting several times in rapid succession. It has been estimated that a vespid sting delivers 2 to 20 µg of venom protein (1).

VENOM CONSTITUENTS

The term *venom* implies the presence of constituents with toxic properties. Hymenoptera venoms are inherently toxic to other members of the same species and to other small creatures, and they serve as a defense against individual or territorial depredation. The omnivorous Vespidae also use the venom in their stings to immobilize and capture live prey, on which they may feed. Honeybees subsist on pollen and nectar and thus sting only in defense, which perhaps accounts for the apparent qualitative differences between honeybee venom and other venoms. Pollen proteins are not present in honeybee venom, although they do appear in honey. Of the Hymenoptera venoms, that of the honeybee has been studied most extensively. Habermann (2) reviewed this subject and enumerated various constituents. Low-molecular-weight vasoactive substances in honeybee venoms include a significant amount of histamine, which also is present in vespid venoms. Honeybee venom contains three major allergenic enzymes, which functionally are phospholipase A_2, a hyaluronidase, and an acid phosphatase (3,4 and 5). Melittin, another polypeptide in honeybee venom, possesses detergent activity; a small polypeptide called apamin has neurotoxic properties. Honeybee phospholipase A_2 is immunologically distinct from enzymes with phospholipase A activity from other sources.

The major vespid venom allergens include a nonenzymatic protein designated antigen 5, as well as proteins with phospholipase A and hyaluronidase functional activity; these constituents have physical and immunologic properties that are different from those of the honeybee venom allergens (4,6,7). It has been shown, however, that there is a low-grade cross-reactivity between yellow jacket and honeybee venoms in a few unusual patients (8,9). The various vespid venoms, in contrast, have a high degree of cross-reactivity and contain essentially the same allergens (3,6,7,10,11). The yellow jacket and hornet venoms are so closely related that 95% of vespid-allergic patients have positive skin tests to all three of the common skin-test preparations: yellow jacket, yellow hornet, and white-faced hornet venoms. *Polistes* wasps are more distantly related to the other vespids, and only 50% of yellow jacket–allergic patients have positive skin tests to wasp venom (12,13).

Although it is now widely accepted that Hymenoptera venoms are the materials to be used for the diagnosis and therapy of insect sting allergy, this has been the case only since 1979. From the early part of the twentieth century, the allergic sensitivity to stings was thought to be directed against an “intrinsic bee protein” present in the insects' bodies and venoms. Benson and Semenov (14) studied a beekeeper who manifested allergy both to stings and to inhalation of material in the air around bee colonies. This patient had become sensitized to venom and developed anaphylaxis when he was stung. He also displayed symptoms of asthma on inhaling the bee body dust, which was stirred up when he worked around beehives. Skin testing this patient gave positive results with both venom and whole-body extracts (WBEs). Some years later, studies with antisera raised in animals showed one or more antigens common to venom and body extracts and antigens common to several Hymenoptera (15,16). These studies notwithstanding, only venoms, not WBEs, can distinguish patients with anaphylactic sting reactions from normal individuals by skin tests (17,18) or *in vitro* tests for specific immunoglobulin E (IgE) antibodies such as the radioallergosorbent test (RAST) (19,20) and leukocyte histamine release (12,21,21a). Some investigators have proposed a live sting challenge before prescribing immunotherapy to assess risk of future reactions (22). WBE preparations retain negligible quantities of biologically active venom antigens (23,24). Most important, WBE is ineffective for preventing allergic sting reactions, and many treatment failures, even deaths, have been reported (23,25). However, WBE of imported fire ants is still used for diagnosis and treatment of this common sensitivity, as it contains most of the relevant allergens (26,27 and 28).

MANIFESTATIONS OF SYSTEMIC REACTIONS

The terminology describing systemic allergic reactions to insect stings may vary, but the events themselves seem fairly straightforward. Although it is simpler to regard reactions as either systemic or local, there is a continuum ranging from the least local response to the ultimate systemic response, death. All the responses that occur fairly promptly after a sting can be explained in terms of our current knowledge of the nature of IgE-mediated reactions: They seem to result from increased vascular permeability, smooth muscle contraction, a vasodepressor effect, or a combination of these manifestations (29). These effects result from the release or production of chemical mediators of anaphylaxis by the mechanisms outlined in Chapter 24. When death occurs, it may be the result of irreversible hypotension, asphyxiation secondary to laryngeal edema, or severe bronchospasm.

The variability of the allergic response to insect stings is not well understood. Many patients give a history of predictable large local reactions to stings. Others have had systemic symptoms on some occasions and local reactions on others. Furthermore, it has been observed that 35% to 60% of sting-allergic patients fail to react when deliberately challenged, even though they may react to subsequent stings (22,30,31,32,33 and 34). This observation is reminiscent of the situations in penicillin allergy and food allergy, in which only half of skin test–positive patients react to a provocative challenge (34,35). Although it is widely believed that allergic reactions become progressively worse with each subsequent sting, systemic reactions to stings tend to recur in a stereotyped fashion; a patient rarely experiences reactions of increasing severity. When successive reactions to stings occur in unimmunized adult patients, the clinical pattern is most often the same as it had been on the occasion of a previous sting (31). In unimmunized children with histories of systemic reactions limited to the skin (e.g., urticaria), the risk of subsequent sting reactions is not the 50% to 60% seen in adults but, rather, 10% to 20% (36). Moreover, the risk of progression to more severe reactions in such children is small.

Systemic allergic sting reactions present the classic manifestations of anaphylaxis. Cutaneous, vascular, or respiratory symptoms may occur singly or in any combination. Cutaneous symptoms are most common (affecting 80%); they are the sole manifestation in 15% of sensitive adults and more than 60% of affected children (37,38 and 39). Almost 50% of children and adults have respiratory complaints. Although uncommon in children, hypotension occurs in more than 60% of adults, and half of these patients experience loss of consciousness. Fatal reactions are rare, although the risk of a fatal outcome increases with age, as might be expected. Fatal anaphylaxis may occur without a history of sting allergy (40).

Large local reactions are perhaps misnamed, as it is the late and prolonged time course that is characteristic. Exaggerated local inflammation, progressive over 24 hours and lasting 2 to 7 days, may cause significant morbidity. These reactions resemble the late cutaneous allergic response described by Umemoto et al. (41) and the late-phase reaction studied by Solley et al. (42), both of which are IgE dependent. From 50% to 80% of patients with large local reactions have detectable venom-specific IgE (43,44 and 45).

Insect stings may result in a variety of unusual reactions involving the neurologic and vascular systems (for example, serum sickness, vasculitis, nephropathies, encephalitis, and other neurologic manifestations including Guillain-Barré syndrome, and peripheral and central neuropathies) (46,47). Large numbers of stings have resulted in rhabdomyolysis and renal failure (48). Immune mechanisms may mediate some of these reactions, but the relation to immediate hypersensitivity is obscure.

INCIDENCE OF SYSTEMIC REACTIONS

Although only 40 deaths per year resulting from insect stings have been reported in the United States (49), there may be many more that are not recognized because the immediate cause of death (e.g., myocardial infarction or stroke) is really a vascular complication of anaphylaxis. Postmortem blood samples have been analyzed to determine if some cases of unexplained death were due to insect-sting anaphylaxis (50,51). Formerly, the only published studies of the epidemiology of insect sting anaphylaxis were surveys of the medical records of Boy Scouts during summer camp. Two such studies found the frequency of systemic sting reactions in children to range from 0.4% to 0.8% (52,53).

There have been reports of the frequency of insect sting allergy in adults (54). In the largest study, a stratified random sample of a population of “normal” factory workers was found to have a 4% cumulative prevalence of systemic allergic sting reactions, and more than 20% had either RAST or skin-test evidence for sensitization to Hymenoptera venom (55). Others have confirmed these findings (56). It seems probable that a significant number of adults develop transient venom sensitivity from stings and fail to have reactions when next stung because the level of their IgE antibody to venom has spontaneously declined. The actual risk for a systemic sting reaction in a person with a positive skin test but no prior sting reaction is unknown. Patients with both positive tests for venom-specific IgE antibody (skin or *in vitro*) and a previous systemic reaction are at high risk, but clearly at less than 100%. Their risk of reaction ranges from 25% to 60% for any given sting (30,31 and 32). In contrast, patients with positive tests for venom IgE and previous large local sting reactions (but no systemic reactions) have a risk of approximately 5% to 10% for future systemic sting reactions (43,57). Because asymptomatic sensitization (positive venom tests with a negative history) may be present in 10% to 15% of the general population (55), routine use of venom skin testing as a screening procedure is of no benefit. The frequency of sting reactions is similar in atopic, nonatopic, and unselected populations (58), but venom sensitivity on skin test seems to occur more frequently in persons with skin test sensitivity to inhalant allergens (55).

Because an obvious prerequisite for sting-induced anaphylaxis is exposure, it is expected that individuals with increased exposure would experience an increased rate of systemic reactivity. In fact, areas supporting agricultural crops that require pollination by honeybees and that also require a relatively large work force for harvesting report that most sting-related reactions are due to honeybees. In other areas, vespids seem to account for more reactions. Beekeepers and their families form a group with a greatly increased risk of sting sensitivity (59). It is significant that professional beekeepers, who are stung regularly (as often as 50 to 100 times a week) seem to experience fewer systemic reactions than do less frequently stung amateurs, or their family members. Furthermore, when beekeepers do experience anaphylaxis, it is usually when they receive their first or second sting of the spring, having not been stung since the previous autumn. One may therefore conclude that frequent, regular stings are associated with the development of immunity but that infrequent stings at irregular intervals may predispose to an increased risk of anaphylaxis.

DIAGNOSIS OF STINGING-INSECT ALLERGY

The diagnosis of insect-sting allergy rests on a convincing history and positive venom skin or serologic tests for IgE antibodies. The clinical history is perhaps the most critical aspect of diagnosis because in the absence of an appropriate history, all other techniques are merely suggestive. The risk of future reactions has not been ascertained in relation to skin tests or *in vitro* tests alone. No diagnostic test can absolutely rule out venom sensitivity or risk in the presence of a convincing history. Because successive stings may evoke differing responses because of induced alterations in the patient's immune status, even an uneventful deliberate sting challenge cannot entirely ensure future immunity.

The method of choice for detecting IgE antibody to venoms is intradermal skin testing with Hymenoptera venoms and venom sac extracts. Five such extracts are available for clinical use: honeybee (HB), yellow jacket (YJ), yellow hornet (YH), white-faced hornet (WFH), and *Polistes* wasp (Pol). Venoms are provided as lyophilized powders, to be reconstituted with special diluent containing human serum albumin as a stabilizing agent. Dilutions of venom more concentrated than 3.0 µg/mL are irritating to a significant number of nonallergic individuals, and 1.0 µg/mL is usually the highest concentration used for skin testing. Approximately 90% of individuals with histories of systemic or large local sting reactions have positive skin tests to one or more venoms at a concentration of 1.0 µg/mL or less. The testing procedure is generally started intradermally at a concentration of 0.01 µg/mL; this dose is preceded by a scratch or prick test if there is concern about provoking a systemic reaction with this small amount of venom. Because sting reactions may have occurred to only one insect type despite sensitization to others, skin testing should always be performed with a complete set of Hymenoptera venoms and a negative diluent control. If all tests are negative, a positive histamine control can demonstrate the ability of the skin to react.

Our experience with more than 1,000 patients has confirmed the findings of Hunt et al. (17) that the diagnosis of nearly 25% of patients with systemic sting reactions would have been missed if the patients had not been tested at concentrations up to 1.0 µg/mL. Furthermore, the degree of skin-test sensitivity has not been consistently correlated with the severity of the sting reaction, as there are many individual patients with positive skin tests only at concentrations of 1.0 µg/mL who have had severe life-threatening sting reactions. Occasionally a patient is seen in whom there is a convincing history of systemic anaphylaxis, yet whose skin tests with the highest venom concentrations yield negative or equivocal results. This condition might be the result of spontaneous loss of the insect-allergic state, attributable to a gradual diminution of IgE antibody production, as occurs in some patients who successfully avoid stings for a long time (37). In patients with a recent episode of sting anaphylaxis, negative skin tests are of unclear significance. Some believe that a period of “anergy” follows a sting reaction. In any case, it would be prudent when in doubt to repeat the skin tests several months later, as some persons may become more clearly positive. There have been reports of reactions to stings in patients with negative skin tests (60); whether the reactions are IgE mediated or anaphylactoid is uncertain. In some individuals with urticaria pigmentosa, after stings, systemic reactions develop, caused by a nonimmunologic release of mediators from the abnormally large numbers of mast cells present (61).

There are many patterns of skin test sensitivity to the Hymenoptera venoms. Honeybee sting allergy is independent of allergy to other insects because honeybee venom is antigenically distinct from all other venoms. Fifteen percent of our adult patients evaluated for sting reactions have honeybee venom sensitivity, and many of these individuals are beekeepers or their families. Honeybee venom allergy is more common in children and in the southwestern United States. More than half of these honeybee-sensitive patients also are sensitive to vespid venoms (17,18). The vespid venoms are highly cross-reactive, so that more than 90% of vespid-sensitive patients have positive skin tests to yellow jacket, yellow hornet, and white-faced hornet, even though most have been stung only by a yellow jacket (13). Half of these patients are also sensitive to *Polistes* venom, and the converse is true of patients with primary wasp-sting allergy (12). Few patients have positive skin tests to only one or two insect types, presumably denoting a uniquely selective response to nonshared antigenic epitopes. Sensitivity to all five Hymenoptera venoms is not uncommon but appears most frequently in careless children and in patients who have had previous WBE therapy (39).

An alternative method for detecting venom-specific IgE antibodies is the RAST, which measures the quantity of such antibodies in the patient's serum. The sensitivity and specificity of the venom RAST are less than those of the skin tests, and certain vagaries may make the RAST test less reliable than the skin test (62). The test depends on the ability of antigen-specific antibody to bind to antigen that is coupled to an insoluble and inert support: paper disks, modified cellulose particles, or other, similar material. Unless care is taken to use a pure, “clean” antigen preparation, nonspecific binding can take place, resulting in false-positive tests. In serum samples in which antibodies are present that belong to both the IgE and the IgG classes, as would be expected to occur in the patient who has been receiving immunotherapy with venom and in a patient who has had a fairly recent sting, the smaller (nanogram) quantities of IgE antibody may have difficulty binding to the allergen disk because of the presence of microgram quantities of IgG antibody. The practical consequence of this type of error is gross underestimation of the amount of IgE antibody present. Technical means are available to compensate for the disparity in concentrations of the two classes of antibody, although they are not usually used routinely with the RAST technique. Thus although the RAST is a good test, we have found it more expensive and less sensitive than the skin test (15% of patients with positive histories and positive skin tests have a negative RAST), and in our clinic, the test is used primarily as an adjunct for evaluating patients with insect-sting allergy.

During studies of the natural history of insect-venom sensitivity, the clinical significance of a positive venom RAST has been reevaluated (63,64). It is possible that individuals with a positive venom RAST and a negative skin test would have had positive skin tests at an earlier time, and that the RAST-positive, skin test–negative state represents one stage in the gradual, spontaneous loss of venom sensitivity. A positive RAST in the presence of a negative skin test is often seen after a patient has undergone several years of venom immunotherapy (M.D. Valentine, unpublished observation). After 2 years of observation, the previously positive RAST became negative in 6% of skin test–positive patients and in 52% of skin test–negative patients. Transient serologic evidence of sensitization after a sting is common, even when the skin tests remain negative. In the studies cited, individuals without prior sting-reaction histories were not at risk for subsequent reactions unless both RAST and skin tests became positive. In view of these discrepancies, we believe that the skin test remains the diagnostic test of choice.

TREATMENT OF ACUTE REACTIONS

Acute reactions to stings are treated symptomatically (65). Most large local reactions can be managed with ice packs, rest, and antihistamines. Extremely large local reactions (e.g., reactions large enough to interfere with normal activities, or if in the head or neck, reactions that hinder breathing or swallowing because of localized edema) may be attenuated by a brief, tapering course of steroids.

Hypotension and hypoxia are two of the manifestations of insect-sting anaphylaxis that require urgent and vigorous treatment. The two drugs of choice are epinephrine and oxygen. Both the a- and b-adrenergic stimulating effects of epinephrine appear to be useful in treating anaphylaxis, causing vasoconstriction, bronchial relaxation, and reversal of the enhanced vascular permeability that results from the release of mediators such as histamine. Vasoconstriction without replenishment of the depleted intravascular volume may further aggravate impaired perfusion of vital organs, and volume replacement with saline or plasma may be necessary to restore normal blood pressure (29). Epinephrine may be ineffective or paradoxically harmful if given to patients who are taking a b-blocking drug. b-Blockers are relatively contraindicated in patients at risk of insect-sting anaphylaxis or in those receiving immunotherapy for whom anaphylaxis, though unlikely, remains a risk.

Antihistamines taken orally may inhibit urticaria and pruritus but are unlikely to reverse shock, laryngeal edema, or bronchospasm; overgenerous parenteral doses of antihistamines may worsen or cause hypotension. The role of glucocorticoids in anaphylaxis remains unsettled, although such drugs clearly cannot by themselves effect rapid reversal of the more serious manifestations. When honeybee stings result in a retained stinger and attached venom sac, the stinger should be flicked away, not pulled. Whether the sac still retains some venom is not known, but it is generally considered advisable to avoid squeezing it as the stinger is removed.

The National Institutes of Health Consensus Development Conference recommended that (a) epinephrine kits be available to all individuals at risk of systemic sting reactions; (b) patients be carefully instructed in the use of such kits; and (c) the lay and professional public be better educated on the dangers of insect stings and the means of treating them (65). A recent survey of allergists and other physicians found that most of those surveyed were unable to provide correct instructions in the use of an epinephrine autoinjector (66).

PREVENTION OF STINGS

Some stings are unavoidable. Certain precautions make others less likely. It is prudent for allergic persons to avoid going barefoot, to eschew perfumed cosmetics and toiletries, to avoid eating and drinking in areas accessible to stinging insects, and to dress in neutral colors, avoiding floral prints. Such bucolic pleasures as grass cutting and hedge trimming should be relegated to nonallergic relatives, willing neighbors, or professionals. Fallen fruit and laden trash cans invariably attract the Hymenoptera and should be avoided. Some allergists have recommended oral thiamine in doses sufficient to render the subject an object of olfactory repugnance. Whether the Hymenoptera shun this odor is not proven, although it is clear that certain aromatic esters attract specific insects (*n*-pentyl valerate has been used as an olfactory bait in commercial yellow-jacket traps).

IMMUNITY FROM VENOM-INDUCED ANAPHYLAXIS

IgG Antibodies

The mechanism of action of allergen immunotherapy is not completely understood. Of the many immunologic responses evoked by immunotherapy, increased production of specific IgG antibodies has been most consistently and directly correlated with clinically successful treatment (67,68,69,70 and 71). Functional “blocking” activity was first found in the sera of pollen-immunized hay-fever patients when it was demonstrated that these sera contained an antigen-specific substance capable of blocking the Prausnitz-Küstner reaction in the skin of nonallergic recipients (72). Later it was shown that “blocking” could be demonstrated by inhibition of antigen-induced histamine release *in vitro*, and that this blocking capacity was entirely contained within the IgG fraction of serum (71). Serum containing high titers of such blocking antibodies with specificity for honeybee venom has successfully conferred passive immunity on individuals allergic to honeybee sting (33,73). It thus seems that IgG-blocking antibodies are at least partially responsible for the protection induced by venom immunotherapy. Venom-specific IgG antibodies can now be accurately measured in the serum with sensitive and specific assays similar to the RAST (74). When determined by this method, the level of venom-specific IgG has been correlated with the degree of protection from systemic reactions to challenge stings and has become a useful test for monitoring the efficacy of treatment (44,75). Observations of more than 200 sting challenges showed that systemic reactions, albeit mild, occurred in only 1.6% of patients with an IgG level greater than 3 µg/mL, whereas those with lower IgG levels had a 16% frequency of reactions (44). Of interest was the fact that there was no such increase in reaction rate in patients who had undergone venom immunotherapy for more than 4 years. Perhaps prolonged venom immunotherapy provides clinical protection by another mechanism that is of late onset but still unknown (76).

History

Initial attempts to use Hymenoptera venoms for immunization were on an entirely empiric basis (77). Investigations of venom therapy were started in patients who were clearly not protected by WBE treatment. The first such patient was gradually immunized with increasing doses of venom until he could tolerate an injection containing venom equivalent to approximately two stings. At that point, his serum was found to contain a high titer of IgG-blocking antibodies that could inhibit venom-induced histamine release from sensitive leukocytes. When he was challenged in the hospital with a live bee sting, he had no systemic reaction (71). This initial success led to the treatment of a small series of patients who also were successfully challenged with live insect stings.

The definitive study of venom therapy was conducted as a double-blind comparison of therapy with Hymenoptera venom, placebo, or WBE (68). Live challenge stings resulted in systemic reactions in 60% of both the placebo-treated and WBE-treated patients but in only 5% of the venom-treated patients. When the placebo- and WBE-treated patients were subsequently treated with venom, they too had a 5% systemic reaction rate. Additional trials of venom therapy in several hundred adults and children confirmed the safety and efficacy of the treatment and helped to establish the optimal regimens for initial and maintenance therapy (44,78).

Indications

Patients are selected for venom immunotherapy on the basis of their history and the results of venom skin or serologic tests (17,64,68). The only clear indication at present is to prevent future anaphylactic sting reactions in patients with a history of such reactions who have positive tests for venom-specific IgE antibodies (79). Patients with negative tests are not candidates for venom immunotherapy; such patients may have had a sting from an insect whose venom is not available for testing or treatment [e.g., bumblebee or sweat bee (80)] or may have spontaneously lost their sensitivity (31). Although some do not advise treatment of adults with mild (cutaneous only) reactions, no prospective data are available that define the risk of withholding treatment in this group. Children with reactions confined to the skin (urticaria, angioedema, or generalized flushing and pruritus) have been found in a prospective study to be at low risk of future reactions of similar severity and at negligible risk of reactions of greater severity (36,38). Although such children can be safely managed conservatively (counseling in sting avoidance, use of epinephrine self-treatment kits), venom therapy should be offered to those children who have had severe reactions.

From 50% to 90% of child and adult patients who have had only large local sting reactions without systemic symptoms have been found to have positive venom tests for IgE antibody (43,81,82) but are not usually immunized, as their risk of a future systemic reaction is estimated at only 5% to 10% (43,81). There is still no test *in vitro*

or *in vivo*, except the sting challenge, that can predict who of this relatively large group will react systemically when stung. In fact, large local reactions occur so commonly that it is of doubtful use to perform venom skin or blood tests on such patients, although a negative test should rule out the possibility of a future systemic reaction. However, children treated for prevention of systemic allergic reactions have been found to experience fewer large local reactions than did a group of comparable, unimmunized children (45).

Initial Therapy

To initiate venom immunotherapy, the physician must decide which venoms to use, the maximal dose to achieve, and the schedule to follow. The choice of venoms is based primarily on venom-test results. Although we currently recommend that immunotherapy include all venoms giving an unequivocally positive test, aiming to provide maximal security to the patient, others suggest that the clinical history can be used to limit the number of venoms used (83). Despite the marked cross-reactivity of the various vespid venoms, we believe the most practical approach to the patient with positive reactions to hornet and yellow jacket venoms is to treat with mixed vespid venoms. It has been shown that treatment of yellow jacket–sensitive patients with mixed vespid venoms provides a better clinical and IgG antibody response than does treatment with yellow jacket venom alone (13). However, when sophisticated laboratory facilities are available, it can often be established with the technique of RAST inhibition that multiple positive serologic tests for venom IgE antibodies are due to cross-reactivity. The most common example of this type of cross-reactivity in Baltimore, Maryland, for example, is a positive test for the *Polistes* wasp resulting from yellow jacket sensitization.

One problem with single-venom therapy is that the IgG response is not so great as with mixed vespid venoms. Complete clinical protection was less frequently observed with yellow jacket venom alone (80% to 85%) than with mixed vespid venoms (98%) (13). In such cases, increased venom doses (up to 200 µg) may be required. There also is evidence that, aside from the obvious antigenic differences, the results of yellow jacket venom therapy are different from the results of therapy with honeybee venom (84). The usual recommended dose to be achieved with the initial treatment schedule is 100 µg of each relevant venom because this regimen provides virtually complete protection to 98% of patients treated. Half-dose (50 µg) treatment may be adequate for some patients but has proved to be inadequate in as many as 20% of patients(67).

The recommended treatment schedule for the venom-sensitive patient is more intensive than traditional regimens for other allergen immunotherapy, achieving the maximal recommended dose with a series of six to eight weekly injections (85). This course, although seemingly accelerated, has been associated with fewer adverse reactions per treatment course and a greater, more rapid increase in venom-specific IgG antibodies than the slower, more traditional regimen. Both regimens, however, are clinically effective (85).

Adverse reactions to venom immunotherapy are much less common than had been feared, occurring no more frequently than with effective immunizing doses of any other allergen. When systemic reactions occur, they often resemble the patient's original sting reaction, usually in the same individual pattern, and may vary in severity from such a subtle subjective sensation as a lump in the throat or lightheadedness to more severe reactions. Systemic reactions to venom injections occur in 5% to 15% of patients during the induction phase of therapy, regardless of the regimen used (85). Although mild systemic symptoms are not infrequently reported, objective reactions requiring treatment occur less often and are only rarely a serious impediment to the therapeutic regimen. Although adverse reactions appear to be less common in children (37), our experience with adults parallels that of Levine (86), who observed systemic reactions to all types of immunotherapy in 15% of his patients, one third of whom required treatment with epinephrine.

Systemic reactions usually occur early in treatment, especially at doses less than 2.5 µg or in the range of 20 to 30 µg. After the IgG response becomes measurable (at doses >25 µg), these reactions are rare. Recurrent problems occasionally require prolonged treatment with submaximal doses until the patient's tolerance improves. However, it may be counterproductive to reduce the dose repeatedly after recurrent systemic reactions, because low-dose therapy can stimulate IgE but not IgG production. In such a case, it is preferable to discontinue therapy for 6 to 12 months and then try again. Alternatively, patients may be pretreated with mediator antagonists with a regimen similar to the one recommended for preventing reactions to iodinated contrast media (87).

Large local reactions to venom injections occur in 50% of patients and only rarely interfere with the progress of therapy. These reactions are seen most frequently (20 reactions per 100 injections) at doses between 15 and 50 µg, when the immune response is not yet optimal. At lower and higher doses, large local reactions are uncommon (five reactions per 100 injections) (88). Slow regimens are associated with the same frequency of reactions as rush regimens, and large local reactions often continue to occur until immunologically effective doses are administered. Furthermore, in contrast to popular belief, large local reactions do not indicate an increased risk of systemic reactions at larger doses. Patients may tolerate these local reactions (unless they are extreme or involve a joint) if they understand that the best way to overcome them is to proceed with the prescribed regimen. Long-term toxicity or side effects have not been observed to date. After 20 years of venom immunotherapy, we have observed no physical or laboratory abnormalities attributable to venom immunotherapy. Routine hematologic, biochemical, and urine tests have remained unchanged. Although it is not known if administration of venom solutions over a much longer term has toxic effects, it is reassuring to note that beekeepers and their families, who experience hundreds or thousands of stings over the course of a year or two, have had no adverse clinical or laboratory results.

Maintenance Therapy

During the first several years of treatment, boosters help to maintain the protective IgG response, which declines gradually if treatment is discontinued. Seasonal therapy is not recommended because it involves as many visits and injections, and as much expense, as would year-round therapy. Once the full dose of 100 µg (of each venom) is attained, the dose is repeated monthly. Although the rate of treatment failure is low, we advocate measuring the serum IgG antibody level against the immunizing venom(s) to confirm that the response has been adequate (44,75). Poor immune responses are seen in fewer than 5% of patients treated with mixed or multiple vespid venoms and up to 10% of patients treated with any single venom. Such patients may require treatment with doses of venom increased 50% or 100% to achieve “adequate” IgG levels.

After six monthly maintenance doses, the interval between doses can be safely lengthened to 6 weeks if the serum venom-specific IgG is 3.5 µg/mL (88). Six months later we recheck the IgG and do so again at 12 to 18 months. Others omit these measurements (89).

The question of when venom immunotherapy may be safely stopped must have a different answer for each patient (90). Many of the immunologic consequences of the treatment have been documented. There is a brisk increase in IgG antibody soon after treatment is started; and this response is maintained in most patients during many years of booster injections. The venom-specific IgE antibody level also increases when immunotherapy is initiated and decreases toward pretreatment levels after the first year of therapy. There is a steady subsequent decline as treatment is continued. Venom skin-test sensitivity also declines in at least half of treated patients but remains unchanged in the others. After 5 years of observation, 5% to 10% of treated and untreated patients lose all evidence of venom-specific IgE antibodies (negative skin tests and RASTs) (45,57). After 8 to 10 years, venom skin tests and RASTs become negative in 25% of untreated patients and in 50% of treated patients (19,91).

It seems reasonable to discontinue treatment if both skin test and serologic evidence of IgE antibodies has disappeared. However, the risk of sting reactions in patients with persistent IgE venom antibodies who stop venom immunotherapy after 1 to 3 years of treatment increases to an unacceptable figure soon after cessation of therapy (31). Within 6 months after venom therapy is stopped in such patients, the serum levels of venom-specific IgG antibodies decline to levels typical of those of untreated patients (57,92).

Although venom therapy is usually associated with an increase in IgG antibodies as IgE antibodies decrease, the relation is not strictly reciprocal. Although it has been recommended that therapy be continued indefinitely or until skin and RAST (IgE) tests are negative, studies suggest that prolonged (4 to 5 years) venom immunotherapy results in a state of resistance to sting reactions that is independent of elevated IgG or suppressed IgE antibodies (91). This finding is in sharp contradistinction to the definite dependent relation sting immunity bears to elevated IgG antibodies earlier in the course of treatment. In patients who have undergone 5 uninterrupted years of venom immunotherapy, sting challenges as many as 5 years after stopping treatment have been well tolerated, with only 5% to 10% of such patients demonstrating a return of clinical sensitivity on challenge. Risk factors for recurrent sensitivity to stings include repeated reactions to immunotherapy and severe initial reactions.

In 1991, Khan et al. noted that successful venom immunotherapy is correlated with the presence of antiidiotypic antibodies. Whether this is an epiphenomenon or bears significantly on the immune state is unclear.

Finally, it seems prudent to reevaluate a patient who, having successfully stopped therapy, is hapless enough to incur repeated stings, as there are as yet no long-term data regarding the risk of resensitization in such patients.

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70 IMMUNOLOGIC LUNG DISEASE

Michael B. Levy, M.D., and Jordan N. Fink, M.D.

Interstitial lung disease encompasses a group of pulmonary disorders that may have features ranging from alveolitis to fibrosis. Pulmonary infiltration with immune effector cells, secretion of effector molecules, and a wide variety of immune responses are characteristic of these disorders. Such responses include the lymphocytic and granulomatous alveolitis of hypersensitivity pneumonitis, the cellular infiltration of eosinophilic pneumonias, and end-stage fibrosis. Both cellular (Th1) and humoral (Th2) immune mechanisms have been implicated in the pathogenesis of these diseases, and defined antigens have been implicated in some syndromes. A review of the clinical and immune aberrations and potential pathogenesis of these major disease entities follows (Table 70.1).

Disease	Agent
Hypersensitivity pneumonitis	Organic dusts, chemicals microorganisms
Eosinophilic pneumonias	
Allergic bronchopulmonary aspergillosis	*Aspergillus fumigatus*
Acute eosinophilic pneumonia	Parasites, idiopathic
Drug-induced eosinophilic pneumonia	Multiple drugs
Chronic eosinophilic pneumonia	Unknown
Vasculitis	Unknown antigen, viral?
Allergic granulomatosis	Unknown
Idiopathic pulmonary fibrosis	Unknown
Fibrotic lung diseases due to inorganic dusts	Silica, asbestos, beryllium

TABLE 70.1. Interstitial Lung Diseases with Suspected Immunologic Origin

HYPERSENSITIVITY PNEUMONITIS

Hypersensitivity pneumonitis or extrinsic alveolitis comprises lymphocytic and granulomatous alveolar and interstitial pulmonary inflammation due to an ever-expanding variety of environmental antigens in organic dusts, chemicals, and drugs (Table 70.2). The immune inflammatory responses may be reversed by avoidance of antigen, or may lead to end-stage pulmonary fibrosis with repeated or continuous exposure to the antigen. Initially described by Ramazzini in 1713 (1), the most common disorder was presented as “farmer's lung” by Campbell in 1932 (2). Clinical features include systemic as well as respiratory symptoms temporally associated with inhalation of the offending agent; however, in only between 7% and 15% of individuals so exposed does the disease pattern develop (3,4). Whereas most patients initially have acute repetitive “flu-type” symptoms, a number of patients also may have “chronic bronchitis” or progressive pulmonary impairment (3,4).

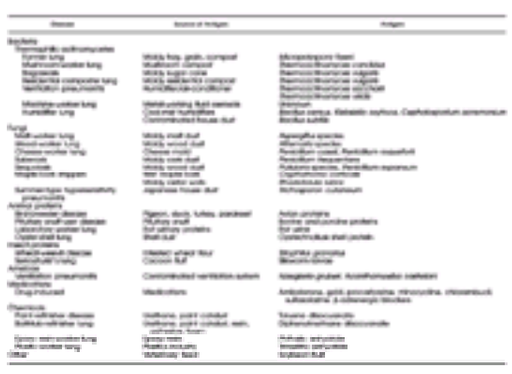

TABLE 70.2. Etiologic Agents in Hypersensitivity Pneumonitis

Clinical Characteristics

The disorder occurs in three forms: acute, subacute, and chronic. The acute form occurs in the majority of patients and is seen as the sudden onset of cough, dyspnea, malaise, myalgia, fever, and chills 4 to 6 hours after exposure to antigen. The episode subsides over the next 8 to 12 hours, only to be repeated with subsequent exposures. With frequent episodes, anorexia and weight loss may develop. Physical examination is characteristic with bilateral end-inspiratory crepitant rales. Cyanosis may be detected, but clubbing is rare. Laboratory examination reveals leukocytosis with a leftward shift, variable eosinophilia, and broad increases in immunoglobulin (Ig) levels except for IgE. Chest radiographs show nodular changes predominantly in the lower lobes (Fig. 70.1). Pulmonary-function changes are characteristic of interstitial disease, with restriction manifested by decreases in forced expiratory volume in 1 second, forced vital capacity, total lung capacity, and diffusion capacity, as well as arterial oxygenation. With resolution of the acute episode, the clinical features resolve, although crepitant rales may persist, and decreases in arterial oxygenation may be detected on exercise for several weeks. Repeated acute attacks are common, but progression of the pulmonary abnormalities is not, except in patients with farmer's lung (5,6).

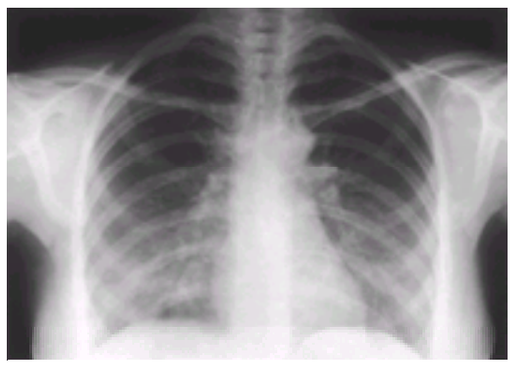

Figure 70.1. Chest radiograph of a 34-year-old patient with recurrent respiratory and systemic symptoms as a result of hypersensitivity pneumonitis due to repeated inhalation and sensitization to thermophilic actinomycetes contaminating the forced-air heating system.

The subacute form of hypersensitivity pneumonitis, which occurs in about 15% of patients, initially shows an almost continuous productive cough with or without purulent sputum, dyspnea, fatigue, anorexia, and weight loss. Acute episodes may occur after exposure to large amounts of antigen. The physical findings are those of interstitial lung disease with diffuse crackling rales, and pulmonary-function abnormalities are mainly of the restrictive type, with decreased diffusion capacity. Laboratory evaluation usually reveals polyclonal elevations of immunoglobulins, low-grade white blood cell count elevations, and normal erythrocyte sedimentation rates. Chest radiograph features include diffuse increases in interstitial markings or nodular formations. Histopathologic studies of the lung (Fig. 70.2) demonstrate lymphocytic interstitial inflammation and bronchiolitis, with maturity of the granulomas increased over the acute form with birefringent materials in some giant cells (7,8). Prolonged avoidance of exposure accompanied by glucocorticoid therapy usually results in resolution of the clinical abnormalities. The need for more aggressive therapy in the subacute form of hypersensitivity pneumonitis suggests that it is a more advanced form of the disease.

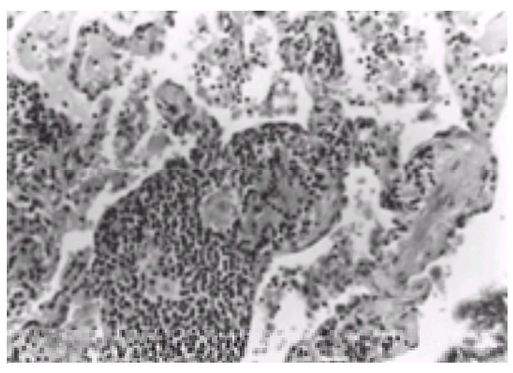

Figure 70.2. Lung biopsy specimen from a pigeon breeder with “bronchitic” symptoms and restrictive defect on pulmonary function. The specimen demonstrates lymphocytic interstitial pneumonitis and granuloma formation.

Long-term exposure to antigen is associated with a chronic and progressive form of the disease. Such individuals initially have progressive dyspnea and cough without systemic features. The disease may progress to respiratory failure with cor pulmonale and may be fatal (9). Physical examination reveals findings of tachypnea, cyanosis, and at times, clubbing of the nails. Cardiac findings of cor pulmonale also may be present. Pulmonary-function studies demonstrate either severe restriction with diminished diffusion capacity or high-grade irreversible obstruction, and these findings are associated with either diffuse fibrosis or features of hyperinflation and honeycombing seen on chest radiographs (Fig. 70.3). Chronic hypersensitivity pneumonitis may be confused clinically with idiopathic pulmonary fibrosis. Pulmonary tissue biopsy may be necessary to differentiate these two entities. Clinical guidelines for the evaluation of hypersensitivity pneumonitis have been published (10).

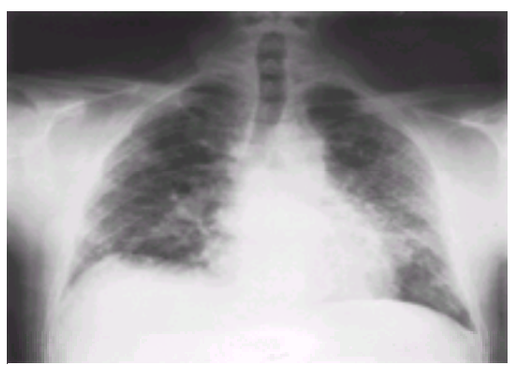

Figure 70.3. Chest radiograph of a farmer with recurrent episodes of respiratory symptoms related to his barn. The radiograph demonstrates the fibrotic picture of chronic hypersensitivity pneumonitis.

Factors Favoring Development of Disease

ANTIGENS

Numerous organic antigens are associated with hypersensitivity pneumonitis, and sensitization develops after repeated inhalation (Table 70.2). At present, there is little information regarding the levels of exposure necessary to cause the disease in susceptible individuals. Thermophilic actinomycetes are the major antigens inducing such diseases as farmer's lung, bagassosis, ventilation pneumonitis, and mushroom worker's lung. These organisms are complex antigens. Immunologic studies have demonstrated that they contain enzymes, glycoproteins, and numerous proteins, all of which may be antigenic, but none of which can be identified as disease specific (11,12,13 and 14). In avian dust–induced hypersensitivity pneumonitis, responsible antigens have been more thoroughly characterized, especially those found in pigeon serum and excreta. Pigeon droppings contain proteases resistant to inhibition by human serum; hydrolases that degrade lung surfactant phospholipid, esters, and elastins; and other enzyme activities that promote inflammation (12,13,15). Recent work has identified a 21-kd protein in pigeon-dropping extract as the responsible agent for the Th1 response in pigeon breeder's disease (16).

Further inflammation-enhancing characteristics of these antigens include their prolonged presence in alveolar macrophages related to their inherent resistance to lysosomal degradation. This allows long-term host immune or foreign-body responses to occur. Some thermophilic organisms can activate complement by either the classic or the alternative pathway, thus inducing inflammatory processes, and such organisms also act as adjuvants in enhancing cellular immune responses (17). Some of the antigens contain endotoxins that also can incite and accelerate inflammation (14). Thus the basic nature of the inhaled dust may be important in subsequent inflammatory processes.

HOST CHARACTERISTICS

The prevalence of hypersensitivity pneumonitis in various exposed populations studied ranges from 3% to 15%, suggesting that host factors must be considered in its development (3,18). The prevalence of farmer's lung, for instance, may vary between countries and even within a country. In the United States, most cases of farmer's lung have been reported in Wisconsin, where there was heavy occupational exposure, with a prevalence in an agricultural population a number of years ago of 3.9%. Since then, changes in farming methods have greatly reduced the frequency of the disease. If there is heavy exposure to contaminated air-conditioning systems, the

risk of disease increases to between 15% and 60% (19). In between 6% and 21% of pigeon breeders, the disease develops (20). Paradoxically, hypersensitivity pneumonitis occurs less frequently in cigarette smokers than in nonsmokers (21), suggesting a role for the damaged alveolar macrophage in protection. Genetic factors have been suggested as important in the pathogenesis of farmer's lung and pigeon breeder's disease, by assessment of major histocompatibility haplotypes in groups of patients (22,23,24 and 25). However, in studies of larger populations when asymptomatic and similarly exposed individuals were evaluated, no significant correlations with disease could be identified (26). Similarly, in studies of other genetic markers such as P blood groups, human leukocyte antigen (HLA)-A, -B, -C, and -DR antigens, and Gm allotypes, there were no correlations with disease (27). In animal studies, however, chronic pulmonary granulomatous inflammation after bacille Calmette-Guérin (BCG) administration was shown to be controlled by thymus-derived $CD8^+$ cells that are cyclophosphamide sensitive (28). Furthermore, the extent of the inflammation was shown by mouse inbreeding studies to be associated with the immunoglobulin heavy-chain locus and with decreased cell-mediated responses to other antigens (29). In addition, some strains of BCG-inflamed mice produce macrophage-derived factors that suppress the inflammation, whereas other strains cannot develop such suppression, and intense and prolonged pulmonary inflammation results (30). This and later work provide evidence that the expansion of Th1 cells, with their subsequent cytokine and chemokine secretion, are involved in granulomatous formation. Mutations described in the interferon-g (IFN-g)-receptor gene with subsequent increased susceptibility to mycobacterial infection have provided additional information toward our understanding of this cytokine/chemokine interaction (31,32).

IMMUNE RESPONSES

As the demonstration of immune responses is the hallmark of disease, immune parameters are most important. Precipitating antibodies that react with the offending antigen are uniformly demonstrated in the serum of ill patients (3,33). However, up to 50% of exposed but asymptomatic individuals also may have such circulating precipitins (3). By macrophage migration inhibition, an *in vitro* technique to detect cell-mediated immunity, the peripheral blood lymphocytes of ill pigeon breeders, but not those of healthy exposed pigeon breeders, were shown to be sensitized to pigeon antigens (34,35 and 36). This technique, therefore, more clearly defined the sick and well pigeon breeder population than did humoral antibody tests and pointed to cell-mediated immune mechanisms in the pathogenesis of the disease, considered as the Th1 type of immunologic response. The development of newer animal models and the further study of bronchoalveolar lavage (BAL) fluids have reinforced this concept.

Immunopathogenesis

Evidence from both human and animal studies indicates that hypersensitivity pneumonitis most likely results from the interaction of the humoral and cell-mediated immune mechanisms. Studies of BAL fluid have consistently demonstrated a predominance of T cells, and particularly increased numbers of T cells (37,38) expressing CD8 and the natural killer (NK) markers CD56 and CD57. Such T-cell populations have been documented in both symptomatic and asymptomatic but exposed groups, with levels higher in the ill individuals. The T cells are activated and respond to stimulation with mitogens and produce cytokines. If antigen exposure persists, the increased $CD8^+$ T-cell population continues, and the number and activity of NK cells increase. With avoidance of exposure, the alveolar lymphocyte population reverts toward normal (38).

Although both symptomatic and asymptomatic but exposed populations have lymphocytic alveolitis, studies of lung suppressor cell function have shown differences. $CD8^+$ T cells obtained from ill pigeon breeders have reduced function in interaction with mitogens or pigeon antigens; cells from exposed but healthy breeders do not (39). These results suggest a failure of immunoregulation in patients with disease.

The number of mast cells is increased in lavage fluids from patients with hypersensitivity pneumonitis (40). Such cells may influence both $CD4^+$ and $CD8^+$ T-cell responses by release of modulating cytokines and may influence pulmonary vascular responses through release of histamine, prostaglandins, or leukotrienes. Because arachidonic acid metabolites can regulate immune cell function and inflammatory responses, they may be important in the development of the interstitial lesions. Evidence suggests that mast cells may be the key in the development of fibrosis (41), and these cells also may be important in the pathogenesis of hypersensitivity pneumonitis.

The presence of serum and alveolar antibodies of IgG, IgA, and IgM isotypes specific for the offending antigen points toward humoral mechanisms in hypersensitivity pneumonitis, especially those leading to a vasculitis. Although vasculitis is not common in alveolitis, immune complexes could initiate inflammation by complement fixation, by releasing inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF) from alveolar cells, by activating pulmonary macrophages and inducing granulomas in pulmonary parenchyma, as well as by participating in the maintenance of chronic inflammation.

Animal model experiments have confirmed the complex nature of hypersensitivity pneumonitis. In early studies with rabbit models of the disease induced by ovalbumin, the development of lesions correlated better with the induction of cell-mediated hypersensitivity than with antibody (18). However, in the guinea-pig model, immune complex–induced pneumonitis that developed after a single antigen challenge could be converted into a granulomatous pneumonitis by repeated challenge (42). Recent work in mouse experimental hypersensitivity pneumonitis has identified that the Th1 cells capable of adoptive transfer are activated/memory T cells, and that Th2 cells do not have this ability (43).

Other experiments have detected high levels of IL-1 in sensitized mice with pulmonary granulomas, which may act by signaling elaboration of other cytokines such as IL-2. The regulation of IL-2 production appears to be under the control of $CD8^+$ cells or macrophages in such animals (44). Recent work in mouse models has shown that the lack of IL-10, which counteracts many of the effects of IFN-g, or the blocking of IL-13 activity, results in a more severe granulomatous inflammatory response (45,46). These studies suggest that activated macrophages and $CD8^+$ cells incapable of suppressing them contribute to the inflammatory environment in the disease.

Other animal studies have shown a role for macrophage-derived arachidonic acid (eicosanoid) metabolites in pulmonary granuloma formation (47). The development of such granulomas has been associated with the generation of leukotrienes C_4 and D_4 and 5- and 12-hydroxyeicosatetraenoic acid (HETE) from macrophages. In addition, the lesions could be suppressed by treatment with antilipoxygenase compounds (48). Such compounds inhibit Ia antigen expression in pulmonary macrophages and inhibition of antigen presentation to T cells, which in turn could reduce the cellular immune responses.

Thus the development of hypersensitivity pneumonitis in animal models and possibly in sensitized individuals may be a complex interaction of cytokines, chemokines, eicosanoid metabolites, immune complexes, and pulmonary macrophages with $CD4^+$ and $CD8^+$ T cells, resulting in the development of granulomas, perhaps related to genetically controlled responses.

Diagnostic Considerations

The diagnosis of hypersensitivity pneumonitis should be considered in patients with histories of systemic and/or respiratory symptoms resembling influenza or bronchitis but temporally associated with a particular environment. Physical examination may suggest interstitial lung disease, and pulmonary-function testing may indicate restriction or obstruction with diffusion defects. In some cases, cautious inhalation challenge with a suspected antigen or purposeful exposure to the suspected environment may be useful in establishing a relationship between an environment and the disease. BAL studies with enumeration of the cellular population or lung biopsy to show a lymphocytic interstitial pneumonitis with granulomas in obtained specimens may be useful.

Several syndromes should be considered when evaluating patients for hypersensitivity pneumonitis. These include the organic dust toxic syndrome (ODTS), which occurs in workers after exposure to dusts contaminated with multiple bacterial and fungal species, humidifier fever, mucosal irritation or “sick building syndrome,” and disorders that cause interstitial lung disease with progressive pulmonary and systemic symptoms. The latter include sarcoidosis, primary pulmonary histiocytosis, and idiopathic pulmonary fibrosis. A careful history, review of pulmonary functions, radiographic studies, and immunologic findings should classify the diagnosis.

Treatment

The treatment of hypersensitivity pneumonitis is avoidance of the offending antigen or environment once it is identified. This may entail the use of protective respiratory devices; alteration of forced-air heating, cooling, or humidification systems; substitution of workplace chemicals; or avoidance of the workplace. Glucocorticoids markedly reduce the pulmonary inflammatory process if parenchymal destruction has not already occurred and return the patient to normal, as long as avoidance measures are instituted. Long-term sequelae are generally minimal once such avoidance is instituted.

EOSINOPHILIC PNEUMONIAS

The eosinophilic pneumonias are characterized by pulmonary infiltration with eosinophils and the clinical effects of those inflammatory cells. Such infiltrations may be due to a variety of known and unknown causes with overlapping clinical features and responses to therapy. The disorder may or may not be associated with bronchospasm (Table 70.3). Whereas pulmonary infiltrates may occur without peripheral eosinophilia, most affected patients usually have a peripheral eosinophil count greater than 500 cells/µL.

Asthmatic	Nonasthmatic
Allergic bronchopulmonary aspergillosis	Loeffler pneumonia
Tropical eosinophilia	Chronic eosinophilic pneumonia
Vasculitis	
Polyarteritis	Drug-induced
Allergic granulomatous angiitis	Eosinophilic granuloma

TABLE 70.3. Patient Characteristics in Eosinophilic Pneumonias

Allergic Bronchopulmonary Aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) occurs as a result of hypersensitivity reactions after the colonization of the respiratory tract with *Aspergillus*, most commonly *Aspergillus fumigatus*. The syndrome occurs largely in atopic patients with underlying asthma, which may be mild or glucocorticoid dependent. It was first described in 1952 (49) and has been characterized by episodic wheezing, fleeting pulmonary infiltrations, sputum and blood eosinophilia, immediate wheal-and-flare skin reactivity, and specific serum IgG and IgE antibodies to *A. fumigatus*, markedly elevated levels of total serum IgE, and central bronchiectasis (Fig. 70.4 and Fig. 70.5) (50).

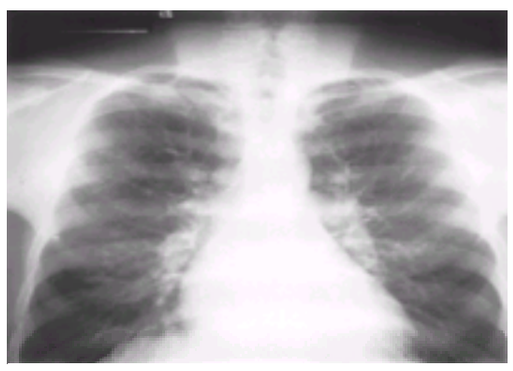

Figure 70.4. Chest radiograph of a patient with asthma, recurrent infiltrates, and elevated serum immunoglobulin E level. The radiograph demonstrates the nodular infiltrates of allergic bronchopulmonary aspergillosis.

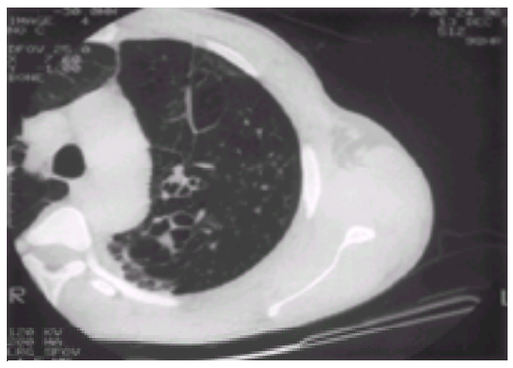

Figure 70.5. High-resolution computed tomography scan of the chest of a patient with allergic bronchopulmonary aspergillosis, demonstrating areas of saccular bronchiectasis.

Although ABPA may be asymptomatic, the disorder should be considered when asthmatic episodes become more severe and are associated with constitutional symptoms. *Aspergillus* organisms are ubiquitous, are widely dispersed in the environment, and thus are not avoidable, nor can a source usually be pinpointed. The spores are abundant in disturbed soil, decaying organic matter, hay or grain piles, and vegetable compost. It is not understood why *A. fumigatus* spores are able to colonize the mucus of patients with ABPA but not that of the many other patients with asthma.

ABPA should be considered in patients with asthma who have immediate wheal-and-flare skin reactions to *Aspergillus* antigens. An elevation of total serum IgE and specific IgE and IgG antibodies is reasonable evidence for the disease. Thin-section computed tomography of the chest should then be carried out to evaluate bronchiectasis (51).

A number of different histopathologic changes may be seen in ABPA, including mucoid impaction syndrome, eosinophilic pneumonia, bronchiolitis obliterans, granulomatous bronchiolitis, and fibrosis, in addition to the expected changes of asthma (52). Thick tenacious mucus includes fibrin, eosinophils, and Charcot-Leyden crystals. *Aspergillus* hyphae may be identified; however, invasion of the bronchial wall is not characteristic. Microscopically there is bronchial wall inflammation with the presence of mononuclear cells and/or eosinophils.

Treatment of the disorder consists of control of the underlying asthma with immunologic or pharmacologic means and resolution of the inflammatory process with glucocorticoids. If such antiinflammatory treatment is not carried out, then progression of the disease, with increasing sputum plugs, further bronchiectasis, and pulmonary fibrosis, is likely. With glucocorticoid therapy, serum IgE levels regress, pulmonary infiltrates disappear, and bronchiectatic areas stabilize (50). Itraconazole may provide effective adjunctive therapy for patients with glucocorticoid-dependent ABPA (53).

IMMUNOPATHOGENESIS

Characteristically, total serum IgE levels are elevated in ABPA. However, the elevation is largely nonspecific, as most of the IgE is not directed toward *Aspergillus* (54). Specific IgG also is necessary to induce pulmonary lesions after *A. fumigatus* inhalation, as shown in passive-transfer animal experiments. No changes of ABPA were induced when animals were given IgG or IgE antibody alone before challenge (55). Circulating immune complexes and activation of complement have been demonstrated (56).

A. fumigatus (Af), as an antigen, has multiple immunoreactive components. Crossed immunoelectrophoresis of culture filtrates of *Aspergillus* with sera from patients with ABPA identifies about 35 precipitin arcs, of which eight to ten show IgE reactivity (57,58).

Characterization of the epitopes has aided our understanding of the immunopathogenesis of the disease. The pathogenesis of ABPA probably depends on specific cytokines and immunoglobulins secreted by lymphocytes on stimulation with different epitopes of those allergens. Asp f 1 has been identified as an 18-kd protein member of the mitogillin family of ribotoxins. With Asp f 1, specific IL-4–producing $CD4^+$ Th2 clones with HLA restriction reacting to two epitopes were demonstrated in patients with ABPA (59). Further evidence for activation of $CD4^+$ T cells was provided when it was shown that soluble IL-2 receptors were increased in the sera of patients with ABPA (60). *Aspergillus* appears to function as an eosinophil growth factor by potentiating IL-5–induced growth and differentiation (61). Asp f 2, a 37-kd

protein, exhibits IgE binding in patients with ABPA (62). In a mouse model, of five peptides from the sequence of Asp f 1, one showed a clear Th1, whereas another showed a Th2 response, suggesting that a number of epitopes of diverse activities are present and may be involved in the pathogenesis through differential cytokine secretions (63).

Acute Eosinophilic Pneumonia

PARASITE INDUCED

In 1932 Loeffler (64) described four patients with peripheral blood eosinophilia and transient pulmonary infiltrates accompanied by minimal clinical findings. It was later recognized that infestation with *Ascaris lumbricoides* and other parasites such as *Toxicara, Strongyloides, Schistosoma, Trichina,* and *Necator* can cause the syndrome (65). The exudative eosinophilic reaction is a response to larval parasitic migration through pulmonary parenchyma. Because emigration from the lung to the gastrointestinal tract is a part of the life cycle of the parasite, the pulmonary lesions are transient.

TROPICAL EOSINOPHILIA

Tropical eosinophilia occurs as a hypersensitivity reaction to filarial infestations with *Wuchereria bancrofti* or *Brugia malayi.* Although infestation with these organisms is usually associated with lymphangitis, fibrosis, and subsequent lymphatic obstruction, in some patients, paroxysmal cough, wheezing, eosinophilia, and patchy pulmonary parenchymal infiltrates develop (66). Donohugh (67) characterized patients with the disease as having a history of a dry irritating nocturnal cough, episodic wheezing, dyspnea, pulmonary infiltrates, and eosinophilia greater than 2,000 cells/µL, serologic evidence of filarial infestations, and a response to diethylcarbamazine. Recently the essential role for IL-5 and eosinophils in *B. malayi*–induced airway hyperreactivity was demonstrated in a mouse model (68).

DIAGNOSIS

The clinical findings of eosinophilic pneumonia due to parasitic infestation include mild cough, low-grade fever, malaise, and anorexia, and are accompanied by chest radiographic features of unilateral or bilateral homogeneous infiltrates in an alveolar/interstitial pattern, often in the periphery. The reaction may be more intense if the burden of parasites is heavy. Serologic evaluations for parasite infestation and examination of stool specimens for ova may be rewarding. Larval stages may, on occasion, be detected in sputum or alveolar lavage fluid.

Because of the transient pulmonary localization of the parasite, the pulmonary lesions also are transient. However, if parasitic elements can be detected, treatment with antihelmintics is warranted. Glucocorticoids may be useful in effecting rapid resolution of the pulmonary infiltrates.

OTHER ACUTE EOSINOPHILIC PNEUMONIAS

When no agent or inciting event can be associated with the onset of eosinophilic pneumonia, the term *pulmonary infiltration with eosinophilia* (PIE) syndrome is often used. The disorder may occur rapidly, demonstrate pulmonary and systemic symptoms, and persist for a variable period. Control is achieved with glucocorticoids, and long-term disability is uncommon.

A more acute eosinophilic pneumonia was reported by Allen et al. (69). No etiologic agent was uncovered in these cases, and the patients had symptoms of an acute febrile illness and clinical hypoxemia, diffuse infiltrations as seen on chest radiograph films, and eosinophilia noted in the BAL fluid. Respiratory failure occurred in two of the patients, and treatment with glucocorticoids resulted in rapid resolution.

Recent studies of the level of IL-5 in peripheral blood and in BAL fluids have found high levels of the cytokine in acute eosinophilic pneumonia, but not in the chronic variety (70).

Drug-induced Eosinophilic Pneumonia

A wide variety of drugs have been described to cause pulmonary infiltration with eosinophils or interstitial pneumonitis (Table 70.4) (71). The clinical features of the reaction vary and generally occur as dyspnea with or without wheezing, fever, or various degrees of cough. There is often a latent period between the use of the drug and the onset of symptoms. Peripheral blood eosinophilia is often present, and eosinophils may be detected in increased numbers in sputum or BAL fluid. The chest radiograph is not specific and demonstrates unilateral or bilateral infiltrates. Examination of a lung biopsy specimen reveals eosinophilic and mononuclear infiltration of the pulmonary parenchyma and alveolar spaces. Glucocorticoids may resolve all of the clinical abnormalities, but cessation of the causative drug remains primary therapy.

Acetylsalicylic acid[b]	Melphelan
Asenicals[a]	Mechanesin[a]
Amiodarone[b]	Methotrexate[a,b]
Azathioprine[b]	Mitomycin[b]
Beclomethasone[c]	Nadolol[b]
Bleomycin[a,b]	Naproxen[a]
Busulfan[b]	Nitrofurantoin[a]
Carbamazepine[a]	Nitrosourea[b]
Chlorambucil[b]	Para-aminosalicylic acid[b]
Chlorpromazine[a]	Penicillin and its derivatives
Chlorpropamide[a]	Phenothiazine[a]
Cromolyn sodium[a]	Phenylbutazone
Cyclophosphamide[b]	Procainamide[b]
Cytosine arabinoside[b]	Procarbazine[b]
Gold salts[a,b]	Propylthiouracil[a]
Hydantoin derivatives[a,b]	Streptomycin[a]
Hydrochlorothiazide[a]	Sulfonamide and its derivatives
Imipramine[a]	Tetracycline[a]
Isoniazid[a]	Toconide[b]
	Vinblastine[b]

[a] Associated with eosinophilic pneumonia.
[b] Associated with interstitial pulmonary infiltration.

TABLE 70.4. Some Drugs Associated with Interstitial Pulmonary Disease

Reactions to nitrofurantoin may be present in an acute or chronic fashion. The acute reaction begins shortly after institution of the drug, as previously described. The chest radiograph may demonstrate pleural effusion along with infiltrative disease, and a lung biopsy specimen shows parenchymal eosinophilic and mononuclear infiltration and, in some cases, vascular involvement. Peripheral eosinophilia and elevation of the sedimentation rate are usual. In the chronic nitrofurantoin reaction, the onset may occur up to 5 years after beginning therapy with the drug. The clinical features of this form include chronic progressive cough and dyspnea, and interstitial fibrosis on chest radiographs may be prominent. Cessation of drug therapy remains the mainstay of treatment, but glucocorticoids may be necessary to reduce progression of the disease process.

Pulmonary eosinophilia can occur after the use of a number of sulfonamides and appears clinically as pneumonitis.

Phenytoin derivatives used to treat seizure disorders and on occasion arrhythmias also can induce a pulmonary disorder. Acute eosinophilic pneumonia associated with fever, cough, and dyspnea may occur days to months after institution of the drug. The symptoms also may be associated with features of a nonspecific dermatitis, hepatitis, and generalized lymphadenopathy, including hilar nodes. Resolution occurs with cessation of the drug.

Chronic Eosinophilic Pneumonia

In 1969, Carrington et al. (72) described a chronic pulmonary eosinophilic syndrome in which no etiologic agent could be detected. The patients initially had fever, sweats, progressive dyspnea, and weight loss. Asthma occurred in 50%. The chest radiographs were characterized as the “photographic negative” of pulmonary edema with peripheral pulmonary infiltrates, but patchy migrating infiltrates, pleural effusion, and cavitation also were seen. Respiratory failure in patients with severe disease has been reported (73). Blood eosinophilia is characteristic, and the sedimentation rate and platelet count may be elevated (74), as may the total serum IgE level (75). Lung biopsy specimens demonstrate interstitial and alveolar infiltrations with eosinophils and multinucleated giant cells with minimal fibrosis. Institution of glucocorticoids is usually associated with resolution or at least a shortening of the course of the disease (72).

The clinical features of bronchospasm, eosinophilia, elevated serum IgE levels, and response to glucocorticoids suggest the involvement of an IgE/mast cell–dependent mechanism of immediate wheal-and-flare reactions, and the mononuclear cell infiltration suggests involvement of the late-onset reaction; however, the

inflammatory action of eosinophils is most likely the key to the tissue damage. BAL fluid analysis has revealed increased levels of eosinophil cationic protein and expression of class II major histocompatibility complex (MHC) molecules, reflecting eosinophil degranulation and activation (76,77). After treatment with glucocorticoids, IL-2 receptor positive and HLA-DR positive T cells decrease in the analyzed lavage fluid (78).

Further work on the biology of the eosinophil and the mechanisms that control its activation, recruitment, and degranulation will help us to understand its role better in these inflammatory disorders.

IDIOPATHIC PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis (IPF) is an interstitial pulmonary disorder of unknown etiology that has been referred to as the *Hamman-Rich syndrome, cryptogenic fibrosing alveolitis,* or *desquamative, usual,* or *lymphocytic pneumonitis.* The patients originally described by Hamman and Rich (79) had a progressive pulmonary disease with associated connective tissue disorders such as rheumatoid arthritis.

IPF is a disease of unknown etiology but has fairly well characterized clinical, laboratory, and histopathologic features. The prevalence is about three to five cases per 100,000 (80), and it occurs more often in patients between ages 40 and 70 years, with a slight predilection for men. The clinical course of the disease varies, but the mean survival time after diagnosis ranges between 3 and 5 years with or without treatment.

Clinical Features

The characteristic clinical features of IPF include the gradual onset of progressive shortness of breath. It occurs at first on exertion, but eventually at rest, and is often accompanied by a dry, nonproductive, persistent cough. Some patients may have a preceding influenza-like illness, and in others, anorexia and weight loss develop. Characteristic physical findings include dry bibasilar inspiratory “Velcro” rales, with clubbing and cyanosis occurring late. Pulmonary-function tests reveal a restrictive pattern with decreased lung volumes and reduced diffusing capacity. As the disease progresses, the restrictive and gas-exchange abnormalities increase. The chest radiograph typically demonstrates diffuse infiltrates, which are most prominent in the lower lobes, where a reticulonodular pattern is characteristic and may be visualized on thin-section computed tomography (81). However, the chest radiograph may appear normal in up to 15% of patients with IPF (82,83). High-resolution computerized tomography (HRCT) is now widely used in the investigation of patients with suspected or known diffuse lung disease (84).

The histopathologic features of the disease are heterogeneous, with a spectrum ranging from an alveolitis to end-stage fibrosis or diffuse honeycombing. Early in the disease, the pulmonary parenchyma may appear normal, and the disease process is spotty. Thus lung biopsy is often necessary for confirmation of the diagnosis. Evaluation of series of lung biopsy specimens has led to classification of the histopathologic features into three broad groups (85,86 and 87). *Desquamative interstital pneumonia* (DIP) occurs as a cellular pattern with an increased number of macrophages and lymphocytes filling the intraalveolar spaces. Interstitial edema also may be prominent (85,87). Fibrosis is not present, and lymphocytes and eosinophils may be found in the alveolar septa. *Usual interstitial pneumonia* (UIP) occurs as a mixed cellular and fibrotic picture and may represent progression of the disease. There is more intense inflammation with derangement of the alveolar walls by edema and injury of endothelial and epithelial cells (85). Fibroblasts proliferate, and there is infiltration of alveolar walls and septa with lymphocytes, eosinophils, and mesenchymal cells. Organized inflammation and fibrosis are present, and with further parenchymal derangements, proteinaceous debris may be found in the intraalveolar spaces. The *end stage* or honeycombing is characterized by replacement of pulmonary parenchyma by cystic spaces lined with cuboidal epithelium and encircled by connective tissue with little inflammation (88). Smooth-muscle hyperplasia, small-airway narrowing, and changes in the pulmonary arterioles suggestive of pulmonary hypertension are features of this phase of IPF.

The recognition of these putative stages of IPF may be helpful in evaluating the prognosis in a given patient. Carrington et al. (72,85) determined that patients with less aggressive disease (DIP) had a lower mortality rate of 27.5%, compared with patients with the more aggressive form (UIP), in which the mortality during follow-up was 66%. Other studies showed that patients with a more cellular pattern on biopsy specimens respond better to glucocorticoid therapy than do those in the fibrotic phase (86).

Thus open lung biopsy is useful in confirming the diagnosis, evaluating the response to therapy, and determining the prognosis of the patient.

BRONCHOALVEOLAR LAVAGE

BAL studies have shown that cell counts from the fluid correlate with the histopathologic features of IPF and also may be useful in predicting the response to therapy (89,90). Patients with progressing IPF and sarcoidosis have increased expression of proinflammatory chemokines such as IL-8 and TNF-a in BAL fluid (91). IL-8 is increased in the BAL fluid and serum of patients with IPF and may reflect the degree of neutrophilic alveolitis (92). Other recent observations indicate that TNF-a may be involved in the recruitment of eosinophils, which are believed to be important in the development of fibrosis (93). The detection of lymphocytosis in BAL fluid correlates with alveolitis without honeycombing, but the detection of neutrophilia and eosinophilia does not correlate with biopsy findings (35). Furthermore, the relative increase in lymphocytes but not other cellular elements in BAL fluid correlates well with glucocorticoid responsiveness. The response of the BAL fluid cellular elements to treatment is variable in spite of clinical improvement (94,95).

Other described abnormalities in BAL fluid include changes in the phospholipid components of pulmonary surfactant with decreased phospholipids, a decreased phosphatidylglycerol-to-phosphatidy1choline ratio, and a relative decrease in the dipalmitoyl form of phosphatidy1choline, as well as the demonstration of lipoprotein macroaggregates (96). These lipid aberrations may correlate with the clinical response of patients with IPF or with the cellularity of the biopsy tissue (96,97).

THERAPY

Since the study of Carrington et al. (85) in 1978, glucocorticoids have been the mainstay of treatment. Between 40% and 70% of patients with IPF so treated reported subjective improvement, but only 10% to 30% had correlative objective improvement (85,98). In general, those patients with the more cellular phase of IPF respond favorably to the drug, probably related to the suppression of the inflammatory process. Because the cellular phase of IPF may represent an earlier and more responsive phase, patients with suspected disease need early diagnosis, preferably detected by open lung biopsy, followed by suppressive doses of glucocorticoid. Regulation of the drug dose should be carried out with periodic chest radiograph studies, pulmonary-function evaluations, and in some cases, BAL studies. The duration of glucocorticoid therapy varies from 3 months to 1 year to lifetime. If the condition of the patient deteriorates during such therapy, glucocorticoid should be reduced or discontinued and alternative immunosuppressive/antiinflammatory therapy should be started.

Cyclophosphamide may be beneficial in reducing the pulmonary neutrophilic inflammation (99,100), thus reducing the gas-transfer disruption across the alveolar membrane. Azathioprine, chlorambucil, colchicine, and vincristine have also been used alone or in combination, with varied results (98,101). Finally, transplantation of a lung or of a heart and lung has been successful in some patients who have uncomplicated disease (102,103). To date, this therapy offers the best prognosis for long-term survival.

PATHOGENESIS

IPF is a chronic inflammatory process of the lung in which inflammation is unregulated by the normal repair processes. As a result, mesenchymal cells proliferate and extracellular connective tissue is deposited, leading to disturbances in gas transfer (80,104). Although the inciting injury is unknown, viral, immune, inflammatory, and genetic processes may be important in the initiation and maintenance of the disease process. Cytokines, which may be divided into proinflammatory, antiinflammatory, and growth-stimulatory groups, play important roles as soluble signaling molecules that dictate and coordinate these inflammatory and immune responses.

A genetic component of IPF has been postulated because of an association of the disease with specific HLA haplotypes. Whereas some studies detected no correlations (105,106), others showed an increased frequency of HLA-B12 (23), HLA-B15, HLA-Dw6 (107), HLA-DR2 (33), and HLA-B8 (108). Additional studies correlated IPF with specific a_1-antitrypsin phenotypes (109), with other genetic disorders such as Gaucher disease (110), neurofibromatosis (111), Neimann-Pick disease (112), and tuberous sclerosis (113), and with familial occurrences in which there is variable penetrance of an autosomal dominant pattern. These findings suggest genetic influences in the disease such as linking of IPF with other inheritable disorders (114,115).

Viral infections may be important. Many patients have a history of a flulike illness before the development of IPF, and some have intracellular inclusion bodies in the alveolar epithelial cells (81,116). Additional studies suggested a relationship between Epstein-Barr viral (EBV) infection and IPF, with increased titers in the serum and BAL fluid of specific IgG against the viral antigens (117,118). However, such studies are not conclusive, as infection with EBV is common in the population.

Immune mechanisms have been implicated in the pathogenesis of IPF because up to 20% of these patients have other connective tissue disorders such as rheumatoid arthritis, systemic sclerosis, myositis, and systemic lupus erythematosus (119,120 and 121). The immune abnormalities in the connective tissue disorders such as hyperimmunoglobulinemia, cryoglobulinemia, elevated antinuclear antibody and rheumatoid factor titers, and decreased complement components have been detected

in IPF (122). Furthermore, circulating immune complexes have been detected in the disease, and levels of the complexes may correlate with the level of pulmonary infiltration (123,124). The latter correlates with immunofluorescent staining of lung tissue, which has demonstrated deposits of immunoglobulins and complement in alveolar walls and capillaries (125). However, the specificity of the antigen(s) coupled to the immunoglobulins has not been identified. These data may represent pathogenetic mechanisms or may merely be epiphenomena.

Other studies suggested that cytokine-mediated activation of immune effector cells may play a role in inflammation and the sustenance of the alveolitis (126). Activated macrophages that can release multiple modulators of inflammation such as leukotriene B, platelet-derived growth factor (PDGF), fibronectin, growth factors, proteases, and reactive oxygen species accumulate in the lung, where inflammatory processes are amplified (127,128,129,130,131,132,133,134 and 135). The macrophages in the lung of a patient with IPF have been shown to be young and recently differentiated, and replicate at a level above those from normal lungs (136). Thus mechanisms that control macrophage proliferation are likely disturbed in IPF.

Other inflammatory cells found in excess in the lungs of patients with IPF include neutrophils and lymphocytes. Neutrophils are attracted and activated by macrophage-derived cytokines and may thus contribute to the inflammatory process. The accumulation of these inflammatory effector cells disturbs the pulmonary parenchymal structure through release or secretion of proteases and reactive oxygen species, and the cells enhance further inflammation by release of cytokines with growth and proliferation capabilities. The production of Th2 cytokines by $CD8^+$ T cells in the BAL fluid of patients with systemic sclerosis is associated with greater decline in pulmonary function (137).

FIBROTIC LUNG DISEASES DUE TO INORGANIC DUSTS

The various mineral-induced pneumoconioses, especially silicosis, berylliosis, and asbestosis, have been of major immunologic interest for years, and immune mechanisms have been considered contributory to the pathogenesis of these diseases. Patients with silicosis inhale dust containing silicon dioxide (SiO_2) for prolonged periods and develop progressive pulmonary fibrosis with clinical pulmonary impairment. At-risk occupations include hard-rock mining, stone crushing, foundry work, sand blasting, and granite work; development of such occupation-associated disease can be markedly reduced by control of the levels of inhalable silica.

The clinical features of silicosis include progressive dyspnea, cough, and sputum. Physical findings may be normal or may demonstrate obstructive or restrictive defects. Chest radiograph features include diffuse rounded opacities predominating in the upper lung fields, eggshell calcification of the hilar nodes, and coalescence of the nodule to form “progressive massive fibrosis” (138). Histopathologic study demonstrates nodules comprised of fibroblasts, macrophages, and lymphocytes surrounding a central core of whorled collagen and reticulin (138). The nodules may coalesce to form the masses of fibrosis. Silica particles may be detected in a central location by electron microscopy, and these particles may play a key role in the activation of alveolar macrophages and induction of the inflammatory process (138,139).

Immunologic evaluation of patients with silicosis has revealed abnormalities in both cellular and humoral responses. Polyclonal elevations of immunoglobulins and the detection of antinuclear antibody, rheumatoid factors, and circulating immune complexes have been described (140). Alterations in $CD4^+$ and $CD8^+$ T-cell numbers and variances in lymphocyte responses to mitogens can be demonstrated in patients with silicosis (141). These abnormalities do not correlate with the clinical status of the patient and may represent secondary effects or the result of immune cascades stemming from alveolar macrophage activation by silica. Such macrophage activation has been demonstrated in animal models of the disease and results in release of proinflammatory cytokines and active oxygen radicals, ultimately enhancing the fibrotic process (142).

In the past, asbestos was widely used in the construction, automotive, insulation, and cement industries. Governmental controls, however, have reduced the use of the material considerably. Asbestosis occurs after the inhalation of fibers that can be deposited in the terminal airways. The clinical features of asbestosis are similar to those of IPF but are usually not apparent until about 20 years after exposure. Progressive dyspnea and cough leading to respiratory insufficiency are frequent findings. Features of interstitial lung disease with end-inspiratory rales, clubbing, and ultimately cor pulmonale are found. Pulmonary-function abnormalities include restrictive and diffusion defects. Chest radiograph abnormalities depend on the degree of involvement and may range from small irregular lower lung-field shadows to more extensive infiltrates that may mimic features of IPF. Areas of pleural thickening, often with calcification, are characteristic, and the occurrence of mesothelioma is most often associated with asbestos exposure. Bronchiogenic carcinoma is increased in tobacco users also exposed to asbestos, suggesting that the mineral may act as a “promoter” (143).

Patients with asbestosis do not have a consistent pattern of immune abnormalities. Some studies indicated reduced circulating lymphocyte numbers, decreased helper-induced lymphocytes, and decreased mitogen responses (144). Other studies showed decreased levels of $CD8^+$ T cells and normal levels of helper and NK cells (141,145). Studies of BAL fluids from patients with asbestosis detected increases in neutrophils, eosinophils, lymphocytes, and macrophages (146,147 and 148). Evaluations of T-cell subsets in the lung fluid detected significant increases in $CD4^+$ T cells (146). In addition, lung-fluid analysis demonstrated both asbestos fibers and fibers coated with various degrees of proteins or iron (ferruginous bodies), the numbers of which correlate with the degree of asbestos exposure (149). Such bodies may activate complement pathways and interact with pulmonary lymphocytes and macrophages to release proinflammatory cytokines. They also may induce release of cellular-activating and fibrosis-promoting factors to enhance further the lesions of asbestosis.

Pulmonary disease due to beryllium may appear as an acute toxic pneumonitis (150) or as a chronic pulmonary granulomatous disorder. Initially described in the fluorescent lamp industry (151), most cases now occur in individuals in mining, milling, or melting occupations or in the formulation of beryllium alloys. An HLA class II marker (HLA-DPB1 Glu69) is strongly associated with the disease. The gene confers increased susceptibility to beryllium in exposed workers (152,153). Symptoms of chronic beryllium disease develop insidiously months to years after exposure. Dyspnea is most common and may be associated with nonproductive cough, with fever, sweats, general malaise, anorexia, and weight loss, as the disease progresses. Physical examination may reveal features of interstitial pulmonary disease, with cyanosis, clubbing, and cor pulmonale occurring in advanced disease. Pulmonary function abnormalities include restrictive and diffusion defects, and chest radiograph features include widespread small reticular opacities, conglomerate nodules, and hilar adenopathy (154,155). The characteristic lesion is that of a diffuse interstitial infiltration of the pulmonary parenchyma along with noncaseating granulomas that may contain calcifications (155). Clinical laboratory features may resemble those of sarcoidosis, with elevated angiotensin-converting enzyme levels, increased gallium uptake on scans, increased levels of calcium in blood and urine, and nonspecific immunoglobulin elevations (156,157). Beryllium is antigenic in humans, inducing cell-mediated responses that can be demonstrated in workers with disease. Results of patch tests with beryllium salts have largely been positive in such workers (158), and blast transformation and production of macrophage migration inhibition with peripheral blood lymphocytes have been demonstrated in exposed but not necessarily ill workers (159). Studies of BAL fluids from ill workers showed increased numbers of T lymphocytes with a predominance of $CD4^+$ cells, which preferentially could be stimulated by beryllium salts (159). Activation of pulmonary $CD4^+$ cells with subsequent release of proinflammatory cytokines and interaction with macrophages might then lead to interstitial infiltrative and granulomatous pneumonitis characteristic of the disease.

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71 ALLERGIC CONTACT DERMATITIS

Stephen I. Katz, M.D., Ph.D.

Allergic contact dermatitis is a very common inflammatory skin disease that represents a form of delayed-type hypersensitivity. Contact dermatitis and primary irritant dermatitis, which results from exposure to irritants, make up almost 40% of all occupationally acquired illnesses (1). Despite the clinical importance of contact dermatitis, knowledge about why a given individual becomes sensitized to a particular chemical is limited. In recent years the role of the skin in the generation of contact dermatitis has become better understood.

ETIOLOGY

Chemical Causes

Allergic contact dermatitis is due to a delayed-type hypersensitivity to simple compounds, usually of low molecular weight, applied to the skin. Approximately 3,000 different compounds have been implicated as causes of allergic contact dermatitis. Haptens vary in their ability to sensitize. Haptens such as 2,4-dinitro-l-chlorobenzene (DNCB), squaric acid dibutyl ester, and diphenylcyclopropenone are strong sensitizers and induce allergic contact dermatitis with a single exposure to no more than 0.1 mL of a 0.5% to 1.0% solution placed on a 2-cm circular diameter of forearm skin. The majority of common environmental or commercial sensitizers require repeated exposures to induce sensitization. For humans, the time from hapten recognition to the development of allergic contact dermatitis is typically 14 days (range, 7 to 20 days). When sensitization has been accomplished from a single exposure to a hapten, the induction site typically flares without the application of a second or eliciting dose 7 to 20 days later. The reason is that very small amounts of the original dose remain in the skin and act as the second (eliciting) stimulus once sensitization has developed. In most clinical cases of allergic contact dermatitis, the original sensitization occurs some time during multiple induction exposures. When the unknowingly sensitized individual is reexposed to the material, contact dermatitis is expressed 1 or 2 days later (the time can be as short as 6 hours or can take up to 3 or 4 days). The important point is that it takes 7 to 20 days for the initial sensitization to take place; but once that has occurred, subsequent reactions typically occur within 8 to 96 hours, depending on the hapten concentration, the manner of exposure, and the individual's degree of sensitization. Most contact dermatitis is therefore an expression of an exposure 1 or 2 days previously. Occasionally, the only exposure that can be documented for a patient with contact dermatitis occurred several weeks before the development of the dermatitis. This finding represents the development of sensitization during the 2-week period, with flaring from the residual of the first exposure. Clinically, this situation is uncommon but important to recognize as a form of contact dermatitis. It has been observed in patients who had contact dermatitis induced by a large exposure to poison ivy or hair dye.

Most patients find it difficult to believe that they have become allergic to commercial products used for years as cosmetics or medications. Modest or weak haptens induce sensitization when repeatedly used under certain conditions (see Factors Influencing Sensitization, below). The product's major ingredient, the components of the delivery vehicle, the preservative system, and fragrances are likely causes of the dermatitis. To complicate matters, allergic contact dermatitis is frequently a superimposed condition on a nonallergic dermatitis. Thus an individual may use over-the-counter topical medicaments for a preexisting condition, and contact dermatitis may develop to an ingredient in the topical medication.

Probably the most common causes of allergic contact dermatitis are plants such as poison ivy, poison oak, and poison sumac. Other common sensitizers include *para*-phenylenediamine, nickel compounds, rubber additives, and numerous biocides used for the preservation of products that contain water. Health-care workers, now required to wear latex rubber gloves, can develop both a delayed and an immediate (urticarial) hypersensitivity to the gloves (see Chapter 73). The rubber additives and antioxidants are typically responsible for the delayed hypersensitivity, although the latex itself can be the cause of contact urticaria or anaphylaxis (2,3 and 4). It is now well understood that certain proteins from the latex itself are mainly responsible for the immediate contact urticaria. The investigation of a latex glove reaction requires clinical skills in both patch testing and immediate skin testing for urticaria. Topical glucocorticoids, often used to treat various forms of dermatitis, have been identified as themselves causing contact dermatitis (5).

Photoallergic contact dermatitis is manifested as an exaggerated response to sunlight and requires exposure to light after the topical application of certain chemicals. Agents that may be responsible for photoallergic contact dermatitis include sunscreens, after-shave lotions, topically applied sulfonamides, antihistamines, certain plants, and antibacterial agents in soaps (although many of these agents have been removed from soaps). This form of photosensitivity must be differentiated from photosensitivity reactions to systemically administered drugs.

Factors Influencing Sensitization

Three important variables influence the development of allergic contact dermatitis on an experimental or clinical level: (a) increasing the concentration of the allergen increases its rate of sensitization (6), (b) increasing the number of exposures leads to higher sensitization rates, and (c) allergen exposure through preexisting inflammation also increases the rate of sensitization. For example, exposure to a potential allergen on an area of irritated skin such as an area of stasis dermatitis will increase its chance of inducing allergic contact dermatitis (7). Strong sensitizers are typically irritating to the skin (creating their own nonspecific inflammation), but not all strong irritants are sensitizers.

CLINICAL PRESENTATION

Clinical Findings

The clinical findings of allergic contact dermatitis depend on the severity of the eruption. Factors that dictate the severity and persistence of lesions include the sensitivity of the individual, the dose of antigenic exposure, and the potency of the antigenic stimulus.

The clinical lesions vary from transient redness to edematous red papules that become vesicular and, at times, even bullous (Fig. 71.1). Itching is one of the hallmarks of the disease and varies in intensity. Any part of the skin that comes into contact with the sensitizing substance may be involved. Characteristically, the dermatitis is, at first, limited to the skin sites exposed to the antigen, although later it may spread to other areas.

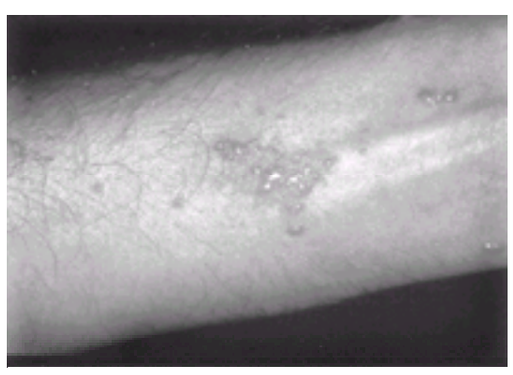

Figure 71.1. Acute allergic contact dermatitis on the arm, showing various-sized blisters.

The course is variable. If the cause is removed, simple redness may fade within several days. However, when vesicles occur, they often rupture, resulting in oozing and crust formation (Fig. 71.2A and Fig. 71.2B). As the inflammation subsides, scaling and some temporary thickening of the skin develop. Continuing exposure to the antigen or complications such as excoriations, infections, or irritation, or allergy to the medicaments applied for the treatment of the primary eruption may perpetuate the dermatitis. Chronic lesions are almost always pruritic and are characterized by lichenification (thickening of the skin with accentuation of skin markings) and scaling without any apparent vesicle formation.

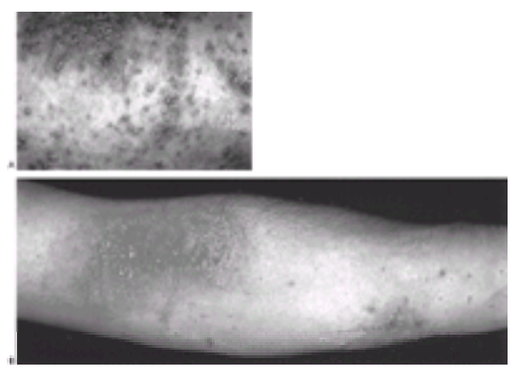

Figure 71.2. A, B: Subacute allergic contact dermatitis on the arm, showing erythema, oozing, and crust formation.

Histologic Features

The histopathologic features of allergic contact dermatitis vary according to the severity of the eruption. Although the findings may be strongly suggestive of contact dermatitis, no pathognomonic features distinguish allergic contact dermatitis from many other forms of dermatitis. Perivascular infiltrates of lymphocytes and monocytes are seen in the upper dermis within 6 hours of exposure to antigen. The infiltration may be accompanied by considerable edema of the superficial dermis. Subsequently, intracellular and intercellular edema develops in the epidermis to form what is known as spongiosis (Fig. 71.3) (8). During these later stages (48 to 72 hours after onset), basophils may constitute about 5% to 15% of infiltrating cells (9). Eosinophils may also be present, albeit to a lesser extent. Fibrin deposition, as well as superficial dermal blood vessel changes, may be seen. More chronic lesions of allergic contact dermatitis are characterized by hyperkeratosis, acanthosis (thickening of the epidermis), and a variably dense mononuclear cell infiltrate in the superficial dermis.

Figure 71.3. Allergic contact dermatitis, acute stage. There is a superficial perivascular infiltrate composed predominantly of lymphocytes and monocytes, edema of the papillary dermis, and epidermal spongiosis with lymphocytes within the epidermis.

Differential Diagnosis

Because allergic contact dermatitis may resemble so many other types of dermatitis (e.g., primary irritant dermatitis, nummular dermatitis, atopic dermatitis, dermatitis herpetiformis, herpesvirus infections), an allergen should be suspected as the cause or as an aggravating factor in dermatitic eruptions. Skin biopsy may be useful in differentiating certain of these diagnoses but, as noted earlier, there is no definitive histologic pattern in allergic contact dermatitis. Characteristic skin changes and a history of exposure often suggest the correct diagnosis. Identifying the site of the initial lesion is helpful in identifying the cause. Identifying the responsible agent, however, may require exhaustive questioning of the patient regarding medical history, occupation, hobbies, household duties, wearing apparel, topical medications and cosmetics, vacation history, and spouse's activities.

Patch Testing

Patch testing with a suspected allergen or with a standard group of common contact allergens may be indispensable if questioning is fruit less. The clinical patch test was initially introduced by Jadassohn (10) in 1896 to demonstrate that allergic contact dermatitis was a clinical entity and could be simulated in individuals who had become sensitized. If a chemical is suspected as the cause of the dermatitis, the material (in the appropriate concentration) is applied to normal-appearing skin (usually the back) under a nonabsorbent adhesive patch and left for 48 hours. In recent years, patch-test kits with materials in their appropriate concentrations have become available (11). A positive patch-test reaction consists of redness with some papules and, at times, tiny blisters. Patch tests should not be performed when patients are experiencing an acute dermatitis because the allergen may worsen the eruption in a very sensitive individual or there may be nonspecific false-positive reactions. A positive result on the patch test, although indicating allergy, does not necessarily identify the agent causing the contact dermatitis. There should be a history of exposure to the test agent in the areas where the dermatitis originally appeared. The North American Contact Dermatitis group has identified the 20 most common allergens by patch testing (Table 71.1) (12). The 20 screening allergens commercially available for patch testing in the United States accounted for only 54% of the patients with positive allergic reactions (12).

Nickel sulfate	Carba mix
Fragrance mix	Diazolidinyl urea
Neomycin sulfate	Lanolin
Balsam of Peru	Imidazolidinyl urea
Thimerosal	Methylchloroisothiazolinone/ methylisothiazolinone
Formaldehyde	Ethylenediamine dihydrochloride
Quaternium-15	*p*-Tert-butylphenol formaldehyde resin
Bacitracin	Rosin (colophony)
Cobalt	Benzocaine
para-Phenylenediamine	
Thiuram mix	

From deShazo R, Kemp S. Allergic diseases: diagnosis and management. Philadelphia: JB Lippincott, 1995; with permission.

TABLE 71.1. The 20 Most Frequent Allergens in the United States

TREATMENT

The most important approach to treatment of allergic contact dermatitis is the identification and removal of the offending agent. Identification is best accomplished by taking a complete history and/or by patch testing. Patients with photoallergic contact dermatitis should avoid exposure to both sunlight and the antigen. In the acute phase of contact dermatitis, such as that caused by poison ivy, gauze or thin cloths dipped in lukewarm water and applied to the lesions are soothing and help stop the oozing; these open wet dressings should be applied for 20 to 30 minutes, 4 to 6 times daily. Blisters may be drained with a sterile needle, but the tops should not be removed. In severe or extensive cases or even in more localized eruptions in which there is facial involvement, early treatment with prednisone administered in an initial dose of 60 to 80 mg daily for 12 to 16 days is recommended. The prednisone dose should be decreased by 10 to 20 mg every 3 to 4 days. Topical glucocorticoids are not helpful in the blistering phase, but when the dermatitis becomes less acute, topical glucocorticoid creams or ointments should be rubbed into the lesions 4 to 5 times a day. Oral antihistamines are used for their sedative effect. Desensitization is generally ineffective in contact dermatitis. Although topical cyclosporine may be effective in preventing allergic contact sensitivity in animals, it is not available for use in humans (13). Various skin-surface protectants are available for use in humans to prevent allergic contact dermatitis to specific allergens, such as poison ivy (14,15).

PATHOBIOLOGY

Allergic contact dermatitis has been studied extensively because it represents a highly specific and reproducible form of delayed-type hypersensitivity. The importance of cellular mechanisms was demonstrated by Landsteiner and Chase (16), who showed that contact sensitivity from a sensitized guinea pig could be transferred to a nonsensitized guinea pig by peritoneal exudate cells rather than by serum. Subsequently, Chase (17) and others (18,19) used a variety of cell sources to conclude that lymphoid cells played the central role in this adoptive transfer. Other studies suggest that serum factors also may be important in the transfer of contact sensitivity (20,21).

Numerous studies have demonstrated the importance of skin in the generation of allergic contact dermatitis and other forms of delayed-type hypersensitivity. Macher and Chase (22) demonstrated that the induction of allergic contact dermatitis requires the interaction of hapten with peripheral tissues (skin). They showed that when hapten was injected into guinea pigs' ears and the ears were excised before a critical 24-hour period, sensitization did not occur. Furthermore, if the ears were excised between 12 and 24 hours after injection of the hapten, specific immunologic unresponsiveness occurred, presumably because of the tolerogenic effect of the rapidly escaping blood-borne hapten. These studies were very much in keeping with those of Sulzberger (23), who demonstrated that skin is a unique site for sensitization, in that when haptens were injected into organs other than skin, specific unresponsiveness ensued. Thus the immunologic outcome, that is, the degree of sensitization or tolerance, is governed by the influences that the routes and the manner (dose, schedule) of exposure exert on helper and suppressor subsets of T cells. When sensitization does occur, usually a downregulating component is generated as well.

Godfrey (24) expanded on prior work on antigenicity by demonstrating that not only was the ability of the hapten to bind covalently with a carrier protein important for allergenicity, but also the chemical's solubility and its local effects influenced the degree of sensitization. Many haptens responsible for allergic contact dermatitis have low molecular weights (<500) and are lipid soluble. The early concepts that induction and elicitation responses are directed toward hapten/carrier complexes remain valid today. The molecular complex recognized by T cells is now known. Studies suggest that haptens bind to endogenous peptides that are associated with class II or class I major histocompatibility complex (MHC) (25,26 and 27). These complexes are well represented on epidermal Langerhans cells (28). An alternative, and not mutually exclusive possibility, is that the hapten binds directly to the class II MHC molecules that are represented on the surface of Langerhans cells (29).

The induction of contact sensitivity involves interaction between T lymphocytes and class II–bearing Langerhans cells (Fig. 71.4 and Fig. 71.5). These latter cells are bone marrow derived but reside within the epidermis and can, in *in vitro* and *in vivo* animal models, replace macrophages and dendritic cells in the induction of various delayed-type hypersensitivity reactions (28). After allergen (hapten) application to skin, there is enhanced class II MHC antigen expression as well as enhanced T-cell stimulatory function by Langerhans cells (30). Indeed, certain haptens are capable of causing a selective and profound enhancement of various messenger RNAs for cytokines produced by the epidermis. Thus interleukin (IL)-lb, derived from Langerhans cells, and IL-10 and tumor necrosis factor (TNF)-a, both derived from keratinocytes, are upregulated in response to hapten application to nonsensitized skin (31). IL-1b and TNF-a are probably both required for the activation and migration of Langerhans cells from the epidermis to the regional lymph nodes (32,33,34,35 and 36). IL-10 downregulates immune responses by directly affecting the antigen-presenting capacity of Langerhans cells (37,38).

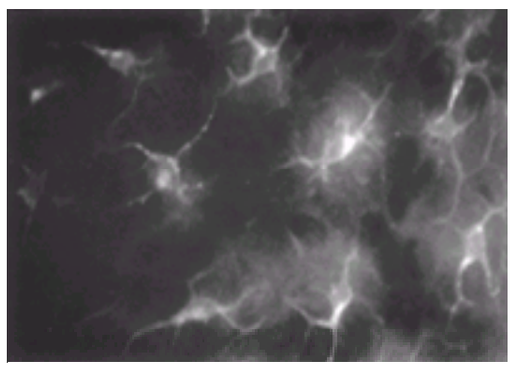

Figure 71.4. Immunofluorescent staining of normal epidermis with major histocompatibility complex (MHC) class II antigen antibody showing dendritic Langerhans cells. The epidermis was separated from the dermis and is viewed *en face*. There is no MHC class II antigen staining of keratinocytes.

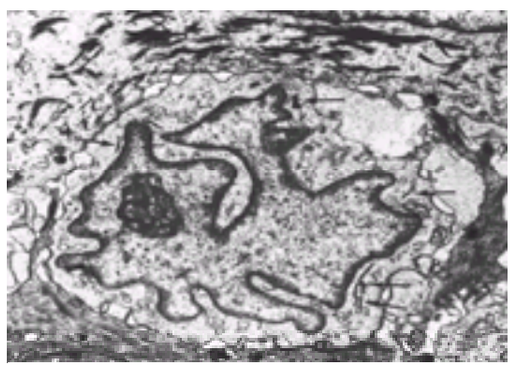

Figure 71.5. Electron photomicrograph of an epidermal Langerhans cell in a milieu of keratinocytes. The cell exhibits a lobulated nucleus, lacks tonofilaments (seen in adjacent keratinocytes), and contains typical cytoplasmic organelles (*arrows*) called Birbeck or Langerhans cell granules.

Macatonia et al. (39) demonstrated that increased numbers of dendritic cells appear in regional lymph nodes 24 hours after hapten application, implying that Langerhans cells migrate from skin into the regional lymph nodes and present antigen therein. Indeed, after intradermal challenge of sensitized animals with electron-dense allergens, Langerhans cells have been observed in dermal vessels resembling lymphatics and in draining lymph nodes (40).

After hapten painting, distinct T-cell subsets (CD8 as well as CD4 T cells) are generated, and both probably contribute to the generation of allergic contact dermatitis. However, it has been known for some time that CD4 T cells are capable of passively transferring contact sensitivity from a sensitized animal to one that has never been exposed to the hapten (41). Moreover, a distinctive subset of CD4 T cells, the Th1 subset that produces IL-2 and interferon-g, is capable of passively transferring contact sensitivity to nonsensitized mice (42,43). Indeed, these effector T cells can be generated by culturing hapten-modified Langerhans cells with virginal T cells (44). More recent studies have implicated CD8 T cells in the generation of contact sensitivity (45).

In addition to effector T cells mediating inflammation, specific CD8 T cells are also generated after sensitization with haptens (46). Thus the degree of immunity

achieved represents a balance between sensitization and tolerance, mediated by a complex suppressor circuit acting on both the afferent and efferent pathways of the delayed hypersensitivity reaction (47). Whether sensitization results in net effector or net CD8 activity depends on the dose and property of the allergens and on the integrity of the epidermal Langerhans cells. Many studies demonstrated, for example, that ultraviolet light irradiation and other physicochemical agents can alter the density or the cell-surface markers of Langerhans cells (48,49 and 50). These alterations may result functionally in the generation of specific immunologic tolerance rather than in sensitization. This tolerance is at least in part due to the generation of hapten-specific suppressor T cells (51). Exploration of various sensitization schedules and methods to alter Langerhans cell functions continues to use these types of information for modulating cutaneous immune responses in humans.

Several studies demonstrated that the dermal infiltrate in the elicitation phase of allergic contact dermatitis in humans consists of an admixture of CD4 and CD8 T cells, with CD4 T cells predominating (52,53). Similar results have, however, been reported for primary irritant dermatitis, suggesting the nonspecificity of these findings (54). This is not too surprising because only a small percentage of T cells infiltrating a cutaneous delayed-type hypersensitivity reaction has specificity for the antigen; the majority of infiltrating cells are therefore nonspecifically recruited (55,56 and 57). Keratinocytes also may participate in the inflammation in contact dermatitis by producing TNF-a and IL-8, by induction of intracellular adhesion molecule-1 (ICAM-1), and perhaps also by inducing the underlying endothelial cells to express various adhesion molecules (58).

FUTURE DIRECTIONS

Allergic contact dermatitis is a significant clinical problem that affects many people. It has also become an important paradigm used to study cell-mediated immune mechanisms both *in vitro* and *in vivo. In vitro* correlates of contact sensitivity have been very informative about the critical role that epidermal Langerhans cells play in the generation and perpetuation of contact sensitivity. *In vivo* models, particularly in mice, have provided important information regarding the dynamics of the cellular responses and have provided numerous opportunities to test various methods for the modulation of immune responses. These methods will continue to be needed to better understand the mechanisms involved in cell-mediated immune reactions in skin, as well as to generate more effective and more specific treatment methods.

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72 ALLERGIC REACTIONS TO DRUGS

Rebecca S. Gruchalla, M.D., Ph.D.

Adverse drug-induced reactions (ADRs) have been defined by the World Health Organization as any noxious, unintended, and undesired effect of a drug that occurs at doses used in humans for prevention, diagnosis, or treatment (1). Because of inadequate reporting methods, it has been and continues to be difficult to determine the actual incidence of ADRs. Several groups who evaluated the extent of underreporting found that in hospitals, including university hospitals, only 6% to 12% of ADRs are reported (2,3).

Despite extensive underreporting, estimates of ADRs have been made. In a recent metanalysis of 39 prospective studies in the United States from 1966 to 1996, the overall incidence of serious ADRs in hospitalized patients was 6.7%; when both serious and nonserious reactions were considered together, this proportion rose to 15.1% (4). These reactions are associated not only with significant morbidity and mortality but also with substantial costs. Bates et al. (5) recently estimated that a 700-bed teaching hospital spends 5.6 million dollars because of ADRs in a single year.

Most ADRs involve the skin. Although most of these reactions are not associated with serious morbidity, they are important because they are the most frequently encountered ADR and are the major reason that drug therapy is discontinued. The Boston Collaborative Drug Surveillance Program, one of the most extensive studies of ADRs, evaluated data from more than 37,000 patients to determine the frequency of cutaneous reactions to drugs commonly used in the hospital (6,7). The data were evaluated in two series, and the overall reaction rate was approximately 2% for both series. Reaction rates were highest for amoxicillin (51 per 1,000 exposed patients) and for trimethoprim-sulfamethoxazole (TMP-SMX) (34 per 1,000 exposed patients); the most frequently encountered reactions were pruritus, morbilliform rashes, and urticaria.

The ADRs that are thought to be immunologically mediated have been designated *hypersensitivity* or *allergic* drug-induced reactions. Although they constitute only 6% to 10% of the ADRs in hospitalized patients (8), they often are serious and potentially life-threatening problems (9). In their metanalysis, Lazarou et al. (4) estimated that allergic reactions accounted for 23.8% of the ADRs identified, and many of these were serious.

Adverse drug-induced reactions are heterogeneous in nature and can be separated into two categories: those that are common and predictable (*type A reactions*) and those that are uncommon and not predictable (*type B reactions*) (10). About 80% of ADRs fall into the first group. In addition to being common and predictable, these reactions usually are dose dependent, are related to the pharmacologic actions of the drug, and occur in otherwise normal persons. Reactions in this category include toxicity, side effects, secondary effects, and drug interactions (reviewed in 11,12).

Less frequently, ADRs fall into the unpredictable drug reaction category. These reactions, in addition to being unpredictable, are uncommon, are typically not related to a drug's pharmacologic properties, and occur in susceptible persons. Reactions in this category include drug intolerance, idiosyncratic reactions, and allergic or hypersensitivity reactions. For details about drug intolerance and drug idiosyncrasy, the reader is referred to the previously mentioned reviews (11,12). This chapter focuses on allergic drug reactions only.

FEATURES OF ALLERGIC DRUG REACTIONS

Although evaluating whether or not a drug reaction involves an immune mechanism is not always easy, several features of allergic drug reactions are common to immunologic reactions in general. Typically, the initial course of therapy is uneventful because there must be a period of sensitization. Thus, if a first-dose reaction does occur, either the reaction is not allergic in nature or there was previous exposure to the drug or to a cross-reacting agent. Reactions are restricted to a limited number of syndromes that are known or are thought to have an immunopathologic basis. Typical drug-induced hypersensitivity reactions include urticaria, angioedema, anaphylaxis, hemolytic anemia, and allergic contact dermatitis, among others. Although many reactions are thought to have an immunologic cause, the actual mechanism involved has yet to be identified. Drug hypersensitivity reactions occur in a small proportion of the population because multiple factors must interact with each other before an allergic reaction is elicited. These factors include the molecular characteristics of the drug, its route of administration, the genetic and metabolic predisposition of the individuals, and environmental factors, such as concomitant infection. Before these important risk factors for the development of drug hypersensitivity reactions are discussed, the current classification system and its limitations are presented.

CURRENT CLASSIFICATION SYSTEMS

In 1963, Coombs and Gell (13) developed a classification system for hypersensitivity reactions that is still used today. Hypersensitivity reactions are classified into one of four categories based on the immune mechanism known or thought to be involved. *Immediate-type hypersensitivity reactions* are mediated by drug-specific immunoglobulin E (IgE) antibodies and include urticaria, angioedema, and anaphylaxis. *Drug-induced cytotoxicity reactions* are mediated by drug-specific IgG or IgM antibodies and include drug-induced hemolytic anemia, drug-induced thrombocytopenia, and drug-induced leukopenia. *Drug-induced immune complex reactions* are mediated by drug-specific IgG antibodies and include drug-induced vasculitis and glomerulonephritis. *Drug-induced T-cell-mediated reactions* are mediated by drug-specific T-lymphocytes and include allergic contact dermatitis and, possibly, drug-induced maculopapular eruptions, bullous eruptions, fixed drug eruptions, and Stevens–Johnson syndrome (SJS). Details of this classification system are provided by DeSwarte in a review (11) of drug allergy.

Unfortunately, most drug-induced allergic reactions cannot be classified easily into one of the Coombs and Gell categories because the responsible mechanisms are not known. Whereas many drug-induced cutaneous and other organ-specific reactions have features consistent with a hypersensitivity mechanism, the actual mechanism has not been identified. Thus, we need to begin thinking “out of the box.” Not only do many drug-induced hypersensitivity reactions not fit nicely into the Coombs and Gell classification scheme, but some reactions most likely involve both immune and nonimmune mechanisms, thus adding to the complexity. Once the underlying mechanisms are better elucidated, a more comprehensive and accurate classification scheme can be developed for allergic drug reactions. In addition, this knowledge will allow us to design more efficient methods to predict and prevent these reactions as well as to manage them if they occur. The elucidation of these mechanisms will not be easy because drug-induced hypersensitivity reactions are uncommon, unpredictable, and not reproducible in animal models.

MOLECULAR CHARACTERISTICS OF ALLERGENIC AGENTS, DRUG METABOLISM, AND ANTIGEN PROCESSING

Because of their macromolecular structure, some drugs are immunogenic in their native form. Drugs in this category include proteins and peptide hormones. Most drugs, however, are of low molecular weight (<1000 Da) and are incapable of inducing an immune response unless they are modified in some way. Because these drugs can and do elicit immune responses, their ability to do so is related to their propensity to combine covalently with large-molecular-weight compounds (14) coupled with successful processing and presentation of the formed immunogenic drug complex by antigen-presenting cells (15).

Our understanding of how drug hypersensitivity reactions occur is based largely on the hapten hypothesis (16,17): Because most drugs are not chemically reactive,

they must be metabolized or “bioactivated” to chemically reactive products. In most instances, drug metabolism is a “good thing,” a type of detoxification process by which drugs are converted from lipid-soluble, nonpolar compounds to more polar, hydrophilic compounds that are cleared by renal or biliary excretion (18). Typically, two sequential biochemical reactions, termed *phase I* and *phase II* reactions, are involved. Phase I reactions involve intramolecular rearrangements and often are mediated by hepatic enzymes of the cytochrome P450 monooxygenase system through oxidation, reduction, or hydrolysis. The products formed may be biologically reactive and more toxic than the parent drug. Typically, however, once these reactive intermediates are formed, they are promptly detoxified in phase II reactions through conjugation with glucuronyl, sulfate, or acetyl groups. Thus, to protect the organism, bioactivation is followed by bioinactivation (19) (Fig. 72.1). In some persons, genetic or environmental factors may perturb the normal balance between these two processes, leading to increased formation of, or decreased elimination of, reactive drug metabolites. If not eliminated, these metabolites may do one of several things. In some cases, they bind to macromolecules, such as lipids, proteins, or nucleic acids, and cause direct cellular damage. A well-known example of this type of direct cellular toxicity is acetaminophen-induced hepatotoxicity (20). Acetaminophen is metabolized to nontoxic metabolites by sulfation or glucuronidation, but a small amount (5%–10%) is oxidized by a number of cytochrome P450 enzymes to *N*-acetylbenzoquinoneimine. Although this reactive metabolite typically is detoxified by conjugation with glutathione, in an overdose situation, the conjugation pathways are saturated, inadequate detoxification results, and hepatic necrosis ensues (19,21).

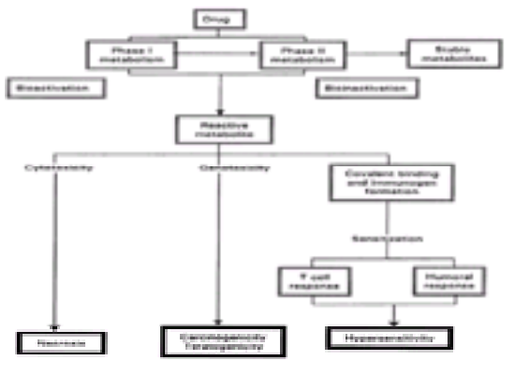

Figure 72.1. Role of drug-metabolizing enzymes in the bioactivation and bioinactivation of drugs. The formation of a chemically reactive metabolite, if inadequately detoxified, may lead to covalent binding to cellular macromolecules, resulting in various forms of toxicity including necrosis, teratogenicity, and hypersensitivity (Adapted from Pirmohamed M, Madden S, Park K. Idiosyncratic drug reactions: metabolic bioactivation as a pathogenic mechanism. *Clin Pharmacokinet* 1996;32:215–230, with permission.)

In addition to being directly cytotoxic, some reactive drug metabolites may covalently bind to, or haptenate, macromolecules, leading to the formation of immunogenic complexes that may then initiate a hypersensitivity reaction. The type of immune response that is elicited toward the hapten depends on the pathway by which the hapten is processed and presented to T cells, and it appears that the particular pathway involved is dictated by the chemical properties of the hapten. Small lipid-soluble molecules (e.g., urushiol) can enter the cytoplasm and can be presented on major histocompatibility complex (MHC) class I molecules for recognition by $CD8^+$ cells via the “endogenous” pathway. In contrast, polar haptens, such as nickel or cobalt, are more likely to be presented on MHC class II molecules for recognition by $CD4^+$ cells (“exogenous pathway”). Some haptens, such as dinitrofluorobenzene, may be processed by both pathways for presentation to both $CD8^+$ and $CD4^+$ T cells (15).

Until recently, little has been known about the antigen-processing pathways involved in drug-induced hypersensitivity reactions. Our increased understanding of drug metabolism and drug-induced diseases, however, is permitting definition of the possible immunologic mechanisms involved in these reactions. Not only are we beginning to be able to identify specific reactive drug metabolites that are formed, but also, through the histologic examination of the particular drug-induced disease, we are able to determine the processing pathways by which these drug-antigens are presented to the immune system.

Persons with morbilliform eruptions to sulfonamides have sulfonamide-reactive T cells in their peripheral blood (22). Moreover, sulfonamide-reactive $CD8^+$ T-lymphocytes have been isolated from sulfonamide-induced bullous eruptions (23). These data lend support to the hypothesis that, at least for some drugs, antigen presentation occurs through MHC class I presentation to $CD8^+$ T-lymphocytes. It is not surprising that this mechanism of antigen presentation may be operative in certain types of drug-induced hypersensitivity because many haptenic drugs undergo intracellular metabolic activation. If not detoxified, these reactive drug metabolites theoretically could conjugate with cytoplasmic proteins. Subsequently, these endogenous proteins would be degraded into drug–peptide complexes, transported into the endoplasmic reticulum, associated with MHC class I molecules, and delivered to the cell surface, where they would be presented to $CD8^+$ T cells. Although this proposed mechanism explains how drug metabolites may be presented to the immune system and how T cells may be involved in drug-induced hypersensitivity reactions, the critical question is whether or not this mechanism plays an important role in drug-induced allergic diseases. Later in this chapter, emerging new data are presented to show that T cells may be central to the pathogenesis of many drug-induced hypersensitivity responses.

PREDISPOSING FACTORS FOR THE DEVELOPMENT OF IDIOSYNCRATIC DRUG REACTIONS

Pharmacologists classify type B ADRs somewhat differently than do allergists, and their scheme actually may be more appropriate for evaluating risk factors for the development of drug-induced reactions. Whereas both groups agree on the definition of type A reactions, pharmacologists use the terms *type B reactions* and *idiosyncratic reactions* interchangeably; that is, they consider all type B reactions to be idiosyncratic because they are unpredictable, occur in a small number of patients, and are not related to the pharmacologic actions of the drug. Although many idiosyncratic reactions have clinical characteristics suggestive of drug allergy, there is no direct evidence that an immune mechanism is operative in all of them. Thus, the term *idiosyncratic drug reaction* is an operational term and does not imply a specific mechanism (24). Since idiosyncratic reactions often times are serious, it is important to elicidate if possible, the chemical, biochemical and immunologic mechanisms responsible for their elicitation.

As pointed out by Park et al. (24), perhaps the most important goal should be to characterize the individual factor(s) responsible for the idiosyncratic nature of the reaction. This information could be used prospectively to avoid drug toxicities by (a) identifying patients who have a genetically determined susceptibility to certain chemical classes of drugs; (b) analyzing cells from identified patients in preclinical experiments with new drugs that are chemically similar to drugs known to cause idiosyncratic reactions; and (c) designing drug analogs that retain the efficacy of the parent drug but that do not elicit idiosyncratic toxicity.

Both drug-related and host-related factors contribute to the development of drug-induced adverse reactions. An important drug-related factor is the presence of certain functional groups in the drug or its metabolite. Hypersensitivity reactions to the penicillins and cephalosporins occur not through the formation of reactive drug metabolites but through a directly reactive b-lactam ring. Because of its strained state, the b-lactam ring readily reacts with nucleophilic groups on proteins (25). This drug-carrier complex is formed in all individuals, but only some mount an immune response.

Other potentially important functional groups are aromatic amino groups. If possible, these groups are avoided during drug design because the metabolism of agents containing them leads to the production of hydroxylamines and nitroso compounds, oxidative metabolites that are unstable and extremely toxic. It is not always possible, however, to “design” a drug that is both clinically effective and chemically inert. Numerous drugs in use today possess an aromatic amino group; these agents include the sulfonamides, procainamide, and the aromatic anticonvulsants. Each of these important therapeutic agents has been associated with idiosyncratic reactions thought to result from the formation of oxidative metabolites (19,21,26,27).

Other drug-related risk factors include the route, frequency, and duration of drug administration. The topical route appears to be the most sensitizing route, and frequent short courses of therapy are more likely to result in the development of an immunologically mediated drug-induced reaction than are longer courses of therapy separated by several years. For an in-depth discussion of drug-induced risk factors, the reader is referred to two reviews (12,28).

Probably the two most important host-related risk factors for the development of drug-induced idiosyncratic reactions are the genetically determined events that dictate how a drug is metabolized and, once metabolized, how or if it is “recognized” in an immune sense. Genetic polymorphisms exist in numerous types of drug-metabolizing enzymes, including those that are responsible for drug oxidation, hydrolysis, acetylation, and conjugation (24). Probably the clearest association between a genetic polymorphism and idiosyncratic drug reactions has been found for *N*-acetyltransferase-2 (NAT-2). Because of this polymorphism, individuals differ in their NAT-2 activity, resulting in the slow- and fast-acetylator phenotypes. Slow acetylation leads to decreased formation of *N*-acetylated nontoxic products and increased formation of metabolically active, toxic products. Thus, persons with this phenotype may be predisposed to developing drug-induced reactions to particular compounds.

Because the liver is a major site of drug oxidation, it would seem to be most vulnerable to drug-induced injury. Balanced against this potentially damaging process is the simultaneous presence of numerous detoxifying processes along with a large glutathione reserve. Despite these “fail-safe” mechanisms, the liver often is involved

in drug-induced reactions. Toxic metabolites are formed that escape bioinactivation. Acyl halides derived from halothane metabolism (29), epoxide metabolites derived from the metabolism of tricyclic antidepressants (30,31 and 32), and hydroxylamines derived from sulfonamide metabolism (33,34) all may induce idiosyncratic hepatotoxicity via an immune mechanism when detoxifying systems are altered. Using an *in vitro* lymphocyte cytotoxicity assay, several groups have shown that persons who have experienced idiosyncratic drug reactions to drugs known to undergo metabolism have a decreased ability to detoxify reactive species formed from the parent compound (34,35). Although these results suggest that these persons have a detoxification defect, the actual genetic defect remains to be identified.

In addition to variations among individuals in drug metabolism, there also could exist variation in both the formation of immunogenic drug complexes and the response to these immunogens once they are formed. Although cell-surface antigens are thought to be more potent immunogens than circulating drug-protein complexes (16), studies in humans have been restricted to the analysis of drug antigens associated with plasma proteins. Lafaye and Lapresie (36) analyzed the amount of penicillin that is covalently bound to albumin in patients taking high doses of penicillin. They found little individual variation in the amount of penicilloyl groups formed. In contrast, Gruchalla et al. (37) found that patients with AIDS who were taking sulfonamide therapy for *Pneumocystis carinii* pneumonia (PCP) prophylaxis differed in their ability to produce sulfonamide-conjugated serum proteins. Moreover, only some of those who possessed these drug-protein serum complexes developed sulfonamide-induced hypersensitivity reactions, a finding that suggests that formation of immunogenic drug complexes is necessary but not sufficient for the development of a hypersensitivity response.

Many idiosyncratic drug reactions have immunologic features and thus, at least in part, are immunologically mediated. They occur only after several days of treatment and recur immediately on drug readministration. An immune mechanism offers a plausible explanation for the seriousness of these idiosyncratic reactions because both cytolytic and inflammatory components can be activated in response to a small amount of antigen or drug hapten located in a target tissue (16). Both the magnitude and the type of the immune response generated demonstrate variations among individuals. For both penicillins and cephalosporins, it appears that drug-induced IgE responses are under genetic control. Adkinson and Wheeler (38) demonstrated in the early 1980s that the immune response to the penicilloyl hapten is genetically restricted. They found that whereas some patients produced penicillin-specific antibodies when high-dose intravenous penicillin was administered for 10 days, most had no serologic response. It was postulated that these “nonresponders” were at decreased risk of developing drug-induced anaphylaxis as well as other types of drug-induced hypersensitivity reactions.

The fact that only a select group of persons is affected is related to their unique ability to mount an immune response, which is dictated by their specific MHC genotype. The MHC genes are the most polymorphic genes in higher vertebrates. This high degree of polymorphism causes some individuals to be more efficient at recognizing certain drug-carrier complexes than others, and thus they are “selected” to mount a vigorous drug-induced response. Associations have been made with specific genes encoded by both the class I [human leukocyte antigen (HLA)-A, -B, and -C)] and the class II regions (HLA-DP, -DQ, and -DR) of the MHC. Examples of such associations include hydralazine-induced systemic lupus erythematosus, which is associated with HLA-DR4 (39); oxicam-induced toxic epidermal necrolysis, which is associated with HLA-A2 and -B12 (40); and sulfonamide-induced toxic epidermal necrolysis, which is associated with HLA-A29, -B12, and DR-7 (40). The finding that immune responses to drugs may be governed by genes residing in both the class I and the class II MHC regions adds further support to the fact that both the endogenous (MHC class I) and the exogenous (MHC class II) pathways of antigen processing are important in the elicitation of drug hypersensitivity responses.

Although the positive HLA associations that have been identified may be used to estimate the relative risk of developing a drug-induced reaction, they do not predict absolutely that a reaction will occur in an individual patient. In the study of Batchelor et al. (39), most patients with hydralazine-induced lupus were HLA-DR4 positive, but 25% of the control subjects were HLA-DR4 positive as well. Thus, although specific genetic associations have been identified, it remains to be seen how these associations will help in predicting the development of drug-induced immune responses.

Finally, because an immune response is initiated, it is not certain that tissue damage will result. The production of antibodies alone is not a pathologic process (24). In some instances, patients may remain asymptomatic despite the fact that they develop drug-specific IgG antibodies during therapy. Thus, other factors that have not yet been elucidated are important in the translation of an immune response into clinical disease.

PATHOPHYSIOLOGY OF ALLERGIC DRUG REACTIONS: EMERGING CONCEPTS

Sites of Drug Metabolism

The liver is the main drug-metabolizing organ. Despite the fact that highly reactive drug metabolites constantly are being generated in this organ, adverse drug-induced hepatic reactions are relatively rare due to the liver's incredible detoxifying capacity. Nevertheless, reactions do occur. Long-term treatment with the diuretic drug tienilic acid may lead to autoimmune hepatitis in some patients; this reaction is associated with the production of autoantibodies directed against the cytochrome P-450 2C9 isoenzyme, which is the enzyme responsible for the metabolism of the drug to its reactive form (41,42). Thus, probably because of their highly reactive nature, the metabolites, immediately after they are generated, covalently bind to the enzyme responsible for their formation. A similar mechanism is operative for both halothane-induced hepatitis (43,44 and 45) and dihydralazine-induced hepatitis (46). Autoantibodies again are directed toward the neoantigen formed by the drug metabolite in conjunction with the cytochrome isoenzyme responsible for its formation.

Since most drug-induced reactions involve the skin, the question arises as to whether extrahepatic drug metabolism is occurring. It is possible that, after being formed in the liver, drug metabolites travel to distant sites where the reaction is manifested; but it is more likely that they are being formed at the reaction site itself. The skin, which is the largest organ in the body, is metabolically active and contains cells that have both phase I and phase II drug-metabolizing enzymes (47,48). Neutrophils, monocytes, macrophages, keratinocytes, and Langerhans cells all have drug-metabolizing enzymes that potentially could lead to the generation of reactive products (49). The skin also is an active immunologic organ, containing numerous cell types that play a strategic role in antigen presentation; this function, along with its metabolic properties, makes it a likely target for drug-induced diseases (50).

T-Cell Immune Responses to Haptens

T cells recognize peptides in the context of MHC class I or class II antigens. Initially, it was thought that T cells recognize only peptides and not haptens and that in the hapten-carrier model T cells recognize the peptide carrier whereas antibodies are specific for the hapten (51). Studies of T-cell responses to trinitrophenyl (TNP) protein derivatives revealed that T cells themselves do recognize and react to haptenated peptides (52,53,54,55 and 56). Whereas the early hapten recognition studies were performed in animal models, more recent studies evaluated T-cell responses to haptenic drugs, such as penicillin and sulfamethoxazole in humans.

In 1992, Martin et al. (57) generated class I-restricted, TNP-specific T-cell clones and then assessed their ability to recognize a variety of haptenated peptides. The hapten-specific, MHC-restricted T cells could recognize TNP-conjugated proteins irrespective of the exact amino acid sequence of the presenting peptide. Instead of recognizing a portion of the carrier protein, these T cells recognized only the TNP hapten. Another interesting finding was that most of the clones reacted to a major type of class I-associated octapeptide that carried TNP lysine in the central position 4. These results suggest that in hapten recognition by T cells, the peptide serves only to anchor the hapten to a defined position on the MHC surface and that it contributes little, if any, to the specificity of the antigenic epitope (Fig. 72.2A) (58,59).

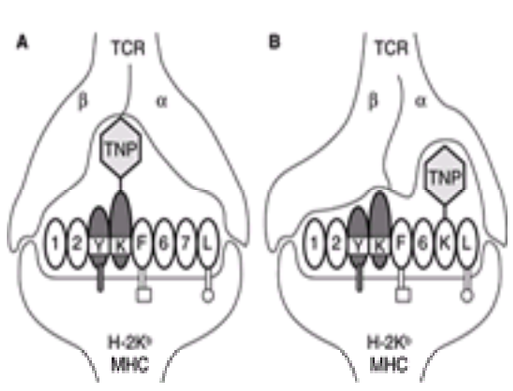

Figure 72.2. Two types of H-2K^b-restricted trinitrophenyl (TNP) determinants. **A:** The immunodominant TNP determinant on H-2K^b contains the hapten bound to lysine in the central position 4 of K^b-associated octapeptides. Many receptors recognizing this determinant predominantly contact the hapten itself in addition to haplotype-specific major histocompatibility complex (MHC) structures but barely interact with side chains of the carrier peptide. **B:** In contrast, T cells specific for peripherally (position 7) modified TNP peptides contact the hapten-peptide via two independent subepitopes: one represented by TNP, the other by amino acids in carrier positions 3 and 4 T-cell receptors (TCRs) a,b. (From Weltzien H, Moulon C, Martin S, et al. T cell immune responses to haptens: structural models for allergic and autoimmune reactions. *Toxicology* 1996;107:141–151, with permission).

In subsequent experiments with synthetic TNP-peptides, T cells specific for hapten peptides that carried TNP–lysine in the peripheral position 7 of the octapeptide were obtained. Although these cytotoxic T-lymphocytes (CTLs) also demonstrated class I-restricted reactivity to TNP, unlike the ones previously characterized, they recognized portions of the carrier peptide. In fact, this peptide reactivity was so strong that target cells that had been pulsed with homologous peptides that were not TNP-modified were lysed. Two subepitopes for these CTLs were identified, one the TNP hapten itself, the other a determinant formed by the side chains of amino acids 3 and 4 in the carrier peptide (Fig. 72.2B) (53,58,59). Thus, by positioning the TNP more peripherally on the octapeptide, a dual TNP and carrier peptide response was generated. In light of these data, it was hypothesized that hapten modification of self-proteins may lead to the triggering of T cells that, once activated, also may react with unmodified self-structures, inducing an autoimmune response.

Other studies demonstrated that TNP peptides also can be presented by class II molecules and that the $CD4^+$ T cells generated recognize TNP in the form of MHC-associated, haptenated peptides with the immunodominant TNP epitopes predominantly independent of the amino acid sequence of the carrier peptide (54). Thus, hapten-induced T-cell responses can be elicited via class I or class II antigen presentation. The type of T-cell response generated would depend on the type of antigen processing that occurs. In the case of skin-sensitizing haptens, one of four mechanisms potentially could be involved: (a) the reactive hapten may modify soluble proteins that then are endocytosed by Langerhans cells, processed, and presented by MHC class II antigens; (b) the reactive hapten may modify soluble proteins that then are endocytosed by Langerhans cells, processed, and presented by MHC-like molecules; (c) the reactive metabolite may bind directly to peptides already associated with MHC class I or class II antigens (no processing involved); or (d) the reactive hapten may penetrate the plasma membrane and modify cytoplasmic proteins that then would be processed and presented by MHC class I molecules (59).

The involvement of T cells in allergic reactions to haptenic drugs in humans could be inferred by the fact that T-cell derived cytokines are necessary for the generation, differentiation, and maturation of B cells that secrete drug-specific antibodies. Moreover, T cells themselves are directly involved in some drug-induced diseases (contact dermatitis), and drug-specific T cells have been isolated from drug-induced skin lesions (60,61 and 62). In addition, *in vitro* T-cell reactivity toward several drugs known to cause hypersensitivity reactions has been demonstrated in patients in whom reactions have occurred. Specifically, reactive human T cells have been found in patients who have developed reactions to penicillin, sulfonamides, nonsteroidal antiinflammatory drugs (NSAIDs), and aromatic anticonvulsants (63,64,65 and 66).

Human T-Cell Responses to Penicillin and Penicillin-induced Allergic Reactions

deWeck et al. (67) demonstrated in the early 1970s that penicillin can cause drug-specific T-cell activation. They found that peripheral blood lymphocytes from patients with clinical penicillin-induced reactions incorporated increased amounts of [^{3}H] thymidine in the lymphocyte transformation test compared with cells from nonallergic persons. When cloning technology became available, antigen-specific T-cell clones derived from the peripheral blood of b-lactam-allergic patients with maculopapular exanthems were shown to be $CD3^+$, $CD4^-$, $CD8^+$, HLA-DR^+ and to produce interleukin (IL-2) and interferon-g (IFN-g) on stimulation (60). Proliferation depended on the presence of both antigen and autologous antigen-presenting cells, and the fact that the clones were $CD8^+$ suggested that antigen recognition occurred in association with class I MHC molecules. This study was one of the first to demonstrate that the penicillin molecule itself acts as a true haptenic determinant in humans and that it appears to form a critical part of the epitope recognized by the T-cell receptor.

Subsequently, Hertl et al. (61) evaluated the actual skin-infiltrating lymphocytes in b-lactam antibiotic-induced vesiculobullous exanthems. Immunohistochemical studies revealed that $CD8^+$ T-lymphocytes were the predominant T-cell subset in these lesions. $CD8^+$ epidermal T-cell clones derived from the cutaneous lesions proliferated in response to penicillin-pulsed autologous antigen-presenting cells but not allogeneic antigen-presenting cells, indicating that the clones were not only antigen-specific but also MHC restricted. In addition, these clones were cytotoxic for epidermal cells, a finding that indicated that T cells might play a role in the keratinocyte necrosis associated with drug-induced blister formation.

While experiments in humans strongly suggests that pencillins, like TNP, form part of the epitopes that contact the antigen-specific receptors of b-lactam-specific T cells in allergic individuals, it has not been clearly shown exactly how penicillin recognition by T cells occurs. To define further the molecular constraints involved in the T-cell response toward penicillin, Padovan et al. (66) constructed “designer” peptides that contained both defined anchor residues for given HLA class II alleles and a lysine-bound penicilloyl group in specific T-cell-receptor contact sites. These allele-specific peptides were used to stimulate a panel of penicillin G-reactive T cells clones from two allergic patients who expressed the required DR allele. In addition to possessing particular amino acids at strategic sites in the peptide that served as HLA-anchoring residues, each peptide contained a lysine residue in a position that corresponded to one of the proposed T-cell-receptor contact sites (68). The lysine residue was derivatized with penicillin G after peptide synthesis, and, depending on the location of this amino acid residue, the penicilloyl hapten was positioned in either a central or a peripheral position of the formed peptide. A detailed analysis of two clones that were generated revealed that, for each, a precise positioning of the hapten on the peptide backbone was required for optimal T-cell stimulation. Because it is unlikely that the penicilloyl-associated peptide sequences that induce penicillin-specific T cells *in vivo* resemble the nonphysiologic sequences of the artificially constructed designer carrier peptides, these data suggest that hapten-specific human T cells, like those of the mouse (57,58 and 59), are characterized by position-specific but carrier-independent hapten recognition.

To determine whether penicillin-specific T cells from allergic patients could recognize other antibiotic agents, Mauri-Hellweg and Padovan and colleagues (65,69) evaluated the cross-reactivity of T-cell lines and clones against b-lactam antibiotics. They found two types of b-lactam-specific T-cell reactivity. One group of patients had a rather restricted recognition profile in that the penicillin-elicited T-cell lines generated from these patients proliferated only to the stimulating penicillin, but not to other b-lactam antibiotics, including cephalosporins, even if the side chain was identical. Thus, for these persons, the structure recognized by T cells appeared to be composed of both the penicilloyl determinant and a portion of the side chain. Cells from the second group of patients were stimulated not only by penicillin G but also by related penicillins. They were not, however, stimulated by cephalosporins. For this group, the penicilloyl moiety, common to all penicillins, appeared to be important for T-cell recognition.

Brander and Padovan and colleagues too found that the immune response to penicillin G was heterogeneous (63,70). On generating T-cell clones specific for penicillin-G or benzyl penicilloyl–human serum albumin (BPO-HSA) from peripheral blood mononuclear cells of penicillin-allergic patients, Brander et al. (63) found that many different penicillin G and BPO-HSA reactive clones were identified, even in a single individual. The clones that were stimulated *in vitro* by penicillin G were either $CD4^+$ or $CD8^+$, class II or class I restricted, and they did not require antigen processing because fixed antigen-presenting cells were capable of efficient antigen presentation. In contrast, T-cell clones that were stimulated by BPO-HSA were $CD4^+$ predominantly, and antigen processing was required for T-cell activation. Both penicillin-G- and BPO-HSA-specific T-cell clones produced a heterogeneous cytokine pattern; most produced high levels of IL-2 and IFN-g and variable levels of IL-4 and IL-5. In the case of penicillin-G, because no antigen processing was required, it was suggested that this hapten may cause T-cell stimulation by directly binding to MHC molecules or to their embedded peptides; this novel finding was subsequently confirmed by others (71). Alternatively, the drug may bind to serum proteins like HSA, which then must be processed and presented by antigen-presenting cells. The fact that the penicillin hapten undergoes multiple modes of antigen presentation to activate different T-cell subsets may be the reason for the extensive clinical heterogeneity of penicillin-induced human immune responses.

Although T-cell cloning studies have provided great insight for our understanding of how human T cells recognize the penicillin hapten, their clinical implications are not clear. Some penicillin-allergic patients produce broadly cross-reactive antibodies that are directed to the common penicilloyl moiety, whereas others produce antibodies that are more selective and that are directed toward the b-lactam side chain (72,73,74,75 and 76). Currently, we try to avoid administering penicillins to patients who have had b-lactam-induced drug hypersensitivity reactions. This therapeutic principle is based on the belief that penicillin-specific antibodies from penicillin-allergic patients may be extensively cross-reactive because they are directed toward the penicilloyl group common to all penicillins. In light of the fact that cephalosporins also contain this common group, they are avoided in penicillin-allergic patients as well. Because the extent to which the penicillins clinically cross-react either with each other or with cephalosporins is unknown, it is difficult to answer the age-old question of whether penicillin-allergic patients can safely receive cephalosporins. It appears that for those reactions mediated by penicillin-specific IgE antibodies, the antibodies are exquisitely specific in most cases because the incidence of cephalosporin reactions in penicillin-allergic patients appears to be low (77,78). Because these reactions do occur, however, and some are severe, it cannot be assumed that cephalosporins will be well-tolerated by all penicillin-allergic patients (79).

Although we can demonstrate the presence of penicillin-specific antibodies or T cells in patients who have had hypersensitivity reactions, we have been unable, for many of these reactions, to determine how, or even if, these immune products are responsible for the clinical manifestations. For penicillin-induced anaphylaxis and urticaria/angioedema, it is clear that penicillin-specific IgE antibodies are responsible. The same is true for penicillin-induced hemolysis: Penicillin-specific IgG/IgM antibodies are responsible; however, the mechanism for many other penicillin-induced reactions is unclear. Specific antibodies of the IgG or IgM class produced during a course of therapy may be clinically irrelevant. In addition, although drug-induced cytotoxic T cells may be responsible for the keratinocyte necrosis in penicillin-induced bullous eruptions, we generally do not understand the role played by T cells in drug-induced hypersensitivity reactions.

Human Immune Responses to Sulfonamides and Sulfonamide-induced Allergic Reactions

Sulfonamides cause a variety of unpredictable reactions, including anaphylaxis, urticaria, angioedema, maculopapular rashes, Stevens-Johnson Syndrome (SJS), hepatitis, nephritis, and blood dyscrasias (80). An immune component is thought to be involved in at least some of these reactions. Sulfonamide-specific T cells (64) and antibodies (81,82 and 83) have been demonstrated in hypersensitive patients, and sulfonamide-haptenated proteins have been found in the sera of treated patients (37,84).

Studies have begun to elucidate the way in which sulfonamides may elicit hypersensitivity responses. Shear and others (33,85) initially showed that, in addition to the usual acetylation reaction that yields nontoxic, easily excretable products, sulfonamides also may be oxidized by a cytochrome P450-dependent pathway to reactive hydroxylamine or nitroso intermediates that *in vitro* are cytotoxic for lymphocytes. In addition, lymphocyte cytotoxicity was more pronounced in persons who had a history of sulfonamide-induced hypersensitivity reactions than in those who had tolerated the drug (33,34,86). Carr and Rieder and colleagues (87,88) corroborated these findings in sulfonamide-allergic patients infected with human immunodeficiency virus (HIV) and also demonstrated that cytotoxicity was significantly reduced in the presence of glutathione. Two hypotheses regarding the increased frequency of sulfonamide-induced reactions in AIDS patients were put forth. Low glutathione levels (89), along with a decreased acetylation ability (90), may predispose these persons to develop increased levels of reactive sulfonamide metabolites that may be directly cytotoxic or that may elicit an immune response through covalent haptenation with serum or cell-surface proteins (Fig. 72.3) (37,91,92).

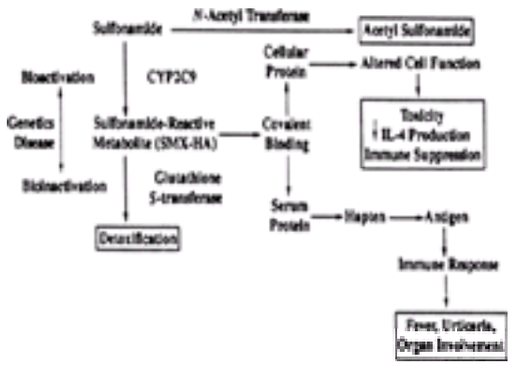

Figure 72.3. Sulfonamide metabolism. The role of sulfamethoxazole hydroxylamine (SMX-HA) in adverse reactions to sulfonamide (From Hess D, Rieder M. The role of reactive drug metabolites in immune-mediated adverse drug reactions. *Ann Pharmacother* 1997;31:1378–1387, with permission).

To dissect the role of T-cell-mediated immune mechanisms in sulfonamide-induced reactions, Kalish and colleagues (22) evaluated antigen-specific T-cell reactivity in patients who had experienced sulfonamide-induced skin eruptions. Using limiting dilution analysis, sulfamethoxazole-specific lymphocytes were detected in the peripheral blood of a patient who had experienced a sulfamethoxazole-induced morbilliform eruption at a frequency of 1 per 170,000 peripheral blood mononuclear cells. This frequency is similar to that observed for urushiol-reactive peripheral blood mononuclear cells in patients with poison ivy eruptions (93). Subsequently, cell lines were generated from sulfamethoxazole-allergic individuals who demonstrated a proliferative response to sulfamethoxazole but not to furosemide, a somewhat structurally similar drug. This study was one of the first to support a role for T cells in sulfonamide-induced reactions, and it corroborated earlier work done by others with penicillin (60,61,67).

Mauri-Hellweg and colleagues (64) extended the preceding studies by analyzing *in vitro* sulfamethoxazole-induced activation of $CD4^+$ and $CD8^+$ T-cell subsets, cytokine secretion, and T-cell proliferation in persons who developed generalized eruptions during sulfamethoxazole therapy. They found that sulfamethoxazole-induced proliferation of peripheral blood mononuclear cells was antigen specific and dose dependent and that IL-5 was the major cytokine secreted. Both $CD4^+$ and $CD8^+$ T cells were activated, and the sulfamethoxazole-specific T-cell clones generated from one patient were of several phenotypes: some were $CD4^+$ only, some were $CD8^+$ only, and some were $CD4^+$ and $CD8^+$. These results both supported and extended the findings of Kalish et al. (22) by showing that not only are sulfamethoxazole-specific T cells involved in sulfamethoxazole-induced reactions but also that MHC class I and class II antigen presentation occurs, a finding reminiscent of the data emerging from the penicillin studies (63,70). It remains to be seen exactly how T cells recognize the sulfamethoxazole hapten because recent data indicate that sulfamethoxazole may be presented to T cells by unconventional pathways (94,95).

Hertl and colleagues (62) demonstrated that sulfamethoxazole-induced T-cell responses were MHC restricted and cytochrome P-450 dependent. Previous studies demonstrated that sulfamethoxazole-induced lymphocyte cytotoxicity was enhanced in the presence of mouse liver microsomes, which are rich in cytochrome P-450-dependent enzymes (86,96). For that reason, dermal T-lymphocytes from a patient with a sulfamethoxazole-induced bullous exanthem were isolated, cloned, and stimulated with sulfamethoxazole in the presence or absence of mouse microsomes. Addition of the microsomes to the culture system caused markedly increased drug-specific T-cell proliferation, a finding that further supports the role of cytochrome P-450-dependent enzymes in the generation of allergenic sulfonamide metabolites. It was demonstrated previously that some patients with sulfonamide-induced cutaneous hypersensitivity reactions have a reduced *N*-acetylating capacity (97). Thus, these results together suggest that polymorphism of drug-metabolizing enzymes is at least one factor that may determine whether a patient is at risk of developing sulfonamide-induced hypersensitivity responses. In addition, because many of these drug-induced reactions are cutaneous in nature, the most relevant drug metabolism may be that which is occurring *in situ*, that is, in the skin (49).

Although Naisbitt and colleagues (98) found that both the *N*-hydroxy and nitroso metabolites of sulfamethoxazole can haptenate cell surface proteins at therapeutic drug concentrations, they hypothesized that under normal physiologic conditions, extensive haptenation probably is prevented due to the presence of plasma thiols. If, however, a balance between drug bioactivation and metabolite bioinactivation, both hepatic and peripheral, is disturbed, such as may occur in patients with systemic disease processes [such as acquired immunodeficiency syndrome (AIDS)], cell surface haptenation may be extensive and may lead to a hypersensitivity response. Sulfamethoxazole metabolites also induce apoptosis in T cells (99) and suppress sulfamethoxazole-specific T-cell proliferation and mitogen-induced antibody responses (100,101). Thus, although these metabolites appear to play multiple roles in sulfonamide hypersensitivity, the mechanisms by which they elicit hypersensitivity responses have not yet been clarified.

Sulfonamide-induced hypersensitivity responses are clinically heterogeneous, a characteristic that should not be surprising in light of the fact that numerous mechanisms appear to be responsible for these reactions. T cells seem to be involved in many of these reactions, but their function is unclear. The same is true for reactive sulfonamide metabolites. Data should evolve that will allow better characterization and thus better diagnosis and treatment of these reactions.

Hypersensitivity Syndrome

ROLES OF DRUG METABOLISM AND MOLECULAR MIMICRY

Drug-induced hypersensitivity syndrome (HSS) is a multiorgan, potentially fatal disorder caused by a select group of drugs that include dapsone and other sulfonamides (102), phenytoin and other anticonvulsants (103,104), allopurinol, (105), and minocycline (106). Symptoms consist of fever, rash, and lymphadenopathy, which typically occur several weeks into therapy. The rash, which often is initially a benign morbilliform eruption, may develop into frank exfoliative dermatitis. Some patients also develop hepatitis, nephritis, eosinophilia and other hematologic abnormalities, and myalgias and arthralgias (104).

The requirement for an induction period after the initial drug exposure only, but not after subsequent exposures, is consistent with an underlying immune-mediated process. In addition, the manifestations are not associated with any pharmacologic properties of the implicated agents. Although an immune process has been implicated, the specific mechanism has not been identified.

The aromatic anticonvulsant agents are metabolized, in part, by cytochrome P-450 to reactive arene oxides, and it has been hypothesized that these substances may be involved in the pathogenesis of drug-induced HSS. Shear and Spielberg (35) and others (107), evaluated lymphocytes from patients with known hypersensitivity to anticonvulsant therapy in an *in vitro* lymphocytotoxicity assay using mouse microsomes as a source of P-450 enzymes for the production of reactive drug metabolites. In contrast to lymphocytes from control subjects, lymphocytes from affected patients demonstrated marked cytotoxicity, and the fact that hepatic microsomes were necessary for the toxicity to occur suggests that reactive metabolites, rather than the parent drug, were responsible. Cells from patients' parents exhibited cytotoxicity levels intermediate between the values for cells from control subjects and patients.

Arene oxides normally are detoxified by epoxide hydrolase (16), and in light of the cytotoxicity studies, it has been hypothesized that this enzyme may be lacking or mutated in persons who develop drug-induced HSS (35,108,109). Characterization of the epoxide hydrolase gene failed to reveal any differences between patients with drug-induced HSS and controls (110), but it remains to be seen whether a defect in the regulatory region of the gene causes altered protein expression. The production of excess reactive metabolites through defective detoxification could contribute to the clinical disease process either by a direct cytotoxic mechanism or by the generation of an immune response after haptenation of protein carriers. Covalent protein haptenation by arene oxide metabolites produced from anticonvulsants or hydroxylamines or by nitroso metabolites from sulfonamides could lead to the elicitation of either a humoral or a cellular immune response.

Infections caused by certain pathogenic agents may stimulate T cells that then initiate a reaction toward cells expressing an epitope that mimics a viral or a bacterial protein (17,111). Epitope mapping studies are in progress to determine whether a similar type of molecular mimicry is involved in the generation of the autoantibodies

present in certain types of drug-induced hypersensitivity reactions (17). Gut et al. (112) provided evidence that molecular mimicry may be playing a role in halothane hepatitis. Antibodies in sera from patients with halothane hepatitis recognize liver trifluoroacetyl (TFA)-protein adducts, which arise on oxidative biotransformation of halothane. These antibodies also recognize the E2 subunit of the mitochondrial pyruvate dehydrogenase complex, and for this reason it has been proposed that molecular mimicry may be occurring between this "self" determinant and the TFA protein adducts. In 1992, Leeder and colleagues (113) identified antibodies to several cytochrome P-450 isozymes that are constitutively expressed as well as inducible in rat liver microsomes in the sera of patients with anticonvulsant-induced HSS. These antibodies were not found in sera from healthy control patients receiving chronic phenytoin therapy without toxicity or patients with hepatic failure not receiving anticonvulsants. Neither the impetus that caused their formation nor their role in disease pathogenesis was elucidated. The conjugation of arene oxide metabolites to self-proteins, in this case, cytochrome P-450 isozymes, may lead to the formation of altered self-proteins that trigger an autoimmune response.

Findings suggest that there may be an association between human herpes virus 6 (HHV-6) infection and drug-induced HSS (114,115). Suzuki and colleagues (114) found HHV-6-specific IgG antibodies, which increased with disease progression, in a patient who had allopurinol-induced HSS. Tohyama et al. (115) described two cases of sulfasalazine-induced HSS that were associated with reactivation of HHV-6. It had been shown that HHV-6 could induce severe infectious mononucleosis (116), graft-versus-host disease (117), and interstitial pneumonitis (118); but this demonstration was the first of a possible association between drug-induced HSS and reactivation of HHV-6. The mechanism by which the virus helps to elicit a drug-induced response is not known. Possibly, HHV-6 reactivation is facilitated by the immunologic events associated with hypersensitivity reactions. Alternatively, drug metabolism may be altered by HHV-6 reactivation and excessive reactive drug metabolites are formed. In light of the facts that T-cell activation is required for reactivation of HHV-6 (119) and that patients with drug-induced HSS have activated peripheral blood mononuclear cells (64), Tohyama et al. (115) hypothesized that drug-induced HSS may result from a two-step process. First, T-cell activation occurs in response to haptenation of tissue proteins by drug metabolites; second, HHV-6 is reactivated by activated T cells and causes symptoms characteristic of infectious mononucleosis and HSS.

Stevens–Johnson Syndrome/Toxic Epidermal Necrolysis

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are serious drug-induced reactions thought by some to be similar disorders of different severity within the spectrum of erythema multiforme. Based on this concept, Bastuji-Garin and colleagues (120) developed a classification scheme of these disorders that is commonly used in the clinical setting. Both diseases have bullous lesions, mucosal involvement, and skin detachment; but they differ from each other by the amount of skin detachment, which is less than 10% in SJS and more than 30% in TEN. About 50% of cases of SJS are drug induced, and 50% are related to an infectious process. About 80% of cases of TEN are drug related. More than 100 drugs have been implicated in these diseases; the most common ones are sulfonamides, aromatic anticonvulsants, b-lactam antibiotics, allopurinol, and NSAIDs (121,122).

Although the mechanisms responsible for SJS and TEN are not known, it is thought that affected persons have an alteration in the ability to detoxify reactive drug metabolites (26,121,123). These may be directly cytotoxic, as previously discussed. Because SJS and TEN have features consistent with immunologically mediated hypersensitivity reactions (timing, linkage to certain HLA genotypes) (40,124,125), metabolites also are thought to elicit an immune response after covalent protein binding.

Because SJS and TEN may be immunologically mediated reactions, efforts were initiated to detect circulating cytotoxic antibodies, circulating immune complexes, or complement deposition. These efforts have been unsuccessful (126). In contrast to these disappointing endeavors, data have been generated that suggest a critical role of T cells in disease pathogenesis (62,127,128,129,130 and 131). Merot et al. in 1986 initially described the presence of T cells in skin biopsy specimens from an individual who had died from drug-induced TEN (127). Miyauchi and colleagues (128) confirmed these findings and also demonstrated that the predominant T-cell type in the dermis was $CD4^+$ and in the epidermis was $CD8^+$. Villada et al. (129) found that the dermal infiltrate in a patient with TEN was composed of activated T-lymphocytes. In addition, an aberrant expression of HLA-DR by keratinocytes was found that, before this study, had been demonstrated in inflammatory skin disorders (132,133). Subsequently, skin-blister fluid from patients with TEN was found to have high in lymphocyte cellularity (130). These cells were $CD8^+$, $CD29^+$ antigen-primed memory T cells ($CD45RA^-$). The fact that these cells expressed CD29, the common b-chain of the very low activation (VLA) adhesion molecule, suggests that these T-cell-associated adhesion molecules may be responsible for mediating T-cell interactions with skin endothelium (134) and with extracellular matrix proteins (135).

Correia et al. (130) demonstrated that blister-fluid cells were memory and not naive T cells, but they did not actually demonstrate that these cells were drug-specific. Evidence to support a role for drug-specific T-lymphocytes was provided by Hertl and colleagues, who generated penicillin-specific $CD8^+$ T-cell clones from cutaneous lesions of two patients with penicillin-induced SJS (61) and very late activation $CD8^+$ T-cell clones from the lesional skin of a patient with trimethoprim/sulfamethoxazole-induced TEN (62). Thus, one hypothesis that may explain the cell necrosis in SJS and TEN is that T-cell recognition of MHC-associated drug antigens leads to cytotoxic T-cell-mediated keratinocyte death (26). The situation is probably not so simple, however. Because the extent of cell death is marked relative to the number of T cells present, other factors such as cytokines, tumor necrosis factor (TNF) in particular (136), and other cell types (131,136,137) most likely contribute to the pathogenic process.

Recently, the final pathway responsible for epidermal cell death in SJS/TEN has become an intensive area of research (138,139 and 140). Since reactive drug metabolites have been implicated in these diseases, death may occur via direct cellular necrosis. However, in light of the data that suggest that an immune mechanism may be involved, and the fact that cytotoxic T lymphocytes and tumor necrosis factor are known to induce an apoptotic signal in target cells (141), apoptosis may be the more likely mechanism responsible. A characteristic pattern of DNA cleavage is the biochemical hallmark of apoptosis. Therefore, to determine if an apoptotic mechanism was involved, two groups evaluated the extent of keratinocyte DNA fragmentation in patients with TEN (138,139). Both found extensive keratinocyte DNA fragmentation indicating that, indeed, cell death was occurring via an apoptotic mechanism. Moreover, Inachi et al. (139) found that apoptosis was mediated by perforin, a cytoplasmic peptide that is believed to be a major "cytotoxic weapon" of cytotoxic T cells. Thus, these results together suggest that epidermal cell death occurs by cytotoxic T-cell-mediated apoptosis.

More recently, Viard and colleagues (140) found that keratinocytes from patients with TEN, in addition to expressing Fas, which is not atypical, also expressed lytically active Fas ligand. In addition, they found that keratinocytes from patients with TEN induced Fas-mediated cell death in Fas-sensitive target cells. They then showed that intravenous immunoglobulin (IVIG) inhibited Fas-mediated cell death by blocking the Fas receptor and that IVIG treatment led to marked clinical improvement in patients with TEN. Thus, as these investigators suggested, it appears that upregulation of keratinocyte Fas-ligand expression is the critical trigger for keratinocyte destruction in TEN. If IVIG proves to be an effective therapeutic modality for this disorder, it may also be effective in other disorders that involve Fas-mediated tissue destruction.

MANIFESTATIONS, DIAGNOSIS, AND MANAGEMENT OF ALLERGIC DRUG REACTIONS

Drug-induced Anaphylaxis

Anaphylaxis is an acute, life-threatening allergic reaction that is characterized by hypotension, urticaria, angioedema, bronchospasm, abdominal cramping, and cardiac arrhythmias, alone or in combination. The mechanism may be IgE mediated or immune-complex mediated, or it may involve non-IgE-mediated direct histamine release from mast cells and basophils. Although the term *anaphylactoid* sometimes is reserved for reactions that are thought not to be immunologically mediated, the clinical symptoms are the same regardless of the mechanism. The drugs most frequently implicated in drug-induced anaphylaxis are b-lactam antibiotics, NSAIDs, sulfonamides, high-molecular-weight agents (e.g., proteins), muscle relaxants, induction agents, and radiocontrast media (142). Some of these agents are thought to cause anaphylaxis via an IgE-mediated mechanism, and drug-specific antibodies have been identified for several of them (81,82,143,144,145 and 146). Other agents, like the muscle relaxants, may cause anaphylaxis both by immune and nonimmune mechanisms (147), whereas radiocontrast media reactions are thought to be caused by nonimmune mechanisms only (148,149). The mechanism responsible for systemic reactions induced by acetylsalicylic acid (ASA) or NSAIDs are generally due to inhibition of prostaglandin endoperoxide synthase 1 (PGHS-1), with consequent overproduction of cysteinyl leukotrienes. These reactions are class related (see Chapter 27). Occasionally, reactions to these agents have features that resemble IgE-mediated reactions (reaction is drug-specific and occurs after two or more exposures to the same drug), but drug-specific IgE antibodies have not been consistently identified (150).

Anaphylactic reactions usually are not difficult to recognize clinically, and, in many instances, the culprit drug is easily identified. During anesthesia, however, when many drugs are given in rapid succession, it is sometimes difficult to determine the cause of the anaphylactic event when one occurs. In addition, many of the administered drugs are known to cause anaphylactic or anaphylactoid reactions. Hypnotic agents and muscle relaxants are the most frequently implicated, along with opioids, benzodiazepines, neuroleptic agents, and nonanesthetic agents, such as antibiotics and latex (151).

Diagnostic tools include *in vivo* skin testing and *in vitro* radioallergosorbent tests for the relevant allergens. For therapeutic agents that are immunogenic in their native form (foreign antisera, hormones, enzymes, toxoids), specific IgE antibodies can be demonstrated by skin prick testing. Because most drugs are of low molecular weight and not immunogenic in their native state, skin testing is generally not useful. Skin tests with agents used in general anesthesia often are difficult to interpret because many of these drugs cause nonimmunologic histamine release. Skin testing is of value in the evaluation of allergy to muscle relaxants (152,153), barbiturates (152,154), chymopapain (155), streptokinase (156), insulin (157), latex (158), and miscellaneous other agents.

Currently, the immunochemistry of only one antibiotic, penicillin, is clearly known. Using both the major antigenic determinant and several minor determinants in testing,

numerous studies documented the presence of penicillin-specific IgE antibodies in persons who previously experienced penicillin-induced anaphylaxis or urticaria and angioedema (143,145,159,160,161,162,163,164,165,166,167,168,169,170,171,172,173 and 174). In contrast to penicillin, valid skin test reagents for detecting IgE antibodies to other antibiotics have not been developed.

In vitro measurements of allergen-specific IgE antibodies most often are performed with the radioallergosorbent (RAST) assay, which measures circulating IgE antibodies by a solid-phase radioimmunoassay. As with skin testing, application of this assay to drug allergy evaluations is limited due to insufficient knowledge of relevant drug metabolites. RAST assays have been developed to measure IgE antibodies to penicillin (major determinant) (175), insulin (157), chymopapain (155), muscle relaxants (146,176,177), thiopental (178), protamine (179), and latex (180). Both false-positive and false-negative test results do occur. False-positive tests may occur because of high nonspecific binding or high total serum IgE antibody levels. False-negative tests may occur because of interference by high concentrations of IgG "blocking antibodies," low assay sensitivity, obtaining blood samples too soon after a reaction, or the presentation of wrong immunogen on the solid-phase matrix. Only a limited number of reliable drug RASTs are available commercially.

An important test that has become available within the last few years is the tryptase determination. Tryptases are neutral proteases stored in mast cell granules, and their detection in serum indicates the likely occurrence of an anaphylactic–anaphylactoid event. Tryptase has a half-life in plasma of approximately 2 hours, and it is not prone to rapid degradation. For these reasons, tryptase determinations are favored over histamine determinations. Matsson and colleagues (181) reported two cases of intraoperative anaphylaxis to anesthetic drugs in which tryptase levels were elevated, and others too (182) reported elevated tryptase levels after drug-induced anaphylaxis. Schwartz and colleagues (183) recommend that tryptase levels be measured 1 to 2 hours after the onset of the anaphylactic episode. Normal levels are less than 2.5 ng per milliliter; levels higher than 10 ng per milliliter are associated with anaphylaxis, and levels may reach as high as 1,000 ng per milliliter (183). Unless the patient has systemic mastocytosis, an elevated tryptase level supports the occurrence of an anaphylactic event (see Chapter 36). Further skin testing or RAST testing may be performed to elucidate the actual agent responsible.

Management of acute anaphylaxis is not discussed in this chapter. For a discussion of this topic, the reader is referred to the *Anaphylaxis Practice Parameter* (184).

Patients who have experienced drug-induced anaphylaxis should not receive the implicated drug again unless it can be ascertained that drug-specific IgE antibodies are no longer present. If, however, future treatment is required with that drug, desensitization may be considered for those patients in whom drug-specific IgE antibodies are known or are presumed to exist. Whereas most drug desensitization protocols have been developed for b-lactam antibiotics (169,185,186 and 187), the principle of desensitization also has been applied successfully to other antibiotics, insulin, chemotherapeutic agents, vaccines, heterologous sera, and other proteins (28,79,188,189 and 190).

Drug-induced Immune-mediated Cutaneous Reactions

Because it is beyond the scope of this chapter to discuss all the various drug-induced cutaneous reactions, only a few that are known or are thought to have an allergic component are presented.

MACULOPAPULAR/MORBILLIFORM ERUPTIONS

The skin is the most frequently involved organ in drug-induced adverse reactions, and, of the skin reactions that are typically manifested, maculopapular or morbilliform eruptions are the most common. Although any drug can elicit a maculopapular eruption, the drugs most frequently implicated are b-lactams, sulfonamides, anticonvulsants, allopurinol, and NSAIDs (12). The eruptions often are generalized and symmetric, and they typically involve the trunk and extremities while sparing the face, palms, and soles. Associated symptoms may include fever, pruritus, malaise, and joint aches. Usually, the lesions arise within a week or two of therapy, but they have been known to appear a few days after therapy has been terminated. Typically, the lesions fade and ultimately resolve when the incriminating drug is stopped. If the drug is continued, in rare circumstances, the eruption may progress to generalized erythroderma or exfoliative dermatitis.

The mechanism underlying drug-induced maculopapular eruptions is not known, and thus few diagnostic tools exist. Most likely, more than one mechanism is operative in light of the variety of drugs that can elicit skin reactions of this type. To date, no role for drug-specific IgE or IgG antibodies has been found (123,191). Because most of these reactions occur several days into therapy and thus are delayed in nature, it has been thought that another immune mechanism, possibly a cell-mediated process, may be participating. Indeed, results from lymphocyte transformation studies support a role for T cells (192,193), and in some persons, patch-test positivity can be demonstrated (194,195 and 196). In addition, several groups have isolated penicillin- and sulfonamide-specific T-cell clones from the peripheral blood mononuclear cells of patients with maculopapular or morbilliform eruptions elicited by these agents (22,65,69,71,94,197). These results suggest that at least some drug-induced maculopapular eruptions may be T-cell mediated.

The treatment of maculopapular or morbilliform eruptions consists of discontinuation of the culprit drug and provision of symptomatic relief. Antihistamines usually are not of benefit because there is no evidence of mast cell involvement in reactions of this type. Because the mechanism is not thought to be IgE mediated, a graded challenge may be considered if the drug is required in the future (79). Readministration is absolutely contraindicated if the previous reaction had features that were consistent with SJS or TEN, however.

URTICARIA

Urticaria is another common drug-induced skin eruption that can be elicited by virtually any drug. The lesions typically are generalized and are characterized by pruritic, erythematous wheals that are evanescent. Drug-specific IgE antibodies may or may not be involved, and, for this reason, skin testing may or may not be helpful (see limitations of skin testing to drugs in preceding "anaphylaxis" section). Although antibiotic-induced urticaria is most likely caused by an IgE-mediated mechanism, some antibiotics, such as vancomycin and ciprofloxacin, may cause urticaria through direct histamine release (198,199 and 200). Therefore, reactions to these agents may occur with the first dose. Other drugs also may cause urticaria through direct histamine release or complement activation.

Treatment of drug-induced urticaria is straightforward. The culprit drug is identified and discontinued. While the lesions are resolving, patients should avoid known mast cell releasing drugs and NSAIDs. If possible, other drugs should be withheld because of the confusion that arises if the urticaria worsens after another drug is added. H_1-receptor antagonists are the mainstay of therapy and may be combined with H_2-receptor antagonists, especially if the urticaria is accompanied by systemic symptoms (201). If the reaction occurs during a course of therapy, the urticaria may be suppressed by antihistamine therapy if there is no alternative agent available. The patient must be monitored carefully, however, and the drug must be stopped if the rash progresses. Anaphylaxis has not been reported during uninterrupted therapy, but it may occur if the drug is discontinued and then readministered 24 to 48 hours later (75).

Drug-induced Allergic Hepatitis

Many drugs can cause liver damage. The most frequent drug-induced manifestation is hepatocyte necrosis; however, some drugs target the bile ducts or canaliculi, causing cholestasis only, whereas others affect sinusoidal or endothelial cells, resulting in venoocclusive disease or fibrosis (202). Still others affect multiple cell types, leading to a particular pattern of liver injury. Although drug-induced liver injury may be classified as hepatocellular, cholestatic or mixed, Lee (202) recommended that these reactions be categorized according to the type of reaction observed because this classification scheme takes into account the histologic changes, the involved cell type, and the clinical manifestations. Because the general topic of drug-induced hepatic reactions is too broad to discuss here, this section focuses on only one immunologically mediated drug-induced liver disease: allergic hepatitis.

Drug-induced hepatitis may be due to direct toxic effects of a drug or its metabolites, or it may result from an immune-triggered event. Severe halothane-induced hepatitis occurs only on secondary exposure and is characterized by fever and eosinophilia along with elevated transaminases. For these reasons, it is thought to be at least partially immunologically mediated (112,203). Drug-specific antibodies and autoantibodies have been detected in affected patients but not in control subjects (204,205). It is thought that halothane-protein adducts formed on the cell surface provide the antigen for immune recognition and that cell lysis is the ultimate event (112). Certain hydrochlorofluorocarbons, agents that are used in industry as substitutes for ozone-depleting chlorofluorohydrocarbons, are structurally related to halothane, and these drugs also may induce an allergic hepatitis similar to that induced by halothane (206). Drug-induced allergic hepatitis also occurs with sulfonamides, anticonvulsants, and other drugs (202).

The diagnosis of drug-induced hepatitis is relatively straightforward if a patient is taking few medications and only one of them is known to cause hepatitis. The situation is usually more complex, however, especially for hospitalized patients who are taking multiple drugs, several of which may be hepatotoxic. Lee (202) recommended that, in these circumstances, the best way to determine the culprit drug is to make a careful timeline of all drugs ingested and to be especially suspicious of those potentially hepatotoxic agents that were begun within 3 months of the onset of the hepatitis.

Treatment of drug-induced hepatitis is drug withdrawal and close observation to ensure that the reaction does not progress. Some agents, such as amoxicillin-clavulanic acid and phenytoin, have been associated with a syndrome in which the reaction worsens after the drug is stopped and several months are required for resolution (207). If the patient worsens clinically or there is laboratory evidence of hepatic failure, the patient must be hospitalized (208). Glucocorticoids may be used to treat those reactions presumed to be allergic in nature; however, their efficacy has not yet been proven (202). Drug rechallenge is not recommended

unless the drug is the only agent that is efficacious in treating a potentially life-threatening disorder.

Drug-induced Immune-mediated Hematologic Reactions

Drug-induced cytopenias include agranulocytosis, hemolytic anemia, and thrombocytopenia; and certain drugs are notorious for causing one or more of these reactions. Some, but not all, of these reactions are immune mediated. High-dose penicillin therapy is associated with hemolytic anemia in some patients because of the recognition of haptenated red blood cells by penicillin-specific IgG or IgM antibodies and subsequent complement activation (209,210). Cephalosporins have been implicated in drug-induced hemolysis, as have nomefensine, methyldopa, and other agents (211,212,213,214 and 215). Depending on the drug involved, the immune response may be directed toward the native drug, drug metabolites, or intrinsic red cell antigens (autoimmune hemolytic reactions) (27). In addition, hemolysis may occur via an “innocent bystander” mechanism in which drug-conjugated plasma proteins elicit antibody formation and the formed immune complexes adsorb to red cell surfaces, leading to complement activation and subsequent hemolysis (216).

Some types of drug-induced agranulocytosis and thrombocytopenia also may be immune mediated (217). The idiosyncratic nature of the reactions, along with their rapid recurrence with drug reintroduction, suggests immune involvement, although direct evidence for this involvement often is limited. Drugs that most commonly cause immune-mediated thrombocytopenia are quinine, quinidine, sulfonamides (antibacterials, sulfonylureas, thiazide diuretics), gold salts, and heparin. Immune-mediated agranulocytosis most commonly has been associated with pyrazolone derivatives, phenothiazines, thiouracils, sulfonamides, and anticonvulsants. Treatment is drug withdrawal.

Drug-induced Immune-mediated Pulmonary Reactions

Drugs can cause an array of adverse reactions in the lung, including alveolar hypoventilation, diffuse alveolar damage, usual interstitial pneumonia, hypersensitivity pneumonitis, pulmonary infiltrates with eosinophilia, noncardiogenic pulmonary edema, pulmonary hypertension, talc granulomatosis, pleuropulmonary disease from drug-induced lupus erythematosus, and bronchospasm (218). Although the mechanisms responsible for these reactions are not clearly known, several have been proposed: direct cytotoxic injury to alveolar capillary endothelial cells; oxidative injury; immunologically mediated injury via drug-induced systemic lupus erythematosus or hypersensitivity reactions; alterations in the lung matrix repair system that results in disturbed repair processes; and alterations in pulmonary capillary permeability caused by drug-related effects on neural control (219,220).

Clinical features that suggest that the participation of an immune mechanism include an acute or subacute onset, rapid resolution when the drug is withdrawn or glucocorticoid therapy is implemented, and the ability to reproduce the symptoms with reintroduction of the suspected agent. Histopathologic features typically are nonspecific. Laboratory findings that suggest the involvement of an immune mechanism include peripheral eosinophilia, elevated IgE levels, and enhanced lymphocyte blastogenesis as reflected by a positive lymphocyte transformation test (218).

An acute pneumonitis that is thought to be immune mediated can appear during treatment with nitrofurantoin, NSAIDs, and sulfasalazine. Clinical features include fever, cough, dyspnea, eosinophilia, and rash in some cases. Nitrofurantoin and sulfasalazine commonly cause interstitial pneumonitis with or without fibrosis, whereas the lesions caused by NSAIDs are more often alveolar. In many cases, lung function is restored after drug withdrawal. Significant pulmonary dysfunction can occur, however, especially if the drug is not discontinued promptly.

Drugs such as gold, procarbazine, and amiodarone induce pulmonary diseases that resemble hypersensitivity pneumonitis (221). Symptoms are both respiratory and systemic and include cough, dyspnea, fever, myalgias, and malaise. Examination of the patient reveals an ill, dyspneic person. Bibasilar rales are heard, and a leukocytosis is usually present. Pulmonary function testing typically shows reduced lung volumes and diffusing capacity. Infiltrates on chest radiogrphy may be interstitial, alveolar, or mixed. Treatment consists of drug avoidance and glucocorticoid therapy (218,221).

Some drugs have been associated with the development of a lupus-like syndrome. The most common agents in this category include hydralazine, isoniazid, procainamide, and the sulfonamides. The most frequent pulmonary manifestations are pleural effusion and pleuritic chest pain, and most patients have a positive antinuclear antibody titer (222). Drug discontinuation is the treatment of choice. Glucocorticoids may decrease the recovery time.

Finally, certain drugs may induce bronchospasm. The most common agents to elicit reactions of this type are the NSAIDs (223) (see Chapter 27).

Drug-induced Immune-mediated Renal Reactions

The two most common drug-induced hypersensitivity renal reactions are interstitial nephritis and nephrotic syndrome. Glomerular abnormalities also may occur in the presence of drug-induced immune complex disease.

Interstitial nephritis has been associated most commonly with methicillin; but other drugs, including other penicillins, cephalosporins, sulfonamides, captopril, furosemide, and thiazide diuretics, also have been associated with this disorder (224). Clinical features include fever, rash, blood eosinophilia, and urine eosinophilia. The patient typically has hematuria, proteinuria, pyuria, and sometimes azotemia. Both cellular and antibody-mediated mechanisms may be involved. Complete recovery typically occurs after drug withdrawal.

The nephrotic syndrome can be induced by gold salts, captopril, NSAIDs, and penicillamine (225). The syndrome is characterized by hematuria, hypoalbuminemia, hyperlipidemia, and edema. The prognosis is generally excellent when the implicated drug is discontinued. Treatment consists of supportive care, including bed rest, and dietary restriction of cholesterol, salt, saturated fat, and protein.

FUTURE DIRECTIONS

The understanding of drug-induced hypersensitivity responses in humans is limited. Not only can many of these reactions not be classified, but also the pathophysiologic processes responsible for them are unclear. Progress in this area has been hampered for several reasons. First, because of species specificity and intraspecies variability in the immune responses elicited to drug haptens, animal studies have not proved to be useful. Second, there is little knowledge of the immunochemistry of most drugs in relation to their metabolites, which represent reactive forms and clinically relevant immunogens. Once the relevant drug immunogens are identified, we should be able to better predict the type of immune response they may elicit and thus be able to understand better the underlying immunopathology of the reactions. Third, there is little understanding of predisposing genetic factors and other factors that allow for the prospective identification of individuals at risk. With improved understanding of the mechanisms responsible for drug-induced reactions, along with a better understanding of relevant risk factors, it should be possible to devise better classification schemes. Many drug-induced reactions, most likely, will involve multiple immune mechanisms or both immune and nonimmune components.

There is a need for enhanced postmarketing surveillance, perhaps a worldwide tracking system, to assess more fully the actual incidence and prevalence of drug-induced hypersensitivity diseases.

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73 NATURAL RUBBER LATEX ALLERGY

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In the past two decades, natural rubber latex allergy has emerged as an important occupational and medical illness. The spectrum of hypersensitivity reactions to rubber products includes both delayed and immediate reactions. Delayed-type hypersensitivity reactions to rubber (1) have been recognized for many decades. Immediate-type hypersensitivity reactions initially were reported in Europe in 1979 (2), and several reports followed in the 1980s (3,4,5 and 6). The first report of latex allergy in the United States appeared in 1989 (7), and by 1992 the U.S. Food and Drug Administration (FDA) had received more than 1,000 reports of serious immediate-type allergic reactions to rubber products, including 15 deaths (8). In the last decade, numerous reports of natural rubber latex allergy have been published. Epidemiologic studies have elucidated the major risk factors for the development of adverse reactions to latex. The clinical syndromes that constitute latex allergy have been well characterized, and considerable progress has been made in determining the proteins and chemicals that cause latex hypersensitivity reactions. This progress led to improved diagnosis of latex allergy and also established methods for determining the levels of contaminants in manufactured rubber products. The management of persons with latex allergy has been refined and has focused on the elimination of exposure in both home and the workplace environments. The pathobiologic mechanisms involved in latex allergy and potential immunomodulatory therapeutic options for affected persons are the subjects of ongoing investigations.

The term *latex* refers to the milky white sap produced by the cells of various seed plants. Latex is a complex colloidal suspension that consists of multiple organic compounds, including proteins, alkaloids, starches, oils, tannins, resins, and gums. The latex from certain plants contains “natural rubber” as a major constituent. Natural rubber is an organic polymer whose chemical structure is cis-1,4-polyisoprene. Most of the world supply of natural rubber comes from the latex of rubber trees (*Hevea brasiliensis*) that are grown on commercial plantations in Southeast Asia and Africa. Natural rubber constitutes about 30% to 50% (wt/wt) of rubber tree latex and is the raw material from which rubber products are manufactured. The term *latex* may also be defined as “a water emulsion of synthetic material.” When used in this way (e.g., “latex” paint), the term does not imply the presence of natural rubber tree latex in the product.

The manufacturing of natural rubber products from raw rubber tree latex is a complex multistep process (reviewed in 9). Proteins in the latex may contaminate the raw natural rubber material and persist in the final manufactured product. Protein contamination occurs to a greater degree in dipped products (e.g., gloves, balloons, condoms) than in molded products (e.g., tires, rubber stoppers) (9). Various compounding agents (e.g., accelerants, antioxidants, and cross-linking agents) are added to the raw material during manufacturing (9). The compounded raw material is heated in the presence of sulfur (*vulcanization*) to catalyze the cross-linking of the polyisoprene chains. Compounding and vulcanization improve the elasticity, durability, and tensile characteristics of the final manufactured products. As with the rubber tree proteins, the chemical compounding agents may contaminate the manufactured end products. Synthetic alternatives to natural rubber exist (e.g., neoprene). Unlike natural rubber, synthetic polymers are not contaminated by protein, but they may contain chemical contaminants (10).

Persons who are exposed to rubber products may become sensitized either to the protein or to chemical contaminants in rubber products. Those with sensitivity to latex proteins can manifest immunoglobulin E (IgE)-mediated immediate-type hypersensitivity reactions while those with a sensitivity to chemical contaminants manifest cell-mediated delayed-type hypersensitivity reactions.

EPIDEMIOLOGY

High-risk Groups

Epidemiologic studies have identified several groups that are at high risk for developing IgE-mediated reactions to natural rubber latex. The occupational use of rubber gloves is the most often cited risk factor for IgE-mediated latex allergy, and health care workers represent the largest group of affected individuals (8,11). The prevalence of latex allergy among health care workers in Europe and North America is reported to be between 2.4% and 12% (3,12,13,14 and 15). The variability in prevalence rates between studies may be accounted for by differences in methods used to define and detect latex allergy. Sensitization of health care workers is believed to occur through exposure to rubber gloves and latex-containing medical devices. Other factors associated with an increased prevalence rate of sensitization among health care workers include a history of atopy or preexisting hand eczema (16). The prevalence of latex allergy in non–health care workers who are regularly exposed to rubber gloves is less well studied but may approach the rates reported for health care workers (17,18 and 19).

Individuals with spina bifida represent the second major risk group for IgE-mediated reactions to natural rubber latex (20,21,22,23,24 and 25). Affected persons are believed to become sensitized by repeated exposures to latex gloves and medical products during surgical procedures and through the use of indwelling latex catheters. Persons with spina bifida have the highest rates of sensitization among high-risk groups; prevalence rates vary between 18% and 50% (20,21,22,23,24 and 25). Undergoing multiple surgical procedures in childhood appears to be an independent risk factor for the development of latex sensitization for persons with spina bifida (25,26).

Rubber factory workers represent another defined risk group with an increased prevalence rate of immediate hypersensitivity to latex proteins. Workers in rubber glove manufacturing plants have been reported to have a prevalence of IgE-mediated latex reactions of 11% (27) and workers in rubber doll manufacturing plants a prevalence rate of 10% (28).

General Population

The frequency of immediate-type hypersensitivity to latex proteins in the general population is unknown. The earliest European studies reported a prevalence of IgE-mediated latex allergy in the general population to be less than 1% (3). More recent studies reported that the prevalence of latex sensitization in occupationally unexposed groups is between 0.12% and 1% (29,30). The prevalence of latex allergy in adults and children who visit allergy clinics is also about 1% (31,32 and 33). The prevalence of latex-specific IgE detected by serologic testing in the general population may be considerably higher. Assays of residual blood samples from donors in the United States and Europe revealed a prevalence of latex-specific IgE of about 6% (34,35). The presence of latex-specific IgE, however, does not indicate the presence of clinically expressed disease (14,36). The significance of a positive serologic test for latex-specific IgE in the absence of symptoms and the long-term prognosis for asymptomatic, sensitized persons are not known.

CLINICAL PRESENTATION

Spectrum of Reactions

The spectrum of hypersensitivity reactions to natural rubber latex is outlined in Table 73.1. Reactions are distinguished by the pathologic mechanism and the causative agent. The most common reaction to rubber products is irritant contact dermatitis (11), which is a nonimmunologically mediated irritation of the skin that is seen most commonly with the use of rubber gloves and is caused by having the hands under occlusion. Glove-donning powder may act as an abrasive to compound the irritation. Irritant contact dermatitis may be further aggravated by defatting of the skin through the use of detergents and alcohol or by exposure to irritating chemicals in the workplace (e.g., antiseptics). Irritant contact dermatitis may range from mild erythema and pruritus to chronic fissuring and hyperkeratosis.

Clinical Entity	Immunologically Mediated	Mechanism	Causative Agent
Irritant contact dermatitis	No	Irritant	Irritants
Aggravation of atopic dermatitis	No	Irritant or preexisting eczema	Irritants
Allergic contact dermatitis	Yes	Cell-mediated	Chemical additives
Contact urticaria/angioedema	Yes	IgE-mediated	Rubber proteins
Immediate disease	Yes	IgE-mediated	Rubber proteins
Rhinitis			
Conjunctivitis			
Bronchospasm			
Anaphylaxis	Yes	IgE-mediated	Rubber proteins
Food-related oral symptoms and anaphylaxis	Yes	IgE-mediated	Plant proteins with homology to rubber proteins

IgE, immunoglobulin E.

TABLE 73.1. Spectrum of Reactions to Natural Rubber

Allergic contact dermatitis is an immunologically mediated cutaneous eruption that is a manifestation of a cell-mediated immune response (1). When seen in association with rubber products, the immune response is directed against one of the chemical additives that exist as contaminants in the end products. Allergic contact dermatitis may be difficult to distinguish from irritant contact dermatitis. Irritant reactions predominantly affect the dorsum of the hand and spare the palmar surface. Weeping, vesiculation, and lichenification of the skin occur more commonly with allergic contact reactions. Both allergic and irritant reactions localize to the area of contact and often have well-defined borders that suggest a contactant as the cause. Persons who have preexisting hand eczema secondary to atopic dermatitis may have aggravation of their dermatitis when their hands are placed under occlusion or are exposed to irritants. Distinguishing active atopic hand eczema from allergic contact dermatitis can be difficult by clinical examination alone and may require diagnostic patch testing to rule out a sensitization to a rubber chemical.

Contact urticaria is an immunologically mediated cutaneous eruption that is a manifestation of an IgE-mediated immune response (11). Affected persons make IgE antibodies to protein contaminants in manufactured latex products. Contact urticaria is a frequent manifestation of IgE-mediated latex allergy (5,16,37,38). In contrast to irritant and allergic contact reactions, which have a delayed onset (usually days), contact urticaria usually manifests within minutes of exposure. The symptoms consist of erythema, pruritus, and the development wheals on areas of contact. Angioedema may be the presenting complaint with contact on mucosal surfaces. Contact urticarial lesions tend to resolve quickly (i.e., minutes to hours) after removal of the inciting agent, in contrast to allergic contact dermatitis, which may persist for days to weeks. Distinguishing between irritant contact, allergic contact, and contact urticaria reactions in persons with glove-related illness is important because the diagnostic testing and treatment options differ for these conditions.

The IgE-mediated reactions to natural rubber latex proteins also may manifest as inhalant disease (38,39 and 40). Inhalation of airborne latex proteins may lead to symptoms of rhinitis or bronchospasm, and deposition of latex proteins on the ocular surface may lead to conjunctivitis, chemosis, and periorbital angioedema. If there is significant systemic exposure or absorption of latex protein through a mucosal surface, anaphylaxis can occur (38). Latex-induced anaphylaxis and death have been reported in association with barium enema examinations; anaphylaxis occurred after the inflation of the latex retention balloon against the rectal surface (41). Systemic anaphylaxis has also been the presenting manifestation of latex allergy in patients undergoing surgical or obstetric procedures; therefore, latex allergy should be considered in the differential diagnosis of anaphylaxis during operative procedures (26,42,43 and 44).

Persons with IgE-mediated sensitivity to natural rubber latex proteins have an increased prevalence of food-related reactions. The prevalence of food-related syndromes in individuals with latex allergy has been estimated to be between 20% and 40% (45,46 and 47). Most studies examining the relation between latex and food allergy have relied on historical information and positive skin tests. Because the existence of the food allergy has not been confirmed by placebo-controlled food challenges, the prevalence rates for food reactions may be overestimated. The spectrum of food-related reactions ranges from localized oropharyngeal reactions (oral allergy syndrome) to systemic anaphylaxis (45,46 and 47). The foods most commonly associated with this condition are chestnuts, bananas, avocados, and kiwi fruit (45). The food-related reactions occur as a result of cross-reacting IgE antibodies that recognize proteins in foods that share homology with natural rubber latex proteins (45).

Laboratory Testing

Laboratory tests available for use in the diagnosis of IgE-mediated latex allergy include skin testing, serologic testing, and provocative testing. Cell-mediated immune reactions to rubber chemicals are confirmed by patch testing.

SKIN TESTING

Skin testing is the preferred diagnostic procedure for persons with suspected IgE-mediated latex allergy. Accurate and reproducible skin testing relies on the availability of a reliable skin testing extract. Latex extracts are currently available in Canada and some European countries. An extract is under development in the United States but is not currently available (Greer Laboratories, Lenoir, NC, U.S.A.). The safety and efficacy of this extract were documented in a study of 324 subjects (304 adults, 20 children) (48). The sensitivity of the extract at a concentration of 1 mg per milliliter was 99% with a specificity of 96% (48). Comparison studies suggested that skin testing is more sensitive and specific than serologic testing (49). In the absence of a commercial extract in the United States, some centers prepare their own extracts from raw latex sap (ammoniated or nonammoniated) or by extraction of manufactured latex products (e.g., latex glove extracts) (50,51 and 52). These extracts must be used with caution because anaphylaxis has occurred during skin testing (51). Furthermore, the protein content of these extracts may vary considerably, depending on the source material used (e.g., raw latex vs. manufactured product) (52,53). Extracts made from gloves that do not contain all the relevant antigens may elicit negative skin test results in sensitized individuals (54). Precautions to minimize risk during skin testing include the initial use of dilute solutions of extract and the use of prick rather than intradermal testing methods. Skin testing should be performed by personnel experienced in skin testing and in the management of anaphylaxis.

SEROLOGIC TESTING

Latex-specific IgE may be measured by enzyme-linked immunosorbent assay (ELISA) or radioallergosorbent test (RAST). In the United States, three FDA-licensed tests are available commercially: the CAP system (Pharmacia-Upjohn, Uppsala, Sweden), the AlaSTAT assay (Diagnostic Products Corp., Los Angeles, CA, U.S.A.), and the HY-TEC assay (Hycor Biomedical Inc., Irvine, CA, U.S.A.). In study of the diagnostic performance of these three assays, the sensitivities and specificities were 75% and 91% for CAP, 79% and 95% for AlaSTAT, and 89% and 67.5% for HY-TEC, respectively (49). The sensitivity of the two most commonly used tests (CAP and AlaSTAT) is less than 80%, indicating that about 20% of persons with clinical latex allergy may not be identified by serologic testing alone.

PROVOCATIVE TESTS

Use testing has been reported as an alternate diagnostic test for persons with suspected immediate hypersensitivity to latex (39,50,55,56). The glove-use test procedure involves placing glove material over a dampened finger or hand for 15 to 30 minutes. The test is usually graded on a semiquantitative scale on the basis of the presence of erythema or wheals. The hand gloved with latex is compared with the one gloved with nonlatex material. Glove-use testing by this method has correlated well with the results of skin-prick testing in diagnosing persons with immediate-type hypersensitivity to latex (50,54).

Provocative testing for latex allergy has been refined and validated (57,58). A two-stage latex glove provocation procedure was used in a recent study of the efficacy of a latex skin test reagent (48). The two-stage protocol involved the use of both a contact (glove-use test) and an inhalant (glove-powder inhalation test) component. Provocative tests were used selectively and were not used in persons with positive skin tests to avoid serious reactions.

The major limitation of inhalation tests is the inability to quantitate the amount of latex protein the test subjects receive. Two exposure chamber devices have been developed to improve latex exposure quantification during inhalation challenges (58,59). These techniques are largely reserved for research purposes because of their technical complexity.

PATCH TESTING

Persons with suspected latex-associated allergic contact dermatitis should be referred for patch testing. The Standard Allergen Series for patch testing, approved by the U.S. FDA, includes the chemicals most commonly associated with allergic contact dermatitis (60). Table 73.2 lists the rubber-associated chemicals included in the Standard Allergen Series. Accelerators account for most contact allergic reactions associated with rubber products (60). Occupationally related allergic contact dermatitis may be due to chemicals other than those found in rubber. Testing with additional chemicals may be required, depending on the exposure history (60). Standard concentrations of chemicals should be used for testing to avoid nonspecific irritant reactions. Patch testing should be performed by experienced personnel to

ensure proper application and interpretation.

Test Material	Composition	Function
Thiuram mix	Tetramethylthiuram disulfide Dipentamethylenethiuram Tetramethylthiuram monosulfide Tetraethylthiuram disulfide	Accelerator
Mercaptobenzothiazole		Accelerator
Mercapto mix	N-cyclohexyl-2-benzothiazole-sulfenamide 2,2-benzothiazyl disulfide 4-morpholinyl-2-benzothiazyl disulfide	Accelerator
Carbamate mix	1,3-diphenylguanidine Zn diethyldithiocarbamate Zn dibutyldithiocarbamate	Accelerator
Black rubber mix	*N*-phenyl-*N*-cyclohexyl-*p*-phenylenediamine *N*-isopropyl-*N*-phenyl-*p*-phenylenediamine *N,N*-diphenyl-*p*-phenylenediamine	Antioxidants

TABLE 73.2. Standard Allergen Patch Test Series: Rubber Compounds

Management

CONTACT DERMATITIS

Therapy for hand dermatitis depends on the severity of the illness. Persons who develop irritant cutaneous reactions should be treated with topical emollients and a reduction in exposure to strong detergents, irritating chemicals, and abrasives. Allergic contact dermatitis can be managed by identifying the causative chemical and eliminating exposure. Therapy directed at the ongoing dermatitis may include drying compresses, oral antihistamines, topical glucocorticoids, and, occasionally, oral steroids. Emollients are used for desiccated and lichenified skin. Significant eczematous dermatitis (cracking and weeping of the skin) may require a temporary work restriction. Persons who react to the chemicals in latex gloves may use non–chemical-containing latex gloves as substitutes. Other alternatives include polyvinyl chloride or nonlatex synthetic gloves. It should be noted that some nonlatex synthetic gloves still contain chemicals that can cause allergic contact dermatitis reactions (10).

IGE-MEDIATED REACTIONS

The treatment of an ongoing immediate allergic reaction to latex depends on the manifestations and severity but conforms to the standard treatment of IgE-mediated reactions. Anyone who has latex allergy should obtain a Medic-Alert bracelet and self-injectable adrenaline (61). The mainstay of the long-term treatment of immediate reactions to latex products is avoidance. Affected persons should be educated about avoidance both at home and in the workplace. Lists of latex-containing devices have been compiled, along with suitable non–latex-containing alternatives (Spina Bifida Association of America, Washington, D.C.). Careful preparation is needed for latex-sensitive persons who require hospitalization or dental or surgical procedures. All procedures on latex-sensitive persons should be performed in a “latex-safe” environment, which is defined as one in which no natural rubber latex product is brought in direct contact with the affected person (61). Management protocols for the perioperative and routine in-hospital care of individuals with latex allergy have been established to safeguard against inadvertent latex exposures (62). Because the prevalence of latex protein sensitization in individuals with spina bifida is high, it is recommended that all medical procedures on these patients, regardless of history, be performed in an environment free of latex (61). Furthermore, to reduce sensitization rates in this group, latex exposure should be minimized.

The prophylactic use of glucocorticoids and antihistamines before surgical procedures remains controversial. Holzman (63) reported on the successful use of latex avoidance without prophylaxis in a large series of children with latex allergy. Others reported the occurrence of anaphylaxis despite premedication, suggesting that prophylaxis may not be effective in preventing reactions if significant exposure still occurs (44,64). Current data suggest that there is no substitute for the strict avoidance of latex (65).

Guidelines for the prevention of reactions to natural rubber products in the workplace have been established (66). These guidelines stress the importance of appropriate barrier protection for workers exposed to infectious or other hazardous material. The use of reduced-chemical and reduced-protein gloves that are powder free is recommended. Institutions that have adopted these recommendations have noted a reduction in airborne latex aeroallergens in the work environment (67,68).

PATHOBIOLOGY

Latex Allergens

The characterization of the proteins present in *H. brasiliensis* latex led to the identification of a number of latex allergens. More than 200 proteins have been identified by two-dimensional electrophoresis of the aqueous phase of rubber tree latex (69). Additional proteins can be identified bound to latex particles present in the sap. Proteins range in size from 5 to 200 kd. Not all latex proteins are allergens; about 50 latex proteins are allergenic (70). Immunoprobing of latex proteins separated by electrophoresis led to the identification of a number of latex allergens (reviewed in 71). The latex allergens that have been given designated names in accordance with the nomenclature system for allergens are listed in Table 73.3. Several other *H. brasiliensis* proteins have been isolated and characterized but have not been yet been classified as major latex allergens (71).

Allergen (Reference)	Alternate Name	MW (kd)	Function
Hev b 1 (57,58)	Rubber elongation factor	14.6	Involved in rubber biosynthesis
Hev b 2 (59–61)	α-1,3-glucanase	34–43	Glucanase
Hev b 3 (58,62,63)	Small rubber particle protein	24–27	Involved in rubber biosynthesis
Hev b 4 (,[3])		100–115	Microhelix component
Hev b 5 (,[3,64,65])		16–17.5	Homology to proteins in kiwi and potato
Hev b 6			
Hev b 6.01 (PR,1,[3])	Profilin	15	Chitin-binding proteins
Hev b 6.02 (,[3])			
	Hevein	5 (N-domain) 14 (C-domain)	
Hev b 7 (,[3],66,67)		45	Homology to patatins; storage proteins in plants
Other allergens			
Hevamine (68)		39.5	Lysozyme/chitinase activity

MW, molecular weight

TABLE 73.3. Designated Allergens from Hevea brasiliensis

Routes of Exposure

Susceptible persons become sensitized to natural rubber latex proteins after repeated exposures. The multiple potential routes of exposure to latex proteins include the following: cutaneous (e.g., gloves), mucosal (e.g., dental procedure or indwelling urinary catheter), percutaneous (e.g., surgical procedure), and inhalant (e.g., inhalation of latex protein on an airborne carrier). The major route of sensitization currently remains unknown. The route of sensitization may differ for different high-risk groups. For example, persons with spina bifida are exposed to latex by repeated orthopedic and urogenital surgical procedures (latex-containing medical devices) and through the use of latex urinary catheters. Persons who use latex gloves occupationally are exposed by direct cutaneous contact and also may be exposed by the inhalation of glove powder that is contaminated with latex protein. The pattern of IgE reactivity to latex differs among different populations with latex allergy (72,73).

The importance of glove powder in the pathobiology of latex allergy must be emphasized. Cornstarch powder can bind latex proteins and act as a carrier for both airborne and contact dispersal of latex allergens (74,75). Studies in hospital centers have identified high levels of latex aeroallergens in areas with extensive use of powdered gloves, whereas areas with minimal use of powdered gloves have substantially lower concentrations of latex aeroallergens (76). These data suggest that glove powder can be a major contributor to airborne latex proteins in hospital environments. Further studies have documented a substantial reduction in latex

aeroallergens in the hospital setting after the removal of high-protein, powdered gloves (68).

Immunologic Response

Serologic testing of persons with latex allergy reveals that most make IgE antibodies to multiple allergens (77). The spectrum of reactivity may differ from one high-risk group to another, depending on the route of exposure (72,73). Serologic testing revealed the presence of IgE antibodies to various food allergens in some persons with latex allergy (45). These antibodies are believed to result from cross-sensitization to food proteins that share homology with latex proteins. Lymphocytes from persons with latex allergic proliferate *in vitro* on exposure to latex proteins, suggesting a role for T-cell-mediated immune regulation in the development of latex allergy (78,79,80 and 81). Allergic contact eczematous dermatitis results from a cell-mediated delayed-type hypersensitivity response to latex-compounding chemical additives.

ANIMAL MODELS

Experimental animals have been used in many roles in the study of natural rubber latex allergy. Immunization of rabbits with crude latex preparations led to the development of polyclonal antibodies that have aided the identification and characterization of latex proteins and allergens (82,83). These reagents also have been used to detect the presence of latex allergens in manufactured latex products (84,85). More specific reagents, such as monoclonal antibodies, have been used for the affinity purification of specific antigens (86,87). Antibodies from animal and human sources also have been used to identify specific epitopes on latex allergens to define allergenic and antigenic determinants of these molecules (88).

Models of allergic pulmonary inflammation induced by latex allergens have been described in mice, guinea pigs, and rabbits (89,90,91 and 92). These studies support a role for IgE and eosinophils in the immunopathogenesis of immediate-type hypersensitivity responses to latex proteins. Other studies examined the use of DNA vaccines with latex allergen transcripts as immunomodulatory therapeutic interventions for latex allergy (93). Animal models also have been used to elucidate the possible role of immune adjuvants (e.g., endotoxin) in sensitization to latex proteins (94). Finally, murine systems have been used to elucidate the role of different routes of exposure on the development of immunologic responses to latex proteins (95).

FUTURE DIRECTIONS

Latex allergy presents unique challenges for rubber manufacturers, federal regulators, and medical investigators. The challenge for rubber manufacturers will be to reduce rates of sensitization by reducing both protein and chemical contaminants in rubber products. Cost-effective alternatives to natural rubber gloves, which maintain an effective barrier protection, are being investigated. Federal regulations mandating the labeling of medical products containing natural rubber were instituted in 1998 (96). Further steps to reduce the exposure of workers to latex proteins and chemicals are required. U.S. FDA approval of latex skin testing extract is anticipated in the near future, and this extract will improve the accuracy and safety of latex testing in the United States. For medical investigators, the focus of current research is on the continued characterization of the major latex allergens. Elucidation of these molecules will improve the diagnosis of latex allergy by ensuring that serologic (e.g., RAST) and skin test reagents contain all the relevant allergens. Allergen characterization also may lead to the development of protein or peptide vaccines for use as immunotherapeutic agents to broaden the treatment options for affected persons.

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74 HUMAN IMMUNODEFICIENCY VIRUS INFECTION

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The acquired immune deficiency syndrome (AIDS) was recognized in 1981, when five unusual cases of *Pneumocystis carinii* pneumonia (PCP) occurring in young homosexual men from Los Angeles were reported to the Centers for Disease Control and Prevention (CDC) (1). An infectious etiology as the cause of AIDS was later suggested by geographic clustering of cases, links among cases by sexual contact, mother-to-infant transmission, and transmission by blood transfusion. The depletion in numbers and function of T-helper cells further suggested that a lymphotropic agent might be responsible. Indeed, a human T-lymphotropic retrovirus that was immunologically related to human T-cell leukemia virus type I (HTLV-I) was detected in lymph node tissue from a French patient who was at risk for AIDS (2). This virus, later named *lymphadenopathy-associated virus*, or LAV, proved identical or highly related to the virus subsequently isolated at the National Cancer Institute termed *HTLV-III* (3,4) and in San Francisco termed *AIDS-associated retrovirus* (ARV) (5). These viruses were subsequently cloned and characterized and now are known as *human immunodeficiency virus type 1* (HIV-1). Molecular and seroepidemiologic studies have consistently demonstrated a near-perfect correlation between the development of AIDS and infection with HIV-1.

Scientific advances in understanding the pathogenesis of HIV infection have proceeded with unprecedented speed. Elucidation of the viral replication cycle led to the rational development of potent antiretroviral agents. Appreciation of the intimate relationship between HIV and the human immune system continues to guide efforts aimed at the prevention and treatment of HIV infection (6). Despite the rapid scientific advances that have been made, the brisk pace of the HIV/AIDS epidemic has continued largely unchecked in most parts of the world. Prevention efforts and the availability of antiretroviral therapies have blunted the expansion of the epidemic in the United States and Western Europe; however, expansion of the epidemic continues in sub-Saharan Africa and Southeast Asia and is now burgeoning in India and in the former Soviet republics.

EPIDEMIOLOGY

Global Pandemic

The global HIV pandemic ranks among the most devastating infectious scourges in human history. Approximately 300 cases of AIDS had been reported by the end of 1982; by the beginning of 1999, more than 33 million people worldwide were living with HIV (Fig. 74.1), and 14 million deaths attributable to HIV infection had occurred since the beginning of the epidemic (7). During 1998 alone, nearly six million persons worldwide acquired HIV infection, and 2.5 million deaths from HIV infection occurred; these statistics highlight the continued expansion of the epidemic on a global scale (7).

Figure 74.1. Global distribution of the estimated number of persons living with human immunodeficiency virus (HIV) infection as of December 1999. [Source: Joint United Nations Program on HIV/AIDS (UNAIDS)].

Phylogenetic analysis of HIV proviral sequences worldwide reveals three major genetic groups of HIVs, the M (major), N (new), and O (outlier) groups. These three HIV groups are most closely related to simian immunodeficiency virus (SIV) sequences (SIVcpz) harbored by chimpanzees of the subspecies *Pan troglodytes* troglodytes (8). The relatively rare O group viruses are concentrated in West Africa in Cameroon, Gabon, and Equatorial Guinea. Group N viruses were isolated in 1998 in two persons from Cameroon (9). Within the M group, related HIV variants are classified into *clades*, or subtypes (currently designated A–J) according to their degree of genetic similarity (10). At the nucleotide level, viral subtypes differ from each other by about 14% in their *gag* (viral core) coding sequences and by about 30% in their envelope coding sequences (10). Subtype C viruses are the most common worldwide, whereas subtype B viruses predominate in North America and Western Europe.

Zoonotic transmission of a retrovirus from chimpanzees to humans in sub-Saharan Africa is the most plausible explanation for the genesis of the HIV epidemic (8). From central, western, and eastern sub-Saharan Africa, the epidemic spread to North America, Latin America, Western Europe, Southeast Asia, and southern Africa. Currently, the heaviest burden of the epidemic is concentrated in southern Africa, India, and Southeast Asia. Although only 10% of the world's population live in sub-Saharan Africa, 70% of the worldwide population who acquired HIV infection in 1998 live there. Of all AIDS deaths since the beginning of the AIDS epidemic, 83% have occurred in sub-Saharan Africa. In the southern African countries of Botswana, Namibia, Swaziland, and Zimbabwe, 20% to 26% of the population between the ages of 15 and 49 years are infected with HIV. The epidemic is also expanding rapidly in South Africa; 15% of new infections in the entire African continent in 1998

occurred in South Africa.

Nearly 1% of the adult population in India is infected with HIV. Although this seroprevalence is lower than in many affected countries, it makes India the country with the largest number of HIV-infected persons (approximately four million). Most HIV infections in India occurred during the 1990s. Thus, an extraordinary burden of HIV-related morbidity and mortality lies ahead in India because the epidemic there is in its early stages. Alarmingly, among women attending a sexually transmitted disease clinic in Pune, India, nearly 14% tested positive for HIV, despite the fact that more than 90% were married and monogamous (11).

Societal changes are fueling the spread of HIV in Eastern Europe and central Asia. Increasing availability and use of illicit drugs and a burgeoning sex-worker population in the republics of the former Soviet Union have contributed to a 25-fold increase in rates of syphilis in the 1990s (7). Although the absolute number of HIV infections in the region is small, the rate of increase is extremely rapid. In this regard, the cumulative number of HIV cases reported in the region increased more than fivefold between 1995 and 1997, with Ukraine, Russia, and Belarus accounting for about 90% of all new cases (12). HIV seroprevalence rates are as high as 70% among injection drug users in the Chinese provinces of Yunnan and Xinjiang, which border poppy-rich regions of Asia. Current estimates place the burden of HIV infections at 300,000 to 400,000 in China, and this number appears to be growing rapidly.

In the United States, between 600,000 and 800,000 persons are infected with HIV (13). Although the incidence of new infections declined substantially in the late 1980s as a result of successful prevention efforts, a plateau of about 40,000 new HIV infections per year has persisted through the 1990s (13). The availability of highly active antiretroviral therapy (HAART) in developed countries led to substantial reductions in the death rate due to AIDS (14,15). Unfortunately, no such benefit has reached the developing world, where AIDS-related mortality continues to exact a devastating toll. In fact, the nine African nations with an HIV seroprevalence greater than 10% will experience a decline in average life expectancy of 17 years by the year 2015. Instead of an average life expectancy that would have been on a trajectory to reach 64 years in the absence of HIV, it will decline to only 47 years (7). Infant mortality rates are rising sharply in these countries as well, largely as a direct result of HIV infections. Finally, most developing countries do not have adequate resources to provide for a growing population of AIDS orphans; 20% to 30% of all children under the age of 15 have been orphaned in nine sub-Saharan countries (16).

Transmission

The transmission of HIV occurs by exposure of the oral, rectal, or vaginal mucosa during sex, by inoculation (i.e., through transfusion of contaminated blood products, use of contaminated equipment during injection drug use, or maternal–fetal circulation), or by breast-feeding. Sexual transmission accounts for more than 90% of HIV infections worldwide. In developing countries, most infections occur as a result of heterosexual contact, with a male-to-female ratio of approximately 1. In the United States and Western Europe, the vast majority of HIV infections that occurred early in the epidemic were associated with homosexual contact. Specifically, unprotected receptive anal intercourse was identified as the strongest risk factor for acquisition of HIV infection in men who reported having sex with men. The demographic characteristics of the epidemic have shifted considerably over the years, with a higher percentage of new infections occurring in injecting drug users and their sex partners. Minority populations are disproportionately affected by HIV; in 1998, AIDS rates per 100,000 population were 66.4 for blacks, 28.1 for Hispanics, and 8.2 for whites (17). Women are also increasingly affected by the HIV epidemic; in the United States, the percentage of AIDS cases occurring in women increased from 7% to 23% between 1985 and 1998 (17). Fewer than 50% of AIDS cases in the United States that occurred in 1998 were in men who have sex with men (17). Behavioral risk factors also shifted over time. In a cohort of men who have sex with men recruited in 1992 through 1994, only 15% of HIV seroconversions that occurred were due to unprotected anal intercourse; many seroconversions appear to occur following activities previously considered to be of lower risk, including oral sex (18).

Transmission of HIV is dependent on a large number of behavioral and biologic factors. The per-contact probability of male-to-female HIV transmission during vaginal sex is approximately 0.1% to 0.2% (19,20); receptive anal intercourse is associated with a considerably higher (i.e., about 0.8%) risk of transmission (18). Not surprisingly, considerable heterogeneity exists in per-contact risk, with some persons becoming infected after only one or several exposures, and others remaining uninfected despite frequent exposure (18,21,22). Some of the many variables that influence the probability of HIV transmission include the genetic background of the potential host, the size and infectiousness of the inoculum, and the local environment in which the exposure occurs. Factors that result in physical disruption of the exposed mucosa, such as the use of vaginal dessicants or the presence of genital ulcer disease, can enhance viral transmission (23,24).

IMMUNE SYSTEM MOLECULES NECESSARY FOR HIV ENTRY

Human immunodeficiency virus enters cells through interactions with the CD4 molecule and one of a number of seven-transmembrane G protein–coupled chemokine coreceptors. In 1984, CD4 was identified as the major receptor for HIV fusion and entry (25,26). Although transfection of CD4 into CD4-negative human cells renders them infectable with HIV (27), murine cells remain resistant to HIV infection despite expression of human CD4. This result suggested that other factor(s) were necessary for HIV fusion and entry, although the identity of these additional factors remained elusive for several years.

In late 1995 and early 1996, several lines of investigation into diverse areas of HIV pathogenesis converged, revealing the identity of several cofactors necessary for HIV entry (Fig. 74.2). Investigators studying HIV suppressor factors secreted by $CD8^+$ T cells reported in 1995 that the CC chemokine macrophage inflammatory protein (MIP)-1a, MIP-1b, and RANTES (regulated on activation, normal T cell expressed and secreted) were major components of $CD8^+$ T cell-derived HIV suppressor activity (28). These chemokines inhibited the infection of activated $CD4^+$ T cells by certain strains of HIV-1, HIV-2, and SIV. Of note, these CC chemokines selectively inhibited infection of cells by different strains of HIV-1. They potently inhibited infection by viral strains that infect macrophages and peripheral blood mononuclear cells [i.e., macrophage (M)-tropic strains] but not strains that infect T-cell lines and peripheral blood mononuclear cells [i.e., T-cell (T)-tropic strains]. In a somewhat unrelated line of investigation, a gene was identified that allowed HIV envelope-mediated cell fusion in the presence of CD4 (29). The encoded protein, called *fusin* (later renamed *CXC chemokine receptor 4*, or CXCR4), is a seven-transmembrane G protein–coupled chemokine receptor. This receptor, together with CD4, was required for cell fusion with envelopes from T-tropic strains of HIV, but was not used by M-tropic envelopes.

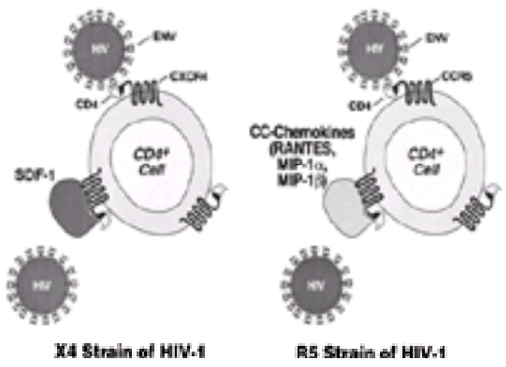

Figure 74.2. Model of human immunodeficiency virus (HIV) coreceptor (CCR5 and CXCR4) utilization and inhibition of HIV entry by coreceptor ligands. Entry of R5 strains of HIV is blocked by the CCR5 ligands macrophage-activating factor (MIP-1)a, MIP-1b, and regulated on activation normal T-cell expressed and secreted (RANTES). Entry of X4 strains of HIV is blocked by the CXCR4 ligand stromal cell–derived factor-1 (SDF-1).

In a separate line of research, Paxton and co-workers were studying a population of persons who had been exposed to HIV-infected partners but remained uninfected (i.e., “exposed-uninfected,” or E-U) (22). They identified two subjects whose $CD4^+$ T cells were refractory to infection with M-tropic strains of HIV *in vitro* but who were easily infectable with T-tropic strains. In addition, cells from these persons produced high levels of CC chemokines, which had been identified as suppressors of infection with M-tropic strains of HIV. Subsequently, a new CC-chemokine receptor, CCR-5, was identified; interestingly, the natural ligands that bind to this receptor were identified as MIP-1a, MIP-1b, and RANTES (30,31). In light of the previous work showing that the CCR5 ligands inhibit cellular entry of M-tropic strains of HIV, the obvious question that arose was whether CCR5 might function as a coreceptor for such strains. A series of five papers simultaneously showed this to be the case (reviewed in 32). Other chemokine receptors, including CCR1, CCR2b, and CCR3, were identified in these reports as potential coreceptors for certain HIV strains. Other chemokine receptors have been shown to be potential HIV coreceptors (reviewed in 32); however, CCR5 and CXCR4 appear to be the most physiologically relevant coreceptors for HIV entry. Strains of HIV are now classified according to their coreceptor utilization preferences; strains that use CCR5 for entry (i.e., most M-tropic strains) are referred to *as R5 strains*, and those that use CXCR4 (i.e., most T-tropic strains) are referred to as *X4 strains* (33).

The interactions between the HIV envelope, CD4, and the relevant coreceptor molecule that is necessary for HIV entry are complex. The HIV envelope glycoprotein (gp) first binds to CD4; this interaction creates a high affinity binding site for the HIV coreceptor as a consequence of a conformational change in the envelope (34,35 and 36). Although signal transduction through the chemokine receptor is not necessary for coreceptor activity (37,38), it may have important effects on viral replication after viral entry (39,40).

REPLICATION CYCLE OF HIV

Interactions between the surface envelope glycoprotein of HIV and the cell surface CD4 molecule and an appropriate coreceptor molecule (generally a seven-transmembrane G protein– coupled receptor; see preceding) result in fusion of viral and cell membranes, with entry of the virion contents into the cellular cytoplasm.

Viral reverse transcriptase (RT) is a product of the polymerase (*pol*) gene, and it synthesizes proviral DNA from the viral RNA genome. Cellular $tRNA^{lys}$ is utilized as a primer for the reverse transcription process. Like RT, RNase H is a *pol* gene product that is necessary for degradation of genomic RNA as viral cDNA is synthesized. Proviral DNA becomes associated with the viral matrix protein (a *gag* gene product), integrase (another *pol* gene product), and RT in a preintegration complex. Nuclear localization signals are present within the gag matrix protein; in addition, the viral Vpr protein appears to play an important role in targeting the preintegration complex to the nucleus (see later). Integrase cleaves chromosomal DNA and facilitates proviral integration.

Transcription of HIV genes is regulated by cis- and trans-acting sequences. The long terminal repeat (LTR) region of HIV DNA contains a TATA box as well as 3 Sp1 binding sites, which function as the major cis-acting transcriptional regulatory sequences. A transcriptional enhancer region is located just upstream from the Sp1 sites, consisting of two nuclear factor-kB (NF-kB) binding sites. These sites represent a prime example of the way HIV is able to utilize the milieu of immune activation (i.e., NF-kB levels are upregulated during T-cell activation) toward its own replicative advantage.

The Tat protein of HIV-1 enhances transcription of viral RNA by binding to the trans-activating response (TAR) region of the viral LTR. The mechanism by which Tat enhances transcription appears to be through its association with cellular factors, including cyclin-dependent kinase Cdk9 and cyclin T1; the complex of Tat and these cellular factors enhances the rate and processivity of RNA polymerase II–mediated transcription (reviewed in 41).

Transcripts of HIV can be grouped into two classes: Multiply spliced RNAs primarily encode regulatory HIV gene products, such as Tat, Rev, and Nef. Unspliced and singly spliced RNAs contain the Rev-responsive element (RRE); these RNAs encode Gag, Pol, and Env products as well as Vif, Vpr, Vpu, and a single-exon form of Tat. Rev binds to the RRE and forms multimers; Rev stabilizes RRE-containing transcripts through a posttranscriptional mechanism, facilitates their transport from the nucleus to the cytoplasm, and enhances their translation (reviewed in 42).

Assembly of new virions requires a number of coordinate biochemical processes. The Gag matrix domain targets the 55-kd Gag polyprotein to the plasma membrane of the infected cell (43) and also facilitates the incorporation of envelope glycoprotein molecules (44). The Gag nucleocapsid domain captures two copies of genomic HIV RNA, which are incorporated into the forming virion (45). The capsid protein associates with cyclophilin A; capsid-mediated incorporation of cyclophilin into the virion may promote uncoating of the viral core after entry into a target cell and is necessary for viral infectivity (46). The viral protease cleaves the Gag-Pol fusion protein; further cleavage of the intact Gag protein by the viral protease yields the viral core proteins that are necessary for virion assembly and maturation. Finally, the maturing virion buds from the cell surface, with envelope glycoproteins protruding outward from the lipid bilayer that is taken from the infected cell.

The HIV genome also encodes a number of other gene products that facilitate the viral replication cycle at a number of different stages. The Nef protein becomes detectable early after transcriptional activation of the HIV provirus. *Nef* mutants of HIV-1 exhibit attenuated pathogenicity, although the mechanisms responsible for this effect remain to be precisely elucidated. Nef downregulates cell surface expression of CD4 by inducing this molecule's endocytosis and lysosomal degradation (47). Other examples of viral receptor downregulation by animal retroviruses are known; teleologically, such an effect is thought to represent a viral strategy aimed at preventing superinfection of target cells. Nef also downregulates major histocompatibility (MHC) class I molecules (48), and this effect may allow HIV-infected cells to evade killing by HIV-specific cytolytic T-lymphocytes (CTLs) (49). Finally, Nef induces secretion of proinflammatory chemokines from HIV-infected macrophages; these chemokines are in turn capable of inducing chemotaxis and activation of resting T cells, an effect that could enhance the availability of suitable HIV targets (50).

Nef also associates with a variety of cellular serine-threonine as well as tyrosine kinases (reviewed in 51). Nef-associated kinase activity enhances serine phosphorylation of gag matrix protein, which in turn facilitates membrane dissociation of the reverse transcription complex during viral entry. These Nef-related effects may be responsible for the enhanced virion infectivity associated with Nef. Finally, Nef can form a complex with the z-chain of the T-cell receptor; this interaction enhances expression of Fas and Fas ligand, sensitizing cells to apoptosis (52,53).

Vif is detectable in the cytoplasm of HIV-infected cells; the protein appears to associate with the plasma membrane and with the cytoskeleton as well (54,55). Vif renders cells permissive for HIV infection, likely by overcoming the activity of an endogenous inhibitor of HIV replication, the identity of which remains undetermined (56). Vif may also be incorporated into virions; it may fulfill roles in virion maturation as well as proper processing of the gag p55 precursor. Products of reverse transcription are unstable in *vif*-defective viruses (57), and the reverse transcription process itself may be inefficient in the absence of Vif. Because Vif does not appear to participate directly in the reverse transcription complex, a defect in the early stages of the viral replication cycle is implied.

Vpu is an integral membrane phosphoprotein. Biologic activities that have been attributed to Vpu include induction of CD4 degradation in the endoplasmic reticulum (58), downregulation of MHC class I molecule synthesis (59), and enhancement of viral particle release from the plasma membrane of infected cells (60).

Despite the absence of a nuclear localization signal, the Vpr protein of HIV-1 localizes mainly in the nucleus of infected cells and facilitates nuclear transport of the preintegration complex (reviewed in 61); this property confers on HIV-1 the ability to infect nondividing cells, such as macrophages. Vpr is incorporated into virions through its interaction with the p6 gag protein. Virion-associated Vpr can arrest the cell cycle at the G2 stage and induce apoptosis in $CD4^+$ T cells (reviewed in 61). These effects are dependent only on viral entry, and not on replication, suggesting a role for defective as well as infectious virions in contributing to $CD4^+$ T-cell dysfunction and death (62).

PATHOGENESIS OF ACUTE INFECTION

The earliest events that occur following exposure to HIV involve the transport of virus from the site of exposure to lymphoid tissue. In a macaque model of SIV sexual transmission, bone marrow–derived dendritic cells (DCs) in the vaginal mucosa were the first cells to contain SIV DNA, which became detectable 2 days after vaginal exposure. In subsequent examinations of lymphoid organs, the pattern of appearance and spread of SIV mirrored the course taken by DC on migrating from peripheral tissues to lymphoid organs (63). In addition to serving as direct targets of HIV infection, DCs are also capable of retaining infectious virions on their surface for extended periods (64). This property is mediated by DC-SIGN [dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin], a DC surface molecule that normally interacts with ICAM-3 on the surface of T cells (65). DC-SIGN also binds with high affinity to the HIV-1 gp120; however, the protein does not serve as an entry coreceptor but rather enhances the viability of surface-bound virions (66). Thus, the role of DCs in the initiation of HIV infection includes capturing virions at sites of entry, carrying HIV to the paracortical regions of lymphoid organs and delivering virus to $CD4^+$ T cells that become activated through their interaction with DCs. In fact, active viral replication occurs in DC–$CD4^+$ T-cell conjugates that are formed *in vitro* (67). Similar conjugates of DC and $CD4^+$ T cells containing HIV antigen have been identified *in vivo* in tonsil biopsy specimens from persons infected with HIV (68), in the peripheral blood in low quantities (64), and in the submucosal tissue after vaginal exposure to SIV (63).

Macrophages and $CD4^+$ T cells are key targets of HIV infection. The viral determinant of cellular tropism maps to the gp120 envelope protein of HIV-1, mostly to the third variable region (V3 loop) (69) that is also the major determinant for coreceptor use (70). Changes in viral phenotype have been observed at different stages of HIV infection. CCR5-utilizing viruses are preferentially transmitted during primary infection and predominate at most stages of infection (71). In about 50% of HIV-infected persons, the appearance of CXCR4-utilizing isolates late in the course of disease heralds accelerated $CD4^+$ T-cell decline and clinical disease progression (72,73). This transition may occur by mutation of only a few amino acid residues predominantly in the envelope V3 loop (74,75). Given the high error rate of RT and the rapid kinetics of HIV replication, the surprising failure of such mutants to emerge until late in the course of the disease process indicates a change in the selective advantage of such a mutation during the course of disease progression. The nature of such a selective advantage is currently unknown; however, it may relate to the ability of X4 viruses to gain cellular entry through an expanded repertoire of coreceptor molecules.

The mechanisms responsible for the restriction of R5 viruses during primary infection is the subject of intense investigation. Freshly isolated Langerhans cells (resembling mucosal DCs) express CCR5, but not CXCR4, on their surfaces (76); this finding suggests that infection of DCs may be a necessary first step in acute infection and that this step is restricted by the expression pattern of HIV coreceptors on DC; however, HIV-pulsed DCs from persons who lack a functional *CCR5* gene are capable of transmitting infection to $CD4^+$ T cells *in vitro* (77). Thus, although infection of DC may be dependent on specific HIV coreceptor expression, the ability of DCs to trap virions on their surface and infect $CD4^+$ T cells that they encounter is at least in part a coreceptor-independent phenomenon.

In animal models of SIV infection, virus can be detected in the peripheral lymph nodes within 1 week of infection (63,78,79). Most of the SIV RNA at this time is associated with individual productively infected cells in lymph nodes. This production of virus by individually infected cells in lymphoid tissue precedes and likely is responsible for the peak of viremia. Coincident with the appearance of SIV-specific immune responses, a decrease in the frequency of productively infected cells is seen during the second week after infection. By 4 weeks after infection, viremia decreases as a result of further elimination of productively infected cells by SIV-specific cell-mediated immune responses and clearance of circulating virions by the formation of immune complexes consisting of virus, antibody, and complement that are

trapped within lymphoid tissue germinal centers (78,79).

Cross-sectional studies in patients evaluated soon after acute HIV infection are similar to the findings in the SIV model (Fig. 74.3). The kinetics of HIV viremia and HIV-specific immune responses in peripheral blood parallel those in the SIV model (80,82 and 83). Data from lymph node biopsies obtained from acutely infected individuals suggest that the SIV model is valid in depicting the early events associated with HIV infection in lymphoid tissue as well (84). In the weeks to months following acute HIV infection, a sharp decline in the frequency of productively infected cells is evident in lymphoid tissue. Germinal center formation within lymphoid follicles becomes pronounced, and viral RNA corresponding to extracellular virions complexed with antibody and complement is detected in the network of follicular dendritic cell (FDC) processes (85,86 and 87). Although the immune system is capable of efficient elimination of a substantial number of productively infected cells in lymphoid tissue, the earliest interactions between virus and host virtually ensure viral persistence. In this regard, massive numbers of virions trapped within the germinal center FDC network as well as latently infected cells, which harbor proviral DNA but do not express viral proteins (and thus elude the immune response), represent potentially continuous sources of virus for *de novo* infection of $CD4^+$ T-lymphocytes that are resident in or migrating through lymphoid tissue (88,89,90 and 91).

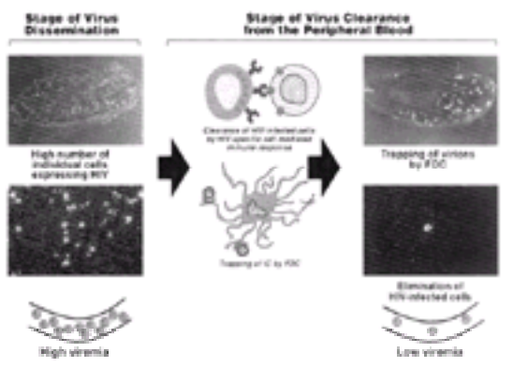

Figure 74.3. Cell-mediated and humoral immune mechanisms involved in the downregulation of viremia following primary HIV infection. Before the emergence of human immunodeficiency virus (HIV)-specific immune responses, large numbers of individual productively infected cells are found in lymph nodes (left; low- and high-power photomicrographs of an *in situ* hybridization assay for HIV RNA in a lymph node), resulting in high levels of plasma viremia. Emergence of cytotoxic T-cell responses against HIV leads to elimination of a large number of productively infected cells (photomicrograph, lower right). In addition, HIV-specific antibodies combine with virions to form immune complexes (IC); complement (C') binds to the immune complexes, which then are trapped in the follicular dendritic cell (FDC) network of expanding germinal centers by complement receptors (photomicrograph, upper right).

VIRAL DISSEMINATION

Like many other invading microorganisms, HIV is transported by antigen-presenting cells to lymphoid tissue. In the case of HIV, however, the host immune system is unable to control and eradicate the infection completely. In fact, in an impressive example of subversion of the immune system, HIV utilizes the activated milieu of lymphoid tissue toward its own replicative advantage. Close contact between immune effector cells as well as high levels of proinflammatory cytokines are characteristic of the lymphoid tissue microenvironment; these conditions favor viral replication in several ways. Activated $CD4^+$ T-lymphocytes migrating through lymphoid tissue serve as ideal targets for *de novo* infection with HIV (91,92). Activation signals such as those delivered by proinflammatory cytokines, found in abundance within activated lymph nodes, are potent inducers of HIV replication in latently infected cells (93,94) and also are able to increase the pool of activated cells that are susceptible to HIV infection (92,95,96 and 97).

Viral dissemination throughout lymphoid tissue is a fundamental pathogenic event during the early phase of HIV infection. Lymphoid tissue remains the most important reservoir of infection throughout the entire course of the disease. The early chronic stage of HIV disease is characterized by heavy concentration of viral load in lymphoid tissue. In this regard, the frequency of infected cells in lymph nodes exceeds that in peripheral blood by fivefold to tenfold; differences in levels of viral replication are generally tenfold to 100-fold (87,98,99). Up to 25% of $CD4^+$ T-lymphocytes present in lymph node germinal centers harbor HIV DNA, further emphasizing the role of lymphoid tissue as a critical reservoir for HIV *in vivo* (88). The continuous state of rapid high-level turnover of plasma viremia derives in large measure from viral replication in lymphoid tissue (100,101).

The concentration of viral load in lymphoid tissue is due in part to the normal process of follicular hyperplasia within lymphoid germinal centers following antigenic challenge (85,102). Expansion of the FDC network within hyperplastic lymphoid follicles is an efficient mechanism for viral trapping via interactions between antibody and complement-coated virions with complement receptors or adhesion molecules on the surface of FDCs (103,104).

During the course of HIV disease progression, there is a shift in the lymphoid histopathologic pattern from follicular hyperplasia to follicular involution (87). This shift in histopathology is associated with important changes in viral distribution. Disruption of the FDC network is characteristic during this period, leading to a decrease in the efficiency of viral trapping in germinal centers and a consequent increase in plasma viremia (87). Sequestration of infected cells within lymphoid tissue also becomes less efficient during follicular involution, leading to an increase in the frequency of infected cells in the peripheral blood. As the $CD4^+$ T-lymphocyte count falls below 200 cells per microliter, there is a tendency for viral load to increase more rapidly in the peripheral blood compartment, leading to equilibration between lymph node and peripheral blood.

Destruction of lymphoid tissue certainly is a major mechanism responsible for the severe immune dysfunction and loss of the ability to inhibit viral replication observed in advanced-stage HIV disease. The ability to maintain an effective immune response to HIV is severely impaired in the absence of intact lymphoid tissue architecture. As a consequence, increased cell-associated viral RNA becomes evident in the paracortical regions of lymph nodes, reflecting increased viral replication. Thus, during progression of HIV disease, there is a reversal in the predominant forms of virus in lymph nodes, with progressive diminution of the extracellular form (i.e., trapped virus) and an increase in cell-associated virus (i.e., cells expressing HIV) (87). In the advanced stage of disease, there is almost total dissolution of lymphoid architecture. Follicular involution, fibrosis, frank lymphocyte depletion, and fatty infiltration herald complete loss of functional lymphoid tissue, contributing to the state of immunodeficiency and the dramatically enhanced susceptibility to opportunistic infections. Disruption of the lymphoid microenvironment during the course of HIV infection remains an enigmatic process with considerable implications for future therapeutic interventions. Productive infection of FDCs by HIV may occur, particularly in the late stages of HIV infection (105); however, most data suggest that productive infection of FDCs is rare during the period of intermediate stage disease, when dissolution of the FDC network begins (104,106). Of note, some of the HIV-related pathologic changes that occur in lymphoid tissue may be at least partly reversible during potent antiretroviral therapy (107,108).

IMMUNE RESPONSES

Similar to most pathogens, HIV induces a broad array of host immune responses in an infected person. A central unanswered question of pathogenesis is how HIV is able to continuously replicate and cause the inexorable decline in immune system function in the presence of these immune responses.

Humoral Immune Responses

Antibodies against the viral core protein p24 develop within weeks of acute HIV infection and may play a role in the decline of plasma viremia associated with primary infection. Loss of anti-p24 antibodies is associated with progression of HIV disease (80,109,110).

Antibodies that neutralize HIV infectivity may be responsible for at least partial control of viral replication *in vivo* (109,110). Neutralizing antibodies may be type specific (i.e., specific for one viral isolate) or group specific (i.e., specific for a broad range of viral isolates). Most type-specific neutralizing antibodies recognize the V3 region of the HIV envelope gp120 protein (111). Neutralizing anti-V3 loop antibodies may prevent conformational changes within gp120 necessary for HIV entry or for cell–cell fusion (112). Group-specific neutralizing antibodies recognize epitopes within the HIV envelope gp41 protein (113), discontinuous conformational epitopes around the CD4 binding site of gp120 (114), or carbohydrate determinants (115). Both type- and group-specific neutralizing antibodies are more efficient in neutralizing laboratory strains of HIV grown in T-cell lines compared with primary isolates grown in peripheral blood mononuclear cells (PBMCs); this is likely due to differential exposure of the V3 loop and CD4-binding domain epitopes among these isolates (116,117). Some neutralizing antibodies interfere with the interaction between the HIV envelope and CCR5, thereby inhibiting cellular entry of R5 strains of HIV (118,119).

Neutralizing antibodies appear to be prognostically relevant to the course of HIV infection (120). The regularity with which viral variants emerge that resist neutralization

suggests that such antibodies are potent impediments to viral replication (121). In nonhuman primate models, neutralizing antibodies are associated with slow rates of disease progression (122) and accelerate clearance of both infectious and noninfectious virions (123); furthermore, passive transfer of SIV-neutralizing antibodies can protect macaques against a subsequent viral challenge (124,125 and 126).

Some anti-HIV antibodies bind to immunoglobulin G (IgG) Fc receptor-positive cells and sensitize them to mediate antibody-dependent cellular cytotoxicity (ADCC) against HIV-infected or HIV-coated cells (127,128). Most of these antibodies are directed against HIV envelope gp120 or gp41 proteins. $CD16^+$ natural killer cells are important mediators of ADCC (129), and monocytes may also mediate this activity (130). Anti-HIV ADCC antibodies develop soon after primary infection and are detectable throughout the course of HIV disease, with some decrease in titers with the onset of AIDS (131). ADCC may also represent an immunopathogenic immune response that may be responsible for $CD4^+$ T-cell depletion during the course of HIV infection. In this regard, a combination of high levels of plasma viremia with high HIV envelope-specific ADCC activity is correlated with rapid $CD4^+$ T-cell depletion; this correlation may reflect ADCC-mediated "innocent bystander" killing of gp120-coated, but uninfected, $CD4^+$ T cells (132).

Cellular Immune Responses

CYTOTOXIC T-LYMPHOCYTES

Classic MHC class I-restricted, HIV-specific, CD8 CTL responses against a variety of HIV target proteins have been demonstrated in HIV-infected persons (133,134). Using standard cell culture methods, a high frequency of HIV-specific CTLs and CTL precursors (up to 1% of peripheral blood T cells) has been observed in asymptomatic HIV-infected patients (135,136). Using human leukocyte antigen (HLA)–peptide tetramers, the true frequency of HIV-specific CTLs may be up to an order of magnitude greater than that detected by standard functional assays (137,138).

A critical role for CTLs in the suppression of viral replication in HIV-infected persons is suggested by the close correlation between the emergence of an HIV-specific CTL response and downregulation of viremia following acute infection (82,83), the association of vigorous HIV-specific CTL responses with slow progression of HIV disease (139,140 and 141), and the decline in HIV-specific CTL activity with disease progression (133,135). Quantitative studies revealed an inverse correlation between the frequency of HIV-specific CTL and levels of plasma viral load (138,142) and between the CTL frequency and the rate of $CD4^+$ T-cell decline (142). A beneficial role for HIV-specific CTLs is also suggested by their presence in the peripheral blood of persons who are frequently exposed to HIV and yet remain uninfected (143,144). Studies in nonhuman primates have demonstrated that monkeys subjected to $CD8^+$ T-cell depletion manifest diminished control of plasma viremia during primary SIV infection (145); in addition, $CD8^+$ T-cell depletion during the chronic phase of SIV infection leads to a prompt and profound increase in levels of viral replication (145,146).

The quality of the HIV-specific CTL response is also an important determinant of the efficacy of these responses in controlling viral replication. The specificity of CTL responses may determine in part their salutary role; in this regard, CTL responses against viral core proteins in particular have been associated with a decreased risk of disease progression (140). CTL recognition of immunodominant HIV epitopes presented by certain MHC class I alleles may result in potent anti-HIV activity (147) and may explain in part the association of certain MHC class I alleles with slower progression of HIV disease (reviewed in 148). Furthermore, the ability to recruit an HIV-specific CTL response composed of a diverse group of T-cell receptor Vb families that recognize multiple epitopes is associated with better control of viral replication and an improved prognosis compared with a narrow CTL response (149,150).

The loss of HIV-specific CTL activity in patients with progressive disease is likely a result of several factors. Viral proteins such as Tat, Nef, and Vpu can downregulate cellular expression of MHC class I molecules that are necessary for CTL recognition of infected cells (48,59,151). Increased expression of killer inhibitory receptors also may inhibit CTL activity (152). Another mechanism responsible for the loss of CTL activity is the selective accumulation of $CD8^+DR^+$ HIV-specific CTLs that lack the interleukin (IL)-2 receptor and are defective in clonogenic potential (153). Finally, the ability of HIV to escape CTL responses by viral mutation or by exhaustion of CTL clones due to high concentration of antigen helps explain the loss of CTL-mediated control over viral replication (154,155).

The host CTL response against HIV is constrained by the ability of the MHC class I alleles to bind to various viral epitopes, and the virus is constrained by the degree to which an escape mutation impairs viral fitness (156,157 and 158). These host–virus dynamics are extraordinarily complex given the large number of permutations of viral epitopes and MHC class I alleles. Viral mutations within CTL recognition epitopes (i.e., "escape mutants") are associated with increased levels of viral replication and progression of HIV disease (159,160 and 161). Viral escape mutants may thrive because of the release of CTL control over their replication and also may inhibit CTL responses against the preescape viral epitope (162,163 and 164). A mathematic model of CTL–virus dynamics was provided by Nowak and co-workers, who described disease progression as a result of viral sequence variation that escapes an immunodominant CTL response and shifts the host response toward a weaker epitope (165). In this scenario, disease progression may be the result of fitness of viral escape mutants outpacing the plasticity of the host CTL response, with slow progression resulting from CTL plasticity overpowering viral escape mutants with limited fitness.

$CD8^+$ T-CELL-DERIVED SOLUBLE SUPPRESSOR FACTORS

A variety of soluble antiviral factors are elaborated by $CD8^+$ T cells. CD8 antiviral factor (CAF) (166,167) is noncytolytic, inhibits viral replication at the level of HIV LTR transcription (168,169) and lacks identity to known cytokines (170). Although CAF activity is non-MHC restricted, its activity is maximal in a syngeneic system (171). CAF activity decreases with disease progression, but it remains potent in long-term nonprogressors (172,173).

The combination of RANTES, MIP-1a, and MIP-1b (the natural ligands for CCR5) are also important soluble antiviral factors, and are secreted by CD8+ T cells as well as by other cell types (28,174). These CC chemokines inhibit viral replication primarily at the level of cell entry. Emerging data support a role for CC chemokines in protecting against HIV infection and disease progression (175,176,177 and 178). Macrophage-derived chemokines (MDC) can potently suppress replication of R5 as well as X4 strains of HIV *in vitro* (179); the role of MDCs in controlling HIV replication *in vivo* is uncertain. Finally, IL-16 has been reported to be a soluble antiviral factor (180). IL-16 inhibits HIV transcription and can potently suppress HIV replication when it is expressed in HIV-infected T cells (181,182); furthermore, IL-16 production has been correlated with protection against disease progression in HIV-infected persons (183,184).

$CD4^+$ T-CELL RESPONSES

The HIV proteins contain helper T-cell epitopes that may be presented by MHC class II alleles (185,186). Recognition of these epitopes by $CD4^+$ T cells results in secretion of cytokines and cellular proliferation. These responses may be associated with abortive infection in persons exposed to HIV but who remain uninfected (187), and they decrease with HIV disease progression (185,186). An inverse correlation has been found between the magnitude of HIV-specific $CD4^+$ T-cell response and levels of plasma viremia (188); these data are consistent with the observed inverse correlation between CTL activity and plasma viremia as well (138). The fate of HIV-specific $CD4^+$ T cells during the course of HIV infection is controversial. Some evidence suggests that these cells are usually depleted in the early stages of disease and that initiation of potent antiretroviral therapy during acute HIV infection may prevent this loss and lead to more effective immunologic control of viral replication (188). Other data suggest that HIV-specific $CD4^+$ T cells are present throughout the course of HIV disease and that their frequency actually declines during HAART-induced viral suppression (189).

CYTOKINES AND HIV DISEASE: DYSREGULATION OF CYTOKINE PRODUCTION

Cytokines that are generated during the host immune response to an infection play an important role in determining the outcome of the infectious process. Dysregulation of the host cytokine network is characteristic of all phases of HIV infection. Many of the observed alterations in cytokine production contribute to HIV pathogenesis by further stimulating viral replication, suppressing the ability of the immune system to mount an efficient antiviral response, and inducing cytokine-mediated cytopathic effects (6,190,191).

Infection with HIV is associated with increased expression of proinflammatory cytokines; high levels of tumor necrosis factor (TNF)-a, IL-1b, and IL-6 are secreted by PBMCs and macrophages from HIV-infected subjects and are found at elevated levels in serum, cerebrospinal fluid, and tissues (reviewed in 191,192). High levels of expression of these cytokines as well as interferon (IFN)-g (193,194) and IL-10 (194), are particularly evident in lymphoid tissue, a major site of HIV replication throughout the course of disease (85,87,88,98).

Another major disruption in the cytokine pattern observed in HIV disease is a progressive loss in the ability to produce immunoregulatory cytokines such as IL-2 and IL-12, which are critical for effective cell-mediated immune responses because they stimulate proliferation and lytic activity of CTLs and natural killer (NK) cells. In addition, IL-12 is essential for stimulating the production of T helper (TH)-1 type cytokines, including IL-2 and IFN-g, which favor the development of cell-mediated immune responses. The T-helper cell type 1 (Th1) limb of cellular immune responses is impaired during the course of HIV infection; although the cause of this TH1 defect in HIV infection is multifactorial, it may derive in part from the ability of the HIV envelope to induce IL-10 production in monocytes and thereby inhibit IL-2 secretion (195). Some investigators have proposed that a dominance of TH2-like responses (i.e., secretion of IL-4, IL-5, and IL-10) characterizes progression of HIV disease (196). Most studies, however, suggest that cytokine dysregulation during the course of HIV infection is complex and cannot easily be classified in terms of TH1

and TH2 polarity (194,197,198).

Effects of Cytokines on HIV Replication

Soluble factors produced by activated PBMCs (93), macrophages (199), and B cells (200) can dramatically upregulate HIV expression in acutely and chronically infected cells of the lymphocytic and macrophage lineage (Fig. 74.4). Cytokines that have been reported to upregulate HIV replication *in vitro* include IL-1b, IL-2, IL-3, IL-6, IL-7, IL-12, IL-15, IL-18, TNF-a, TNF-b, and the colony stimulating factors (CSF) macrophage (M)-CSF and granulocyte-macrophage (GM)-CSF (reviewed in 191; see also 201,202,203,204,205 and 206). IFN-a, IFN-b, and IL-16 (184,207,208) are primarily suppressors of HIV production, whereas other cytokines, such as IL-4 (209), IL-10 (210,211), IL-13 (212), IFN-g (213,214), and TGF-b (215,216), reduce or enhance viral replication, depending on the infected cell type and the culture conditions. The effects of a particular cytokine are often greatly influenced by the activity of other cytokines present in the lymphoid tissue microenvironment (211,217,218).

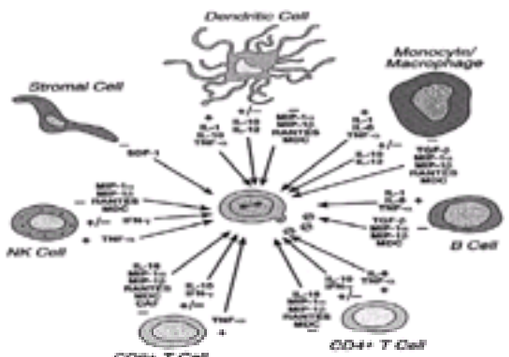

Figure 74.4. Cytokine networks that regulate human immunodeficiency virus (HIV) replication. Cytokines that enhance viral replication (+), inhibit viral replication (–), and enhance or inhibit viral replication depending on the conditions (+/–) are shown.

Proinflammatory cytokines, particularly TNF-a, are considered the most potent HIV-inducing cytokines, and their mechanism of action is relatively well understood. Both TNF-a and IL-1b activate the cellular transcription factor nuclear factor (NF) kB (219,220), a strong inducer of HIV LTR-mediated transcription. IL-6 increases HIV expression primarily by a posttranscriptional mechanism; however, IL-6 can synergize with NFkB-inducing cytokines to enhance HIV transcription (217). The production of HIV by macrophages or PBMCs stimulated with physiologic inducers of proinflammatory cytokine production, such as bacterial endotoxin or IL-2, can be partially or nearly completely abrogated by the addition of anti-proinflammatory cytokines (221,222), neutralizing antibodies to the cytokines (201), or receptor antagonists (ra), such as IL-1ra (201). In cultures of HIV-infected macrophages, the viral-suppressive activity of several cytokines, such as IL-10 and TGF-b, is attributable largely to their ability to inhibit the secretion or activity of HIV-inducing proinflammatory cytokines (215,221,222). HIV production by infected T cells is sensitive to both the anti-proinflammatory and the anti-proliferative activity of such cytokines (223).

Chemokines, or chemoattractant cytokines, are produced by numerous cell types and may influence HIV replication by binding to chemokine receptors that also serve as HIV coreceptors (224). Chemokine production, induced during inflammation, is enhanced by CD40 ligand stimulation (225) and by several cytokines, including TNF-a, IL-1b, IL-2, and IL-15 (226). Chemokines that bind to CCR5 suppress HIV replication *in vitro* in PBMCs from asymptomatic HIV-infected persons harboring predominantly R5 strains of HIV (174) but not in PBMCs from persons with more advanced disease harboring predominantly X4 strains of HIV (174,227). Similarly, HIV isolates obtained longitudinally from persons with rapid disease progression exhibit reduced sensitivity to inhibition by CCR5 ligands *in vitro* over time (227,228). Ligation of chemokine receptors in different cell types may have different effects on HIV replication; CC chemokines inhibit replication of R5 strains of HIV in $CD4^+$ T cells but actually may enhance replication of these strains in monocytes and macrophages (229,230). Unanticipated effects of manipulating the chemokine–chemokine receptor axis also have been observed, including CC-chemokine-mediated upregulation of CXCR4 expression and enhancement of replication of X4 strains of HIV *in vitro* that is dependent on intracellular signal transduction (39,40).

ROLE OF CELLULAR ACTIVATION IN HIV PATHOGENESIS

The end result of HIV infection is profound immunodeficiency; however, paradoxically, HIV infection is associated with hyperactivation of the immune system throughout most of the course of disease. HIV subverts the immune system by inducing immune activation and utilizing this milieu toward its own replicative advantage (6,191,231).

The replicative cycle of HIV infection is achieved most efficiently in activated cells (232,233 and 234). Cytokines that induce T-cell activation can contribute further to viral replication by inducing a state of productive infection in latently infected resting T cells (235). Several lines of evidence suggest that HIV replication *in vivo* is dependent on antigen-driven activation of $CD4^+$ T cells. HIV-infected persons with intercurrent infections experience transient increases in plasma viremia that correlate with the degree of induced immune activation (236); similar observations have been made in HIV-infected persons who received immunizations against various pathogens (95,96 and 97).

Analysis of viral quasispecies and immune responses within lymphoid tissue from HIV-infected persons reveals that, within individual splenic white pulps, a restricted number of individual antigen-specific immune responses occurs (defined by the analysis of T-cell receptor Vb gene usage), and each of these areas contains a single or limited number of HIV quasispecies (237). These data support the theory that, within the context of individual antigen-specific immune responses, a single quasispecies of HIV, which was present at initiation of the reaction, spread among the newly activated T cells. Thus, it is likely that the continuous daily production of HIV occurs in newly activated $CD4^+$ T cells that are being driven by antigen-specific activation (237,238).

IMMUNE DYSFUNCTION DURING HIV INFECTION

The pathogenesis of HIV disease is a complex, multifactorial process (191,231). A wide array of immune system deficits is associated with HIV infection; abnormalities in the function of all limbs of the immune system, including T- and B-lymphocytes, antigen-presenting cells, NK cells, and neutrophils, have been described.

$CD4^+$ T Cells

$CD4^+$ T-cell dysfunction and depletion are hallmarks of HIV disease. The proximate cause of the susceptibility to opportunistic infections observed with advancing disease is the defects in T-cell number and function that result directly or indirectly from HIV infection. In addition to the decrease in IL-2 production and IL-2 receptor expression, the percentage of $CD4^+$ T cells expressing CD28 (i.e., the major costimulatory receptor that is necessary for normal activation of T cells) is reduced during HIV infection compared with cells from uninfected persons (239). $CD28^-$ cells do not respond to activation signals and express markers of terminal activation, including HLA-DR, CD38, and CD45RO (240). In addition, $CD4^+$ T cells from HIV-infected persons express abnormally low levels of CD40 ligand (241).

A variety of mechanisms, both directly and indirectly related to HIV infection of $CD4^+$ T cells, are likely responsible for the observed defects in T-cell function. Interference with CD4 expression by HIV gp120 (242), Nef (47), and Vpu (58) may impair the ability of the infected $CD4^+$T cell to interact with appropriate MHC class II molecules. Preferential infection by HIV of $CD4^+$ memory cells or the preferential susceptibility of these cells to the cytopathic effects of HIV infection may explain in part the loss of memory responses to soluble antigens and the consequent increase in the risk of infection with opportunistic organisms (243,244).

The relatively small fraction of T cells that is infected with HIV in seropositive subjects at any given time militates against direct infection as the sole mediator of the immunopathogenesis of HIV infection (see later) (98). In addition, $CD4^+$ T-cell dysfunction is evident even in the early stages of HIV infection, before quantitative depletion of this key cellular subset (245). A role for HIV envelope in the immunopathogenesis of HIV disease has been suggested by a number of studies (reviewed in 246). The presence of measurable levels of circulating soluble gp120 in HIV-infected subjects (247) and the accumulation of high concentrations of virions (both infectious and defective) in lymphoid tissue (85,87,248) underscore the potential for envelope to contribute to T-cell dysfunction in a process distinct from infection of $CD4^+$ T cells.

The HIV envelope glycoproteins bind with high affinity to the CD4 molecule and to a number of chemokine coreceptors. Intracellular signals transduced by HIV-1 envelope have been implicated in several immunopathogenic processes, including anergy (249,250), syncytium formation (251), apoptosis (252), and inappropriate cell

trafficking (253). The molecular mechanisms responsible for these abnormalities include dysregulation of the T-cell receptor phosphoinositide pathway (254), p56lck activation (255), phosphorylation of focal adhesion kinase (256), activation of caspase-3 (257), downregulation of the costimulatory molecules CD40 ligand and CD80 (258), and activation of the mitogen-activated protein (MAP) kinase and *ras* signaling pathways (259). Most of these aberrant signals were presumed to result from interactions between HIV envelope glycoproteins and CD4; however, more recently, it has become clear that HIV envelope also may transduce intracellular signals through coreceptor molecules such as CCR5 (253,256,260) and CXCR4 (261).

Direct Infection as a Cause of $CD4^+$ T-Cell Depletion

The observations that $CD4^+$ T cells are the principal targets of HIV infection *in vivo* and that HIV infection of $CD4^+$ T cells *in vitro* causes cytopathicity led to a reasonable assumption that direct infection of $CD4^+$ T cells *in vivo* results in their depletion. Quantitative studies of the frequency of HIV-infected cells *in vivo* suggest, however, that single-cell killing by direct infection with HIV may not be the predominant mechanism of $CD4^+$ T-cell depletion (98,262). Although it increases with disease progression, the frequency of HIV-infected peripheral blood $CD4^+$ T cells rarely exceeds 1 in 100, even in patients with AIDS (262). Viral burden and levels of virus expression are far greater in lymphoid tissue compared with peripheral blood (87,88,98); however, these levels, even in lymphoid tissue, do not appear to be sufficiently high to account for $CD4^+$ T-cell depletion solely by direct mechanisms.

Multiple mechanisms of cell death appear to be operative after infection of a $CD4^+$ T cell with HIV. Accumulation of reverse-transcribed viral DNA in the cytoplasm (263), interference by viral RNA with normal cellular RNA processing (264,265), intracellular interactions between the viral envelope gp120 and CD4 molecules (266), compromise of cell membrane integrity by the budding virions (267), and HIV-induced increases in the concentration of intracellular monovalent cations (268) all may play roles in $CD4^+$ T-cell killing.

In addition, HIV-infected cells may die as a consequence of viral-specific immune responses that occur before the cell succumbs directly to viral infection. Multiple effector mechanisms may be involved in the killing of HIV-infected cells, including CTL responses, ADCC, and NK cell responses.

Indirect Mechanisms of $CD4^+$ T-Cell Depletion

SYNCYTIUM FORMATION

The molecular events associated with viral entry that lead to fusion between the viral coat and cell membrane involve the interaction of the HIV envelope glycoprotein, CD4, and a co-receptor molecule. Similar events may occur when an infected cell bearing HIV envelope glycoprotein molecules on its surface encounters an uninfected $CD4^+$ cell with an appropriate coreceptor. Fusion between infected and uninfected cells, resulting in multinucleated giant cells, or syncytia, has long been observed *in vitro* (269,270). Syncytia have been observed only rarely in tissues obtained from HIV-infected persons (102); thus, it is unlikely that syncytium formation is a major pathogenic mechanism of $CD4^+$ T-cell depletion *in vivo*.

AUTOIMMUNITY

Autoimmunity may occur during the course of HIV infection as a result of molecular mimicry by viral components. Highly homologous regions exist in the carboxy terminus of the HIV-1 envelope glycoprotein and the amino terminal domains of different HLA-DR and DQ alleles (271). Sera from a substantial number of HIV-infected persons react with the shared determinant of gp41 and MHC class II: These sera can inhibit normal antigen-specific proliferative responses and also eliminate class II–bearing cells by ADCC (272). Similar instances of molecular mimicry between HIV-1 envelope constituents and host proteins, which may result in pathogenic autoimmune responses, include the collagen-like region of complement component C1q-A (273), MHC class I heavy chains (274), HLA-DR4 and DR2 alleles (275), variable regions of the T-cell receptor a-, b-, and g-chains (275), Fas (276), functional domains of IgG and IgA (275), denatured collagen (277), and a number of nuclear antigens (278). Finally, immune recognition of normal cellular components may be induced by their association with HIV; such phenomena may be responsible for the emergence of CTLs that are specific for autologous $CD4^+$ T cells (279).

INNOCENT BYSTANDER PHENOMENA

Immune responses that target HIV determinants on infected cells also may contribute to the elimination of uninfected cells with HIV proteins (e.g., gp120) bound on their surface. Targeting of such “innocent bystander” cells by antibody and cellular immune responses has been described (127,280).

HIV-MEDIATED INHIBITION OF HEMATOPOIESIS

Failure of normal hematopoiesis is an obvious candidate mechanism to account for depletion of $CD4^+$ T cells during HIV infection. A subset of $CD34^+$ progenitor cells express CD4 and HIV coreceptors and are infectable *in vitro* with HIV-1 (281,282). Furthermore, a substantial minority of HIV-infected patients with severe $CD4^+$ T-cell depletion have a reservoir of HIV-infected $CD34^+$ progenitor cells (283).

Although the role of direct infection of $CD34^+$ progenitor cells in CD4+ T-cell depletion remains controversial, a large body of evidence suggests that viral proteins and HIV-induced cytokines can impair the survival and clonogenic potential of these cells (284,285). The subnormal mobilization of $CD34^+$ cells into the peripheral blood following treatment with GM-CSF in HIV-infected persons provides further evidence of reduced hematopoietic capacity and reserve in HIV infection (286).

Disruption of the thymic microenvironment (287) and HIV-induced thymocyte depletion also may contribute to the failure of $CD4^+$ T-cell production. Thymic epithelial cells normally secrete IL-6, which in turn can increase HIV replication in infected cells (288). Subpopulations of thymic $CD3^-CD4^-CD8^-$ cells (i.e., “triple negative” cells) are susceptible to infection with HIV *in vitro* (289), and thymic $CD3^-CD4^+CD8^-$ progenitor cells from HIV-infected patients are infected *in vivo*. Finally, uninfected thymocytes from HIV-infected persons are primed for apoptotic death, suggesting that indirect mechanisms of defective thymopoiesis are operative as well (290).

APOPTOSIS

Aberrant intracellular signals transduced by HIV may prime $CD4^+$ T cells for apoptosis, resulting in depletion of these cells during the course of HIV infection. Acute infection of T cells with HIV *in vitro* can induce apoptosis (291), and T cells from HIV-infected patients undergo enhanced rates of apoptosis *in vitro* compared with normal T cells, particularly following activation (292,293). Cross-linking of CD4 followed by ligation of the T-cell receptor is sufficient to induce apoptosis, suggesting that uninfected $CD4^+$ T cells can be depleted inappropriately on encountering antigen if CD4 had been cross-linked by gp120 (252).

Both Fas-dependent and Fas-independent pathways of apoptosis may be triggered by HIV. HIV-mediated Fas-independent apoptosis of $CD4^+$ T cells was shown *in vitro* by several groups (294,295); enhanced susceptibility of $CD4^+$ T cells to Fas-dependent apoptosis has been reported as well. Mechanisms that may contribute to this susceptibility include upregulation of Fas and Fas ligand (296,297); upregulation and activation of caspase-1 (298), caspase-3 (257), and caspase-8 (299); activation of cyclin-dependent kinases (300); and downregulation of the anti-apoptotic Bcl-2 protein (301). Viral gene products that have been associated with enhanced susceptibility to apoptosis include the viral envelope (252,296,302), Nef (52,53), Tat (296), and Vpu (303).

The susceptibility of uninfected “bystander” cells to apoptosis is a possible mechanism by which a large number of cells may be eliminated during HIV infection. In fact, studies in lymphoid tissue from HIV-infected persons suggest that most apoptosis occurs in uninfected bystander cells (304,305). Macrophages are efficient mediators of $CD4^+$ T-cell bystander apoptosis. HIV-induced upregulation of Fas ligand expression on macrophages and enhanced secretion of TNF-a from these cells appear to be responsible in part for this phenomenon (306,307). Another mechanism of bystander apoptosis may involve upregulation of CD62L on lymphocytes by HIV (308). These HIV-exposed lymphocytes home to lymph nodes and undergo apoptosis on signaling through homing receptors such as CD62L, CD44, and CD11a (309).

It remains uncertain whether HIV-induced apoptosis plays an important role *in vivo* in $CD4^+$ T-cell depletion. The frequency of apoptotic $CD4^+$ and $CD8^+$ T cells as well as B cells is significantly higher in lymphoid tissue from HIV-infected persons compared with uninfected controls (304,310). The relationship between the frequency of apoptotic cells and the stage of HIV infection remains controversial. Some data indicate a positive correlation between the stage of HIV disease and susceptibility of peripheral blood T cells to apoptosis (311), and another study demonstrated low frequencies of apoptotic cells in HIV-infected long-term nonprogressors (312). Other studies, however, found no correlation between apoptosis and stage of disease (302,304). Compelling evidence that apoptosis may play a role in HIV pathogenesis comes from animal models wherein an increased frequency of apoptosis in $CD4^+$ T cells is seen in primates infected with pathogenic strains of SIV but not in primates infected with nonpathogenic strains of SIV (313).

CLINICAL FEATURES

Studies prior to the advent of HAART revealed that the median time between primary HIV infection and the development of AIDS was approximately 10 years (314). In the acute and early stages of the disease, symptoms resulting from immune hyperactivation predominate; however, with the inexorable depletion of $CD4^+$ T cells that characterizes disease progression, overt cellular immunodeficiency and its many complications develop (Fig. 74.5). The 1993 CDC classification of HIV infection consists of clinical categories and quantitative $CD4^+$ T-cell categories (Table 74.1) (315).

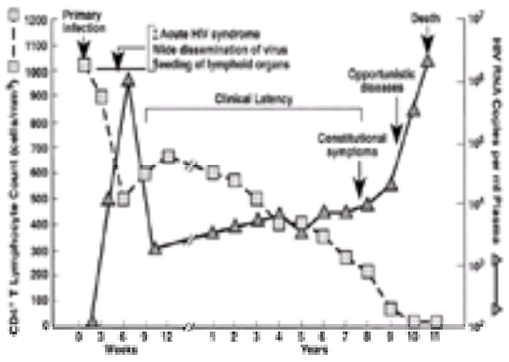

Figure 74.5. Natural history of human immunodeficiency virus (HIV) infection. High levels of viremia during acute infection often are associated with a sharp decline in $CD4^+$ T-cell numbers in the peripheral blood. Emergence of HIV-specific immune responses results in downregulation of viremia and partial recovery of the $CD4^+$ T-cell count. A long period of clinical latency ensues during which levels of plasma viremia may remain relatively low, and the $CD4^+$ T-cell count declines slowly. As the $CD4^+$ T-cell count falls below 200 cells per microliter, the risk for opportunistic diseases increases. In the late stages of disease, sharp increases in viremia and sharp declines in $CD4^+$ T-cell counts occur. (Adapted from Fauci AS, Pantaleo G, Stanley S, et al. Immunopathogenic mechanisms of HIV infection. *Ann Intern Med* 1996;124:654, with permission.)

CD4 Cell Categories	Clinical Categories* A: Asymptomatic, Lymphadenopathy, or Acute Infection	B: Symptomatic,* Not Category A or C	C: Clinical AIDS*
>500/μL (>29%)	A1	B1	C1
200–499/μL (14–28%)	A2	B2	C2
<200/μL (<14%)	A3	B3	C3

AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency.
* Categories A3, B3, and C1-3 are defined as AIDS.
† Examples of these symptoms include bacillary angiomatosis; thrush; vulvovaginal candidiasis that is persistent, frequent, or poorly responsive to therapy; cervical dysplasia or carcinoma in situ; constitutional symptoms such as fever or diarrhea of greater than 1-month duration; oral hairy leukoplakia; multidermatomal or recurrent herpes zoster; immune thrombocytopenic purpura; listeriosis; pelvic inflammatory disease; and peripheral neuropathy.
‡ Candidiasis of the esophagus or respiratory tract; invasive cervical cancer; extrapulmonary coccidioidomycosis; extrapulmonary cryptococcosis; cryptosporidiosis; enteropathy; cytomegalovirus infection; herpes simplex: with mucocutaneous ulcer longer than 1-month duration, or bronchitis, pneumonitis, or esophagitis; extrapulmonary histoplasmosis; HIV-associated dementia; wasting syndrome; isosporiasis; Kaposi sarcoma; central nervous system lymphoma; non-Hodgkin's lymphoma; pulmonary tuberculosis; disseminated *M. tuberculosis*, *M. avium*, or *M. kansasii* infection; nocardiosis; *Pneumocystis carinii* pneumonia; recurrent bacterial pneumonia; progressive multifocal leukoencephalopathy; recurrent *Salmonella septicemia*; extrapulmonary blastomycosis; toxoplasmosis.

TABLE 74.1. AIDS Surveillance Case Definition for Adults and Adolescents

DIAGNOSIS AND LABORATORY MONITORING

Serologic tests for antibodies to HIV form the mainstay of laboratory diagnosis of HIV infection. A solid-phase enzyme immunoassay (EIA) containing most HIV antigens has a sensitivity of greater than 99.5% for the detection of antibodies to HIV. Although the EIA is extremely sensitive, false-positives are not uncommon in a number of underlying conditions; therefore, a positive EIA test must always be followed by a confirmatory test. A western blot assay is the most reliable confirmatory serologic test for the diagnosis of HIV infection. A negative western blot demonstrates the absence of reactivity against HIV components. A positive test demonstrates reactivity to at least two of the following HIV proteins: p24, gp41, gp120, and gp160 (316). Indeterminate tests may be the result of reactivity to antigens that cross-react with HIV determinants; however, indeterminate results may also be a reflection of recently acquired infection and an evolving antibody response. Therefore, clinical follow-up and repeat serologic testing are essential in these cases.

The ideal laboratory marker of HIV disease activity should be easily and reproducibly measurable in all persons who have the disease. Furthermore, it should worsen with progression of disease and improve with positive responses to therapy. Although no ideal marker currently exists, several have been of critical importance in the clinical management of patients, especially with regard to establishing standards of care for initiation of antiretroviral therapy and prophylaxis against opportunistic infections.

Markers of Immune Depletion and Immune Dysregulation

Because $CD4^+$ T cells are the primary targets of HIV infection and because depletion of $CD4^+$ T cells is the immunologic hallmark of HIV disease progression, measurement of $CD4^+$ T cells should be an excellent marker of HIV infection. Indeed, studies of the natural history of HIV infection demonstrated that the $CD4^+$ T-cell count is a powerful predictor of the short-term risk of developing an AIDS-defining illness (317,318). $CD4^+$ T-cell counts frequently increase in response to antiretroviral therapy, and this salutary response has been used as a criterion in the licensing of new anti-HIV drugs.

Markers of generalized immune activation such as serum levels of b_2-microglobulin and neopterin are valid markers of HIV disease progression (319) but are now largely of historical interest only. Other immunologic markers of disease activity include levels of soluble TNF receptors (320), proliferative responses of T cells to anti-CD3 antibodies *in vitro* (321), delayed-type hypersensitivity response to recall antigens *in vivo* (322), and the percentage of peripheral blood $CD8^+$ T cells bearing the activation marker CD38 (323).

Markers of Viral Load

Before the development of currently available sensitive molecular techniques for the quantitation of HIV RNA molecules, direct culture of HIV from plasma or mononuclear cells was found to correlate with stage of disease (324,325). Changes in levels of culturable plasma viremia further serve as reliable indicators of activity of antiretroviral agents (326); however, these assays are extremely labor intensive and less sensitive than currently available molecular diagnostic tests. Measurement of p24 antigenemia is a relatively insensitive assay; however, positivity of a p24 antigen test does herald accelerated decline in $CD4^+$ T-cell counts and the likelihood of progression to AIDS (327).

The frequency of HIV-infected cells can be quantified by using polymerase chain reaction (PCR) for the detection of HIV-1 DNA, and this measurement has some predictive value with regard to HIV disease progression (262). Measurement of cell-associated HIV mRNA in peripheral blood, although technically demanding, has been shown by several groups to serve as an independent predictor of disease progression. Particularly impressive is the ability of this marker to predict disease progression in patients with early stage HIV disease and relatively high $CD4^+$ T-cell counts (328). Determination of the splicing pattern of HIV mRNA in PBMCs can add further predictive value with regard to disease progression. In this regard, a disproportionate increase in unspliced HIV mRNA is most predictive of accelerated loss of $CD4^+$ T cells and clinical disease progression (329). Furthermore, decreases in levels of unspliced HIV mRNA in lymphoid tissue correlate well with decreases in plasma viremia during antiretroviral therapy (100). This finding, combined with the fact that HIV mRNAs are orders of magnitude more abundant in lymph node mononuclear cells compared with peripheral blood mononuclear cells, supports the concept that changes in plasma viremia during antiretroviral therapy are reflective of changes in cell-associated viral mRNA production in lymphoid tissue (100).

The ability to assess the level of viral replication with quantitative assays of HIV RNA in plasma (i.e. plasma viremia) has revolutionized the use of laboratory markers for HIV disease. Measurement of the $CD4^+$ T-cell count yields information regarding the degree of immunodeficiency and short-term risk of opportunistic disease (i.e., a reflection of the damage already sustained by the immune system). In contrast, quantitation of HIV RNA in plasma yields information that predicts the rate and severity of immune deficiency. Technology currently available for reproducible measurement of plasma viremia in clinical specimens includes PCR, nucleic acid sequence based amplification (NASBA), and branched DNA (b-DNA) assays. PCR and NASBA are both cycle-based target amplification systems, whereas b-DNA is a

single-step signal amplification system that uses multiple HIV-specific nucleic acid target probes, each of which can be bound to an amplifier molecule containing multiple oligonucleotides. Each oligonucleotide in this array in turn can hybridize to an enzyme-labeled complementary probe, and detection is accomplished by addition of substrate. Each of these assays (PCR, NASBA, and bDNA) is able to detect as few as approximately 20 to 50 molecules of HIV RNA per milliliter of plasma, and each has a dynamic range of several orders of magnitude.

The enhanced sensitivity of molecular amplification assays compared with conventional viral detection techniques and the application of these assays to lymphoid tissue led to the observation that substantial levels of viral replication occur, even during the early, asymptomatic stages of HIV infection (87,330,331). The predictive value of plasma viremia as a valid, independent marker for disease progression was demonstrated in the Multicenter AIDS Cohort Study, where subjects were stratified by quartiles according to baseline plasma viremia levels (Fig. 74.6). The proportion of patients who progressed to AIDS within 5 years after entry into the study with plasma viremia levels in the lowest through highest quartiles was 8%, 26%, 49%, and 62%, respectively (332). Further validation of the use of plasma viremia measurement as a predictor of disease progression was provided by the AIDS Clinical Trials Group, which found that baseline measurements of plasma viremia as well as changes in viremia over time were predictive of disease progression (333,334). The combination of $CD4^+$ T-cell counts with measurements of plasma viremia has the greatest predictive power for HIV disease progression. In this regard, the likelihood of developing AIDS within 3 years given a plasma HIV RNA level of more than 30,000 copies per microliter is 33% with a $CD4^+$ T-cell count of more than 500 cells per microliter, 43% with a CD4+ T-cell count of 351 to 500 cells per microliter, 64% with a count of 201 to 350 cells per microliter, and 86% with a count of fewer than 200 cells per microliter (335). Given a $CD4^+$ T-cell count of 351 to 500 cells per microliter, the likelihood of developing AIDS within 3 years increases from virtually nil with a plasma viral load of fewer than 3,000 copies per milliliter, to 8% with a viral load of 3,000 to 10,000 copies per milliliter, to 16% with a viral load of 10,000 to 30,000 copies per milliliter, and to 43% with a viral load more than 30,000 copies per milliliter (335).

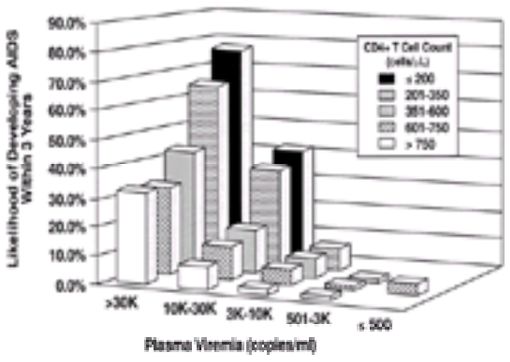

Figure 74.6. Likelihood of developing AIDS within 3 years, given the indicated levels of plasma viremia and peripheral blood $CD4^+$ T-cell counts.

The impressive decreases in plasma viremia during combination antiretroviral therapy reported by many groups suggested that this measurement might serve as a valid marker not only for predicting progression but also for monitoring therapy in HIV-infected persons. The link between decreased plasma viremia in patients receiving therapy and improved clinical outcome was established by the Veterans Affairs Cooperative Study Group on AIDS (336). Finally, data indicating that decreases in plasma viremia during therapy are reflective of decreases in viral replication in lymphoid tissue further validate the use of plasma viremia as a laboratory marker of a critical aspect of HIV pathogenesis (100).

Levels of plasma viremia become predictive of disease progression after stabilization of viremia (i.e., achievement of a viral load "set point") in the months following primary HIV infection. Mellors and co-workers found that the level of plasma viremia at 6 months following seroconversion was significantly higher in patients who developed AIDS within approximately 5 years of follow-up (74,000 HIV RNA copies/mL) compared with levels in those who did not develop AIDS during the same period (19,000 HIV RNA copies/mL) (337). A plasma viremia level of greater than100,000 HIV RNA copies per milliliter at 6 months following seroconversion was associated with an odds ratio of 10.8 for the development of AIDS. Schacker and co-workers found that levels of plasma viremia obtained 0 to 4 months after seroconversion were highly variable and did not correlate with subsequent clinical outcome; however, the level of plasma viremia at 4 to 12 months after seroconversion was a strong predictor of $CD4^+$ T-cell depletion during follow-up (338).

The development of sensitive, quantitative assays for plasma viremia and the ability of this marker to predict progression of disease and to serve as an indicator of therapeutic efficacy have added an important dimension to decisions regarding initiation and maintenance of antiretroviral therapy in HIV-infected patients. The variation in plasma viremia by several orders of magnitude in patients with early stage HIV disease with similar $CD4^+$ T-cell counts allows for the initiation of early therapeutic intervention in those who are most likely to have disease progression. Current recommendations regarding the initiation and maintenance of antiretroviral therapy rely heavily on the two best laboratory markers for HIV disease progression: the $CD4^+$ T-cell count and the level of plasma viremia (339,340).

Acute Infection

Although more than half of primary HIV infections (and perhaps up to 90%) are accompanied by symptoms, most of these infections are not recognized in the acute setting (341). The differential diagnosis of primary HIV infection is extremely broad given the variable presentations associated with the syndrome. Fever, rash, pharyngitis, and lymphadenopathy are common during primary HIV infection, as they are in many "flulike" and "mononucleosis-like" illnesses (342). Myalgias, arthralgias, diarrhea, nausea, vomiting, headache, hepatosplenomegaly, weight loss, thrush, and neurologic symptoms also may occur. Among patients who develop symptoms during primary HIV infection, the mean duration of symptoms is 3 weeks (343).

Plasma HIV RNA levels vary widely in the setting of primary HIV infection, from thousands to millions of copies per milliliter (338). After reaching a peak, plasma HIV RNA levels decline by about 6.5% per week over a 4-month period (338). A steep decline of 5.2 cells per microliter per week occurs in the peripheral blood $CD4^+$ T-cell count over the 5 months following primary infection (338). The peak level of HIV RNA may have prognostic value (337), and persistently high levels after 4 to 6 months add to the predictive power toward more rapid progression of disease (337,338).

Early Stage Disease

The natural history of HIV infection includes a long period (i.e., about 10 years) of clinical latency between the time of primary infection and the development of symptoms indicative of advanced immunodeficiency. Even during the period of clinical latency, many patients experience fatigue as well as generalized lymphadenopathy. Syndromes indicative of depressed cell-mediated immunity in HIV infection, which are not AIDS defining, generally begin to appear as the $CD4^+$ T-cell count falls below 500 cells per microliter. Examples include oropharyngeal and recurrent vulvovaginal candidiasis, bacillary angiomatosis (usually due to infection with *Bartonella henselae*), recurrent or multidermatomal herpes zoster, listeriosis, infections due to *Rhodococccus equi*, pelvic inflammatory disease, oral hairy leukoplakia associated with Epstein–Barr virus (EBV), cervical dysplasia (usually associated with human papillomavirus infection), constitutional symptoms such as unexplained fever or diarrhea lasting more than 1 month, idiopathic thrombocytopenic purpura, and peripheral neuropathy (315).

Late-stage Disease

As the $CD4^+$ T-cell count falls below the level of approximately 200 cells per microliter, the loss of integrity of cell-mediated immune responses allows ubiquitous environmental organisms with limited virulence (e.g., *Pneumocystis carinii* and *Mycobacterium avium*) to become life-threatening pathogens. Conditions indicative of severely depressed cell-mediated immunity from HIV infection constitute the CDC surveillance case definition of AIDS. These conditions include fungal infections (e.g., esophageal or pulmonary candidiasis, extrapulmonary cryptococcosis, histoplasmosis, or coccidioidomycosis, and pulmonary or extrapulmonary *Pneumocystis carinii* infection), viral infections (e.g., cytomegalovirus retinitis, esophagitis, pneumonia, myelitis, pancreatitis, or adrenalitis; and herpes simplex virus bronchitis, pneumonia, esophagitis, or chronic skin ulcers), bacterial and mycobacterial infections (e.g., pulmonary tuberculosis, extrapulmonary infection from any mycobacterium, recurrent bacterial pneumonia, and recurrent *Salmonella* septicemia), protozoal infections (e.g., gastrointestinal syndromes from infection with *Cryptosporidium* or *Isospora*; and toxoplasmic encephalitis), neoplasia (e.g., invasive cervical cancer; Burkitt, immunoblastic, or primary central nervous system lymphoma; and Kaposi sarcoma), as well as HIV-related encephalopathy, progressive multifocal leukoencephalopathy (due to reactivation of JC virus), and wasting syndrome (315). HIV-associated diseases indicative of severe impairment of cell-mediated immunity but not included in the current case definition of AIDS include chronic microsporidiosis, gastrointestinal infection with *Cyclospora cayetanensis*, disseminated *Penicillium marneffei* infection (endemic to Southeast Asia), cerebral or disseminated *Trypanosoma cruzi* infection (endemic to Latin America), relapsing or chronic visceral leishmaniasis, anal carcinoma, and EBV-positive cases of leiosarcoma, leiomyosarcoma, and Hodgkin disease (344).

The degree to which an infection or reactivation syndrome can be considered opportunistic can be inferred by the $CD4^+$ T-cell counts at which it occurs (318). Oral candidiasis and tuberculosis tend to occur as the $CD4^+$ T-cell count falls into the 250 to 500 cells per microliter range. Cryptosporidiosis generally does not occur until the $CD4^+$ T-cell count is fewer than 200 cells per microliter. *Pneumocystis carinii* pneumonia, disseminated *Mycobacterium avium* complex infection, cryptococcosis, and toxoplasmosis are indicative of more severe immunodeficiency. At the more extreme end of the opportunistic disease spectrum, cytomegalovirus (CMV) retinitis is usually diagnosed after the $CD4^+$ T cell count has fallen below 50 cells per microliter. The steep increase in the risk of developing AIDS-defining illnesses associated with a $CD4^+$ T-cell count of fewer than 200 cells per microliter led to the 1993 revision of the CDC definition of AIDS, which includes a low $CD4^+$ T-cell count (<200 cells/µL) as an AIDS-defining criterion (315).

The incidence of AIDS-defining illnesses in industrialized nations has decreased sharply since 1996. In the United States, the AIDS mortality rate decreased by 47% between 1996 and 1997 and 20% between 1997 and 1998 (17,345). These decreases in AIDS-associated mortality can be attributed to a number of factors, including continued progress in prophylaxis against opportunistic infections (Table 74.2) (346), improved access to health care, and improved antiretroviral therapies (see later).

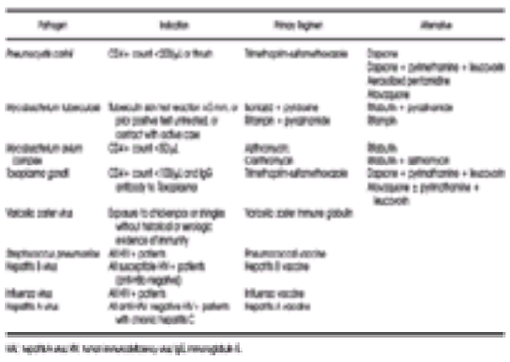

TABLE 74.2. Prophylaxis Recommendations to Prevent Opportunistic Diseases

Determinants of the Rate of Disease Progression

Myriad host and virologic factors determine the extremely variable rates of disease progression that are observed among HIV-infected persons. A small percentage of HIV-infected persons experience no evidence of disease progression over a prolonged period; these long-term nonprogressors provide clues regarding factors associated with disease progression. Although most cases of long-term nonprogressive HIV infection may be associated with attenuated strains of HIV (347,348), most data suggest that viral attenuation is rare among long-term nonprogressors and that host factors play a dominant role in determining the state of nonprogression (139,349,350 and 351).

Host genetic factors strongly influence the rate of disease progression in HIV infection. Numerous different mechanisms may be responsible for the observed associations between certain HLA haplotypes and different rates of HIV disease progression (reviewed in 148). The ability of certain HLA molecules to present immunodominant viral epitopes effectively to generate cell-mediated immune responses may explain an association with slow disease progression. Conversely, other HLA molecules may promote immunopathogenic responses associated with more rapid disease progression.

Mutations in the *CCR5* gene have a large impact on the ability of HIV to be transmitted and on HIV disease progression in HIV-infected persons. A 32-base pair (bp) deletion in the *CCR5* gene (*CCR5-D32*) results in the production of a truncated version of the receptor that is not expressed at the cell surface and therefore does not serve as a functional coreceptor for HIV infection (352). Molecular epidemiologic studies in HIV-uninfected populations have found that about 20% of white persons of northern and western European background are heterozygous for this mutation and 1% are homozygous; the frequency of the *CCR5-D32* allele appears to be exceedingly rare in Asian and African populations (353,354). Among HIV-infected white persons, no subjects homozygous for *CCR5-D32* were initially identified, suggesting complete protection from infection; however, rare cases of infection of homozygous individuals subsequently have been reported, likely reflecting transmission of non-R5 strains of HIV. Although *CCR5-D32* heterozygotes are not protected against HIV infection, they are afforded a modest degree of protection against disease progression and are overrepresented among cohorts of HIV-infected long-term nonprogressors (354,355,356,357 and 358). The mechanism of delayed disease progression in *CCR5-D32* heterozygotes may involve a lower viral load "set point" following acute HIV infection and a slower rate of $CD4^+$ T-cell depletion (355). These effects may be due to a decrease in expression of CCR5 on $CD4^+$ T cells from *CCR5-D32* heterozygotes (359,360), allowing less efficient spread of HIV infection to new target cells (361); however, it is clear that *CCR5-D32* is not the sole determinant of delayed progression of HIV disease (358).

Mutations in chemokine receptor and chemokine genes other than *CCR5-D32* also can confer protection against HIV infection or partial protection against disease progression. A rare point mutation in the *CCR5* gene (m303) results in a premature stop codon, preventing the expression of a functional coreceptor; the *CCR5D32/m303* genotype confers a high degree of resistance to HIV infection, similar to that afforded by homozygosity for *CCR5-D32* (362). Polymorphisms in the CCR5 promoter also influence rates of disease progression (363,364,365 and 366). Polymorphisms in the *CCR5* gene can be grouped into a number of haplogroups based on an evolutionary-based classification system; interestingly, several of these haplogroups have race-specific effects on HIV disease progression (365,366). A mutation in the *CCR2* gene involving a conservative amino acid change in a transmembrane domain of the receptor results in a significant delay in disease progression among HIV-infected persons (367,368 and 369); this effect may be related to the ability of the mutant form of the CCR2 protein to form heterodimers with CXCR4 and CCR5, thus limiting the functional availability of these important HIV coreceptors (370).

A polymorphism in the RANTES promoter is associated with enhanced transcription of the RANTES gene; not surprisingly, this polymorphism is also associated with a slower rate of HIV disease progression (371). A mutation in the 3' untranslated region of the *SDF-1* gene, *SDF1-3'A*, also plays a role in protecting against disease progression in HIV-infected persons (372). Homozygosity for the *SDF1-3'A* allele may be associated with high levels of expression of SDF-1, which may occupy CXCR4 and thereby inhibit replication of X4 strains of HIV. Consistent with this hypothesis, the protective effect of the *SDF-1* mutation is observed late in the course of HIV disease, when T-tropic strains may predominate. Downregulation of coreceptor expression or upregulation of chemokines also may recapitulate phenotypes similar to those created by the genetic polymorphisms described previously (22,175,176,177 and 178,360).

Qualitative and quantitative features of the host immune response that is mobilized against HIV are important determinants of the rate of disease progression as well. In this regard, studies of long-term nonprogressors suggest important roles for CTL, $CD8^+$ T-cell suppressor factors, $CD4^+$ T-helper responses, ADCC, and neutralizing antibody responses in protecting against HIV disease progression (see Immune Responses).

UNIQUE ASPECTS OF PEDIATRIC HIV DISEASE

Since the implementation of routine screening for HIV infection in blood products, pediatric HIV infection has been transmitted through two primary routes: perinatal transmission and sexual transmission to adolescents. Each mechanism establishes a unique clinical situation, with challenges in treatment and understanding pathogenesis.

Perinatal HIV disease transmission is particularly important. If no antiretroviral prophylaxis is used in a country with little breast-feeding, 20% to 30% of infants born to HIV-infected women will be infected. Of those infections, 30% to 50% occur *in utero*, whereas the remainder are thought to occur at the time of delivery (peripartum). If the mother breast-feeds, an additional 10% to 15% of children may become infected. Antiretroviral therapy can be effective at preventing transmission. Vertical transmission rates as low as 2% to 3% have been reported using combination antiretroviral therapy of the mother and RT inhibitors in the child. Cesarean section may also reduce transmission rates, particularly if optimal antiretroviral therapy is not possible. Breast-feeding substantially diminishes the positive benefits of perinatal prophylaxis.

Once HIV is established in an infant, virus replication rapidly achieves very high levels. HIV RNA concentrations of a million or more virions per milliliter are typical. This high level of replication probably reflects a combination of an immature neonatal immune system that responds poorly to HIV and the large number of $CD4^+$ lymphocytes found in infants and thus available to the virus. Newly infected adults also have a burst of early, high-level replication. Adults control the virus within a few months, whereas most infected infants continue to have high viral loads for years.

Infants tend to sort into two categories: rapid and slow progressors. Patients in the rapid progressor cohort develop early opportunistic infections, particularly *P. carinii* pneumonia and *Candida* infections, encephalopathy, wasting, and recurrent invasive bacterial infections. Children who go through the first year of life with no serious

events and their $CD4^+$ lymphocyte count intact may live for years with no problems. Their progression appears much like the adult disease, with a typically slow onset of symptoms. Unfortunately, there is no good way to predict early in life which children will have rapid or slow progression. Both groups have high viral loads, and although the rapid progressor group on average has a higher level of HIV replications, there is enough overlap between the cohorts that HIV viral RNA concentrations are not prognostically useful as they are for adults. One unique aspect of HIV disease in children is its effect on growth. Children have symmetric delays in weight and height growth so that they appear “normal” (correct weight for height) but younger than their calendar age.

Antiretroviral therapy in children is challenging for several reasons. Infants do not take any medicines easily, and many parents of HIV-infected children have difficulty remembering to give the medicines. Many of the medications are not available in liquid preparations that can be titrated to the infant or child's size, and some of those that are available as liquids have unpleasant tastes. Children may have unique pharmacokinetics that do not mimic the adult situation as a result of enzymatic immaturity, lack of gastric acidity, or other problems. Finally, the high viral load found in young children may mean resistance to the antiretroviral drugs can develop relatively quickly. In addition, some infants may inherit virus quasispecies with high levels of resistance from their mothers, particularly if their mothers have had prolonged previous antiretroviral therapy.

The issues in adolescence are somewhat more straightforward. Many infected adolescents acquired their HIV sexually; so the infection when found is typically proximate to the time of infection. The virus may be less genetically diverse, the reservoirs of infection less established. These potential therapeutic advantages are counterbalanced by the fact most adolescents are not compliant patients. Many do not take their medications regularly, they miss appointments, and they disregard medical authority figures. Peer interactions often mean more than communications from adults. Therefore, there is an important social overlay to the management of HIV in teens.

ANTIRETROVIRAL THERAPY

Recent progress in understanding the pathogenesis of HIV disease, combined with the development of potent antiretroviral agents, has resulted in an abundance of treatment options for HIV-infected patients. The recognition that virus continuously replicates in lymphoid tissue throughout the course of HIV infection, even during the clinically latent stage, strengthened the rationale for early intervention (87,88). Quantitative estimates of the extraordinary rate of viral replication *in vivo* further strengthened the rationale for early therapy with combination antiretroviral regimens (373,374 and 375). Combinations of antiretroviral agents that are capable of profoundly suppressing levels of plasma viral load for prolonged periods are referred to as *highly active antiretroviral therapy*, or HAART. Significant declines in AIDS-related morbidity and mortality rates are due in large measure to the wide use of HAART (14,15). Optimal use of antiretroviral agents, however, remains a rapidly evolving field, with many obstacles that need to be addressed.

Viral Replication Cycle and Antiretroviral Agents

Several steps in the viral replication cycle can be targeted by agents that are either currently available or under investigation (Fig. 74.7). Currently available antiretroviral drugs target either the viral RT or protease enzyme; however, investigational agents that target the entry and fusion step of the viral replication cycle, as well as agents that target the viral integrase, are in various stages of development. Another promising strategy targets the conformational changes in gp41 that are necessary for fusion of HIV with the target cell membrane (376).

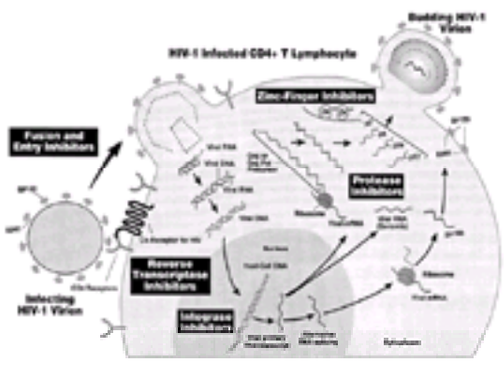

Figure 74.7. Targets of antiretroviral therapy. Drugs that are currently licensed for use in human immunodeficiency virus (HIV) infection include reverse transcriptase inhibitors and protease inhibitors. In development are inhibitors of HIV entry and fusion, inhibitors of HIV integrase, and zinc finger inhibitors.

Inhibitors of viral RT were the first agents to be developed for the treatment of HIV infection; they remain important components of the anti-HIV therapeutic armamentarium. Most RT inhibitors are nucleoside analogs; these agents act as DNA chain terminators by virtue of their structures that lack the 3' hydroxyl moiety necessary for chain elongation. Nonnucleoside RT inhibitors are also used clinically. RT inhibitors as a group suffer from several disadvantages, including their association with multiple neurologic and hematologic side effects, the rapidity with which they select for resistant viral variants when used alone, and their lack of effect on cells already infected with HIV, which no longer require RT to complete the viral replication cycle.

The HIV-1 protease cleaves the Gag precursor polyprotein into the p24 and p17 virion components necessary for viral infectivity. Development of HIV-1 protease inhibitors based on the crystal structure of the protease was a triumph for rational and targeted drug design (377,378). The combination of agents that can inhibit *de novo* infection of cells (i.e., RT inhibitors) with agents that lead to the release of defective, noninfectious virions from cells already infected by HIV (i.e., protease inhibitors) results in potent antiviral activity. Protease inhibitors, in combination with RT inhibitors, have rapidly become the drugs of choice for the treatment of HIV infection.

Integration of reverse-transcribed viral DNA (provirus) into the host DNA is facilitated by the viral enzyme integrase. The determination of the crystal structure of integrase (379) has stimulated a search for inhibitors of this enzyme, which hopefully will lead to the availability of clinically useful agents.

Targeting the “accessory” proteins of HIV has been hampered by the difficulty in delineating the precise biochemical activities of these proteins. A clinical trial of the Tat antagonist Ro24-7429 failed to demonstrate antiviral activity for this agent (380); however, agents targeting the HIV-1 Rev and Nef proteins are being developed.

Delineation of the role of chemokine receptors as HIV entry cofactors and of the role of the chemokine ligands of these receptors as potential inhibitors of HIV cellular entry led to the development of new therapeutic strategies (381). Chemokine analogs that bind to HIV coreceptors and prevent entry of virus without transducing an intracellular signal represent an attractive class of compounds because they are unlikely to elicit inappropriate inflammatory signals. Such a strategy is highlighted by molecules that bind to CCR5 and block infection of cells with R5 strains of HIV without delivering intracellular signals (reviewed in 382). Several small molecules (AMD3100, a bicyclam; T22, a peptide derivative of polyphemusin II; and ALX40-4C, a highly cationic oligopeptide) that compete with SDF-1 for binding to CXCR4 have also been developed. These compounds bind specifically to CXCR4 and inhibit cellular entry of X4 strains of HIV; they also inhibit SDF-1-mediated intracellular calcium mobilization (reviewed in 382). Finally, molecular genetic approaches have been taken to prevent cell surface expression of HIV coreceptors, thus recapitulating the *CCR5D32* phenotype (383,384).

Another therapeutic strategy that inhibits HIV fusion and entry is targeted at the requisite conformational changes within the HIV envelope glycoproteins (385). After interaction with CD4, dramatic conformational changes occur in both the gp120 and gp41 envelope components. Within gp41, a central trimeric coiled coil structure is formed; this structure is essential to the process of fusion with the cell memebrane, and represents an excellent therapeutic target (386,387). In fact, a peptide, T20, which prevents the assembly of the coiled coil structure, has shown promise as a clinically useful antiretroviral agent (376).

Treatment of Primary HIV Infection

Treatment of HIV infection before its wide dissemination to lymphoid tissue and hence prior to establishment of the viral load “set point” is an approach supported by current understanding of the pathogenesis of early events in HIV infection (388). Unfortunately, the pool of resting, latently infected $CD4^+$ T cells appears to be established during the earliest stages of acute infection; this process is not abrogated even by initiation of therapy during the symptomatic phase of acute infection (389). Although these data militate strongly against the possibility of eradicating HIV before its wide dissemination during primary infection, the window of opportunity for treatment of primary HIV infection actually may extend to 12 to 18 months following acute infection; this possibility is based on the persistence of high numbers of productively infected cells in lymphoid tissue during this period, despite attainment of the apparent set point of plasma viremia (84).

A trial of zidovudine therapy versus placebo given for 6 months following primary HIV infection revealed that patients assigned to active treatment maintained higher CD4+ T-cell counts, lower levels of plasma viremia, and slower progression to minor clinical endpoints (e.g., oral candidiasis, herpes zoster, and oral hairy leukoplakia) over a mean follow-up period of 15 months (390). Treatment of patients with recent (i.e., within 90 days) HIV infection with a combination of zidovudine, lamivudine, and a protease inhibitor can suppress plasma viremia to fewer than 20 HIV RNA copies per milliliter for a prolonged period (>2 years) (391); initiation of HAART during acute HIV infection may prevent the loss of HIV-specific $CD4^+$ T-cell proliferative responses (188).

Treatment of Chronic HIV Infection

Early studies of zidovudine monotherapy demonstrated improvements in laboratory markers of HIV disease activity and delayed progression of disease in both symptomatic patients and in asymptomatic persons with relatively low $CD4^+$ T-cell counts (reviewed in 392). Subsequent studies demonstrated that combination therapy with two nucleoside analogs was generally superior compared with monotherapy (393,394,395 and 396).

More potent combination antiretroviral drug regimens, such as zidovudine plus lamivudine, which are capable of prolonged suppression of viral replication, became available in 1995 (397). The addition of protease inhibitors to the antiretroviral armamentarium in 1996 resulted in a dramatic enhancement in the ability to suppress viral replication. The protease inhibitors saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir are all potent antiretrovirals, particularly used in combination with nucleoside analog RT inhibitors (NRTIs). Such combination therapy is now considered the treatment of choice for HIV infection (339). When used in combination with NRTIs, protease inhibitors typically suppress plasma viral load to undetectable levels in the majority of patients for more than 48 weeks. In addition, significant reductions in the rate of progression to AIDS or death have been demonstrated for indinavir and ritonavir (398,399).

The decision to initiate antiretroviral therapy should be made jointly by patient and physician. The decision should be driven in part by data regarding the natural history of disease progression at various levels of $CD4^+$ T-cell counts and plasma viral loads (332,335). Treatment is indicated for patients with symptomatic HIV disease regardless of $CD4^+$ T-cell count and viral load (339). Treatment for asymptomatic patients is generally indicated when the $CD4^+$ T-cell count is below 350 cells per microliter or the plasma viral load is greater than 30,000 copies per milliliter as determined by a bDNA assay (339); however, some physicians and patients choose to initiate antiretroviral therapy earlier or later than recommended in official guidelines. Risks and benefits to early initiation of antiretroviral therapy are presented in Table 74.3; however, unfortunately, no data are yet available regarding the effects of early initiation of HAART on delaying disease progression or improving survival.

Potential benefits
Control of viral replication and mutation; reduction of viral burden
Prevention of progressive immunodeficiency; potential maintenance or re- construction of a normal immune system
Delayed progression to AIDS and prolongation of life
Decreased risk of selection of resistant virus
Decreased risk of drug toxicity
Possible decreased risk of viral transmission
Potential risks
Reduction in quality of life from adverse drug effects and inconvenience of current maximally suppressive regimens
Earlier development of drug resistance
Transmission of drug-resistant virus
Limitation in future choices of antiretroviral agents due to development of resistance
Unknown long-term toxicity of antiretroviral drugs
Unknown duration of effectiveness of current antiretroviral therapies

AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus.

TABLE 74.3. Risks and Benefits of Early Initiation of Antiretroviral Therapy in the Asymptomatic HIV-Infected Patient

Many treatment options now exist for the HIV-infected patient. A regimen should be selected that will afford a high likelihood of long-term profound suppression of plasma viral load and a significant increase in the CD4+ T-cell count. Many other factors deserve consideration, including the pill burden associated with a particular regimen, dosing frequency, food requirements, convenience, toxicity, and drug interaction profile. Recommendations regarding specific regimens can be found in Table 74.4 and in the Department of Health and Human Services/Henry J. Kaiser Foundation Guidelines for the Use of Antiretroviral Agents in HIV-Infected Adults and Adolescents on the worldwide web at *www.hivatis.org*.

TABLE 74.4. Recommended Antiretroviral Agents for the Treatment of Established HIV Infection

A change in the antiretroviral regimen may be indicated when a suboptimal reduction in viremia occurs after initiation of therapy, when viremia reappears after suppression to undetectable levels or increases from a nadir of suppression, or when the $CD4^+$ T-cell count declines significantly. When any of these events occur, it is important to determine whether the underlying cause may be drug intolerance or difficulty with adherence to the drug regimen, suboptimal drug levels due to malabsorption or drug–drug or drug–food interactions, or development of drug resistance. If drug resistance is suspected, it is appropriate to change the regimen entirely to drugs that have not been used previously and to which cross-resistance is not anticipated. More targeted changes within a drug regimen may be guided by the use of resistance assays (see later); however, this approach requires further clinical validation. If a regimen is changed because of intolerance or toxicity, it is appropriate to substitute one or more alternative drugs of the same potency and from the same class of agents as the agent suspected to be causing the problem.

Although sustained suppression of viral load is achievable in a high proportion of patients treated with HAART regimens in the clinical trials setting (i.e., nearly 80% at 2 years) (400), only 50% or fewer respond adequately in urban clinic settings (401,402). Predictors of success include lower baseline viral load, higher baseline $CD4^+$ T-cell count, initiation of therapy with multiple drugs simultaneously, and adherence to the drug regimen (400,401,402,403,404,405 and 406). Although the use of HAART regimens has greatly improved the prognosis for HIV-infected persons (14,15), a large number of challenges to the optimal treatment of HIV infection remain.

Adherence

Antiretroviral regimens are complex, consist of multiple agents, and must be taken indefinitely in most cases. Many of the drugs have significant side effects and toxicities (see later). The sheer number of pills required in many regimens (i.e. >20 pills per day) poses a significant challenge to adherence even for the most committed patients. In addition, many antiretroviral agents, particularly protease inhibitors, have complex dosing requirements in relation to food intake.

Lapses in adherence provide windows of opportunity for virus replication and enhance the probability of the emergence of drug-resistant virus. In the INCAS drug trial of nevirapine, zidovudine, and didanosine, missed doses of antiretroviral drugs on 28 or more days during the 52-week study significantly predicted a lower likelihood of achieving durable viral suppression (403). In a study of antiretroviral therapy in an urban clinic setting, missed clinic appointments were significantly associated with failure to suppress viral load over a 1-year period (402).

Side Effects and Toxicites

Gastrointestinal disturbances, such as nausea, vomiting, or diarrhea, are commonly induced by several of the NRTIs and by all of the protease inhibitors. Peripheral

neuropathy may be caused by most of the NRTIs, and rash and Stevens–Johnson syndrome may occur with the use of any of the nonnucleoside reverse transcriptase inhibitors (NNRTIs). Protease inhibitors have a number of drug specific toxicities, such as the association of nephrolithiasis and indirect hyperbilirubinemia with indinavir; nausea, vomiting, and diarrhea and paresthesias with ritonavir; nausea and diarrhea with saquinavir; and diarrhea with nelfinavir. In addition, several class-specific adverse effects are associated with the use of protease inhibitors. In this regard, numerous metabolic derangements involving lipid metabolism are of serious concern with the use of protease inhibitors. Triglyceride and cholesterol levels may become elevated to dangerously high levels. Insulin resistance, hyperglycemia, and even frank diabetes mellitus may occur. Additionally, peripheral fat wasting in the setting of central fat accumulation as well as “buffalo humps” reminiscent of Cushing disease may occur (407,408 and 409). These metabolic derangements may be related to homology between HIV protease and two proteins, cytoplasmic retinoic-acid binding protein type 1 and low-density lipoprotein (LDL) receptor–related protein, that regulate lipid metabolism (410). Finally, a large number of drug interactions may occur with the use of NNRTIs and protease inhibitors, all of which alter cytochrome P-450 metabolism.

Resistance

Failure or loss of suppression of plasma viremia in the setting of good adherence and adequate drug absorption may be due to the emergence of drug-resistant virus. A single nucleotide substitution at position 103 of the RT gene can significantly reduce the activity of the NNRTIs against HIV replication. Similarly, a single nucleotide substitution at position 184 of the RT gene confers resistance to lamivudine. Resistance to some of the NRTIs, such as zidovudine, follows a relatively orderly sequential path whereby accumulation of serial mutations in the RT gene confers increasing levels of resistance to the agent (411); for other drugs in this class, the pathways to resistance are less clear.

Resistance to protease inhibitors is a complex phenomenon that has important implications for drug therapy. The HIV protease is a 99 amino acid protein; that over half of these residues have been reported to be associated with drug resistance is testimony to the genetic plasticity of HIV. Some of these mutations are strongly selected under the pressure of protease inhibitor therapy; however, even when drugs select for unique mutations associated with drug resistance, a great deal of *in vitro* and clinical cross-resistance may still be observed between drugs (412,413). Other mutations are compensatory in nature and can partially restore replicative fitness that is lost with primary mutations. In fact, distant mutations in the protease cleavage site in the HIV p7/p1 core components create a more optimal substrate for a protease enzyme that is compromised by protease inhibitors (414).

Genotypic and phenotypic resistance assays are now available from commercial sources (415). Genotypic assays generally provide information about the presence of mutations in HIV genes (i.e., RT and protease) that may have been selected for during drug therapy. Phenotypic assays evaluate the replication of virus or of a viral construct containing genes from the patient's virus in the presence of antiretroviral drugs. Interpretation of these assays is fraught with difficulty (415). The tests are insensitive to minor viral variants that may be important *in vivo*. In this regard, viral variants harboring resistance mutations to drugs given in the past may be present at frequencies well below the limits of detectability using these assays. Thus, although a positive resistance assay argues strongly that a particular drug will be ineffective, a negative test by no means assures the drug's utility (415).

Preliminary data suggest that resistance testing can be used to optimize the design of antiretroviral regimens. In this regard, one study demonstrated that viral load suppression over a 3- to 6-month period was superior when genotypic resistance assays were used in helping to formulate antiretroviral regimens (416). Another study showed that phenotypic drug resistance may be detectable before viral load rebound becomes evident during drug treatment (417). These data suggest that drug resistance assays will likely be an important adjunct in the formulation of antiretroviral regimens in the future.

Persistent Viral Replication

The beginning of the HAART era saw a great deal of optimism that, with prolonged suppression of viral load, eradication of HIV infection might be possible. Chun et al., however, reported the presence of unintegrated HIV DNA in lymphocytes from HAART-treated patients who had experienced sustained suppression of plasma viral load for an average of 10 months; because of the short half-life of unintegrated viral DNA, this finding argued strongly that ongoing low-level HIV replication was occurring in spite of undetectable levels of plasma viral load (418). Support for this same conclusion came from studies showing persistence of HIV transcription in PBMCs from patients with sustained suppression of plasma HIV viral load (419,420). In addition, two other studies demonstrated evolution of proviral DNA in patients receiving HAART with undetectable viral loads, again indicating ongoing viral replication (421,422). Finally, the rapid rebound of plasma viral load that occurs after cessation of HAART following prolonged suppression of plasma viremia also likely indicates ongoing replication during HAART (423,424).

Persistent viral replication in the setting of apparently effective HAART may be due to intermittently suboptimal drug levels (425,426), poor penetration of drugs into various tissue compartments such as genital secretions (427,428), or suboptimal potency of the drug regimen. Clearly, more potent antiretroviral agents that are capable of inhibiting a broader array of HIV target gene products would be welcome additions to the antiretroviral armamentarium. In this regard, inhibitors of the viral entry and fusion process are in clinical trials (376), and inhibitors of the HIV integrase are in development.

Latent Reservoirs of HIV Infection

On initiation of HAART, plasma viral load declines in several phases (373,374 and 375). The first phase of rapid exponential decay reflects the short half-life of free virions and of recently infected virus-producing cells. After the first several weeks of therapy, a slower second phase of viral load decay occurs with a half-life of approximately 14 days. This phase of decay is thought to reflect attrition of longer-lived, chronically infected cells (429). Extrapolation of this decay suggested that treatment of HIV infection with a potent combination drug regimen that completely suppresses viral replication for approximately 3 years may eradicate infection (429). A practical limitation of this estimate is the assumption of complete suppression of viral replication, which is beyond the capability of currently available HAART regimens (418,419,420,421 and 422).

Another major limitation of the time-to-eradication estimate is the presence of a reservoir of latently infected resting $CD4^+$ memory T cells that persists during HAART. HIV may enter resting $CD4^+$ T cells, and a limited degree of reverse transcription of the viral genome may occur in these cells. This period of preintegration latency may last hours to days; in the absence of an activation signal, the viral nucleic acid loses its capacity to initiate a productive infection. If these cells become activated, however, reverse transcription may proceed to completion, followed by nuclear import, and by integration of proviral DNA into cellular DNA (232,233 and 234). In most $CD4^+$ T cells that become infected with HIV in the *in vitro* setting of a sufficient degree of activation, integration and productive infection occur, followed by cell death. A small fraction of productively infected $CD4^+$ T cells may escape cell death and revert to a resting state in which HIV infection is latent (i.e., postintegration latency). Chun and colleagues estimated the size of this latent reservoir of HIV *in vivo* by examining highly purified resting $CD4^+$ T cells from both blood and lymph nodes of HIV-infected persons. Using a PCR assay specific for integrated proviral DNA, they estimated that the frequency of latently infected resting $CD4^+$ T cells was approximately 0.05%. This frequency was quite similar in the peripheral blood and lymph node compartments (90,430), reflecting the fact that resting memory $CD4^+$ T cells may circulate continuously between various lymphoid tissue compartments. Subsequent micro-coculture analysis revealed that approximately one tenth of latently infected resting $CD4^+$ T cells harboring integrated HIV DNA carried replication-competent HIV (90).

Although the size of the pool of latently infected resting $CD4^+$ T cells *in vivo* harboring replication-competent HIV appears to be quite small, it may nevertheless constitute the most important obstacle to eradication of HIV infection. Latently infected cells are not subject to elimination by the host immune response because of the absence of expression of viral antigens on the cell surface. Furthermore, integrated proviral DNA cannot be targeted by currently available antiretroviral agents. Therefore, the natural half-life of latently infected resting $CD4^+$ T cells may be the rate-limiting step in eradicating HIV infection. These suppositions are substantiated by several independent studies that attempted to recover replication-competent HIV from cells of patients receiving HAART for prolonged periods. In these studies, virus was recovered from $CD4^+$ T cells in essentially all patients receiving HAART with levels of plasma viremia that were below the limit of detectability for up to 94 weeks (418,431,432). To estimate the half-life of this pool of latently infected resting memory $CD4^+$ T cells, Finzi and colleagues performed longitudinal analyses of the number of these cells harboring replication-competent HIV in 20 patients receiving HAART whose plasma viral load remained suppressed to fewer than 200 copies per milliliter over a mean of 14.4 months (433). The decay of the latent pool of $CD4^+$ T cells was extremely slow, with an estimated half-life of 44 months; given a conservative estimate of the size of the latent pool, this leads to an estimated time-to-eradication of 60 years in patients receiving HAART (433). It is possible, however, that the half-life of the latent pool was overestimated if transient low-level viral replications were reseeding the reservoir over the course of the study. As more potent HAART regimens become available, more accurate assessments of the decay of the reservoir of latently infected cells should be possible.

Strategies aimed at long-term control of HIV infection will need to focus on the pool of latently infected cells that represent a long-lived reservoir capable of “reigniting” viral replication during periods of suboptimal antiretroviral therapy. Antiretroviral regimens that are highly potent and more easily administered should be part of such a successful strategy; however, this approach could be combined with interventions, such as IL-2 therapy, aimed at activating latently infected cells in the setting of HAART as well as enhancing HIV-specific immune responses (235). In this situation, latently infected cells might be driven to a state of productive infection and die; infectious virus released from these cells would be unable to infect new cells under the cover of HAART. In fact, Chun and colleagues found that patients who received IL-2 in addition to HAART had a significantly lower frequency of latently infected $CD4^+$ T cells harboring replication-competent virus compared with patients who received HAART alone (434). The mechanism of this effect is under investigation; however, unfortunately, even patients who harbored a frequency of latently infected cells that was below the limits of detection experienced prompt viral rebound when HAART was discontinued (435). These data highlight the importance of other reservoirs of smoldering virus replication or latently infected cells in the body (436,437).

CONCLUSION

Within a relatively short time, AIDS has emerged as a leading cause of morbidity and mortality worldwide. As the epidemic matures and plateaus in certain parts of the world, it emerges and accelerates in others. The extraordinary burden of the epidemic that has devastated sub-Saharan Africa will likely next be realized in India, central Asia, and Eastern Europe.

The pathogenesis of HIV infection is a complex, multifactorial process involving viral and host factors. HIV is a formidable opportunist of the human immune system, as illustrated by its ability to utilize several receptors to gain entry into its target cells. Furthermore, HIV is uniquely able to utilize the milieu of an activated immune system to its own replicative advantage; thus, activation and mobilization of the immune response, which are intended to thwart the virus, rather fuel its replication and spread.

Advances in the treatment of HIV infection have been made at a pace that is unparalleled in the study of human viral diseases. Nevertheless, some sobering obstacles remain to the optimal treatment of HIV-infected persons. More than 90% of HIV-infected persons live in the developing world, where expensive combinations of antiretroviral agents are far beyond the capacities of local per capita health care expenditures. Even in developed countries, it is clear that, although HAART regimens have vastly improved the prognosis of HIV-infected patients, they are not curative, and their use is fraught with difficulties including numerous toxicites and drug interactions. To deal effectively with HIV infection, new antiretroviral agents that target different stages of the viral replicative cycle will be needed, as well as immunorestorative strategies that will allow more complete reconstitution of the immune system in the setting of effective antiretroviral therapy.

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75 EPSTEIN-BARR VIRUS

David A. Thorley-Lawson, Ph.D.

Epstein-Barr virus (EBV) was the first candidate human tumor virus to be identified. Since its discovery, it has been found in a number of important malignancies, primarily of B lymphocytes (Burkitt, Hodgkin, and immunoblastic lymphoma) and mucosal epithelium (nasopharyngeal and gastric carcinoma). It also is the accepted cause of infectious mononucleosis. Despite its pathogenic potential, the virus infects virtually the entire human race and persists benignly within the vast majority of individuals for their whole lives. In the last few years, tremendous strides have been made in understanding how EBV causes cells to grow, but only recently have we begun to understand why. EBV latently infects resting B lymphocytes, in the nasopharyngeal mucosal epithelium, and causes them to proliferate so that they can differentiate into resting memory B cells–the site of life-long persistent infection. When this process becomes deregulated, malignancies can arise. This chapter discusses benign infection and the known EBV-associated diseases. We describe how EBV has interwoven its biology with that of the mature B lymphocyte, first to establish and then to maintain a persistent infection within the B-cell arm of the immune system. The virus achieves this despite an aggressive and ongoing immune response against the virus and virus-infected cells. This model is used as a basis for discussing the origins of the EBV-associated diseases.

History and Background

EBV is classified as a g herpesvirus and has a tropism for B lymphocytes. It was discovered through its association with Burkitt lymphoma (BL) (1), a common tumor of children in tropical Africa. The distribution of BL closely follows that of hyper- and holoendemic malaria (2). This led to the suggestion that BL might be caused by an infectious agent spread by the malarial mosquito. Subsequently, BL tumor cells were propagated in tissue culture and a fraction of the cells found to contain viral particles that resembled, morphologically, members of the herpesvirus family (3). Thus was discovered the first candidate human cancer virus. It is ironic to note that, almost four decades later, no causal link has been demonstrated between malaria and either EBV transmission or BL development and that the role of EBV in BL remains a mystery.

After the virus was identified, there followed a series of elegant studies exploiting immunofluorescence assays that used human sera to detect viral antigens expressed by BL tumor cells (4). It was discovered that people developed antibodies to EBV during the course of infectious mononucleosis (IM) (5). This established the causative role of EBV in IM. Subsequently, a strong association was found between elevated antibody titers to EBV and the development of nasopharyngeal carcinoma (NPC), whereas a weaker association also was found with Hodgkin disease (HD) (4). Both associations have subsequently been confirmed by more sophisticated immunohistochemical and molecular biologic techniques.

In parallel with the studies on disease association, other investigators uncovered the fundamental biologic properties of the virus that established it as a *bona fide* tumor virus. The virus was isolated from EBV-positive cell lines (6) and from the throat washings (7) and peripheral blood cells of infected individuals (8). No matter the source, the virus was capable of infecting normal B lymphocytes in culture and causing them to proliferate indefinitely. This process is called immortalization or growth transformation. The cells do not produce infectious virus; therefore the infection is latent. These studies established that EBV alone has the capacity to drive the proliferation of normal cells, thus accounting for its being a risk factor in the development of neoplastic diseases. Immortalization is a powerful experimental tool not only for studying EBV latency, but also for studying human biology. It allows the rapid establishment of cell lines from any individual of interest for subsequent biochemical and/or genetic studies.

Finally, it has been shown in certain New World monkeys (9), severe combined immunodeficiency disease (SCID)/hu mice (10), and in individuals who are immunosuppressed (11,12 and 13), EBV can directly give rise to B-cell tumors. These tumors contain EBV and resemble the proliferating cell lines obtained by infection *in vitro*.

Because EBV is (a) so ubiquitous, (b) capable of driving cellular growth, and (c) infects B lymphocytes, it has been suggested as a candidate causative agent for a number of neoplastic and autoimmune diseases. It is now accepted that EBV has a role in gastric carcinoma (14) and rare T/natural killer (NK) cell lymphomas (15). More controversial and yet to be substantiated is its involvement in invasive breast carcinoma (16), hepatocellular carcinoma (17), and systemic lupus erythematosus (18). Earlier suggestions that EBV plays a role in rheumatoid arthritis and chronic fatigue syndrome are generally discredited (19,20).

Since the time of its discovery, the focus on EBV has been its ability to transform B cells and its relationship to human cancer. Only in recent years has it become apparent that EBV has a less-threatening side. It infects >90% of the human population, persists for their whole lives, and in the vast majority of cases, remains benign. Persistent infection is demonstrated by the continuous presence of infected B cells in the peripheral blood and the shedding of infectious virus in saliva (21). The virus persists by establishing a latent infection in resting memory B cells (22). Here the virus is invisible to immunosurveillance and not a pathogenic threat to the host because no viral genes are expressed (23).

Recent advances in our understanding of the molecular biology of growth transformation *in vitro* and the specificity of viral infection *in vivo* allow a synthesis that begins to explain how EBV establishes and maintains a persistent infection and what the risk factors are that lead to EBV-associated disease.

EPSTEIN-BARR VIRUS INFECTION IN THE HEATHY HOST

Most studies of primary infection have been performed on IM patients on the assumption that IM is simply an amplified version of asymptomatic infection (24). This is untrue (see later). IM is a pathologic, atypical form of primary infection. Normally, EBV does not aberrantly deregulate the behavior of infected B cells *in vivo*. It initiates, establishes, and maintains persistent infection by subtly using various aspects of normal B-cell biology. Ultimately this allows the virus to persist within memory B cells for the lifetime of the host (25,26).

Entering the B Cell: The Earliest Events in Primary Epstein-Barr Virus Infection

It is generally believed that EBV is spread through salivary contact (27). The lymphoid system that surrounds the nasopharyngeal region includes the adenoids and tonsils and is called Waldeyer ring. Together with the overlying epithelium, it forms a continuous structure referred to as the lymphoepithelium (28). The epithelium is invaginated to form crypts, below which resides the lymphoid tissue (Fig. 75.1). Deep in the crypts, the epithelium can be very thin. It is likely that the virus, in saliva, enters the crypts (Fig. 75.1) and crosses the thin layer of epithelial cells to infect B cells that reside below. What happens next is assumed to recapitulate what is seen *in vitro*. EBV infects resting B cells *in vitro* and drives them to become latently infected, proliferating B blasts (29,30). This form of latency is referred to as the growth program (31) or latency III (32). The only population that expresses the growth program in tonsils are naive B cells (33); therefore the target of the incoming virus is the

resting naive B cells that reside in the mantle zone immediately below the epithelium (Fig. 75.1).

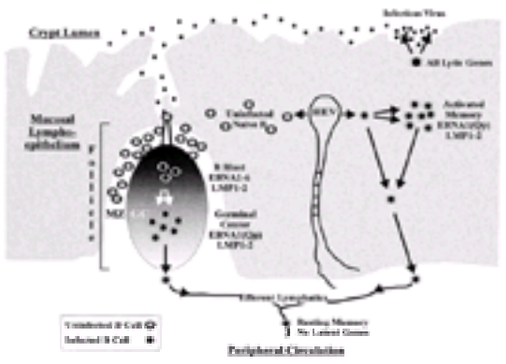

Figure 75.1. A representation of mucosal lymphoepithelium in the nasopharyngeal region. The events and structures depicted here are described in detail in the text and in diagrammatic form in Fig. 2. *MZ*, mantle zone; *GC*, germinal center.

Because the target for EBV infection is a resting cell, the virus must initiate latent gene transcription in a quiescent environment. It infects cells through the interaction of the viral glycoproteins gp350/220 with CD21 (34) and gp42/gH/gL with major histocompatibility complex (MHC) class II (35). CD21 is the receptor for the C3d component of complement and forms part of a multimeric signal-transduction complex with CD19, CD81(TAPA-1), and Leu-13 (36). Cross-linking of surface molecules by the viral glycoproteins provides the signal to move the resting B cells from G_0 to the G_1 phase of the cell cycle and allows expression of the first latent proteins (EBNA2 and EBNALP) (37). EBNA2 is a transcription factor that activates the promoters necessary to produce all nine of the latent proteins expressed in the growth program [reviewed in (38)] (Table 75.1). The result is that the infected B cell becomes an activated lymphoblast and begins to proliferate. The nine latent proteins include six nuclear proteins [EBV nuclear antigens (EBNAs)] and three membrane proteins [latent membrane proteins (LMPs)]. The presence of EBNA2 is diagnostic for the growth program (Fig. 75.1 and Fig. 75.2B). Several of the latent proteins have potent growth-promoting activity and can act as oncogenes. These include EBNA2 (39), EBNA3c (40), and LMP1 (41).

Latent Gene[a]	Function
EBNA1	Tethering and replication of the viral genome
EBNA2	Transcription of latent viral and cellular genes
EBNA3/EBNA3a	Unknown
EBNA4/EBNA3b	Unknown
EBNA5/EBNALP	Augmentation of EBNA2 function
EBNA6/EBNA3c	Cell-cycle progression
LMP1	Cell survival/TNFR family-type signaling
LMP2a/TP1	Cell survival/BCR-type signaling
LMP2b/TP2	Unknown
EBER1[b]	Unknown
EBER2[b]	Unknown
BARF0/BART[c]	Unknown

[a] Where more than one nomenclature is used the alternates are given.
[b] Small nonpolyadenylated RNAs. No protein.
[c] No known protein to date.

TABLE 75.1. The EBV Genes Expressed in Latently Infected Cells

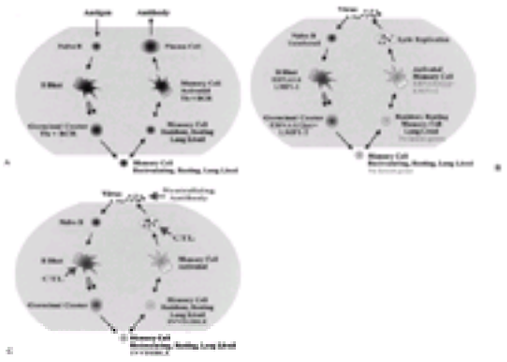

Figure 75.2. A diagrammatic representation of the current model of the establishment and maintenance of Epstein-Barr virus (*EBV*) persistent infection. *The gray shaded area* represents the lymphoepithelium. **A:** The major events involved in the response of naive B cells to antigen. The details are described in the text. **B:** How EBV has exploited normal B-cell biology, shown in **A**, to establish and maintain infection. The cells labeled in *blue* represent the major pathway involved in establishing a persistent infection. The *orange* cells represent the major pathway involved in long-term maintenance. The details are described in the text. Note that the overall consequence of all this activity is that the numbers of latently infected recirculating memory B cells remain extremely stable for many years. **C:** The major targets of the immune response. Note that despite these potent responses, the virus continues to be shed, and B blasts expressing the growth program are continuously present in the lymph nodes such as the tonsil.

In addition to the nine latent proteins, EBV-infected lymphoblasts express two small nonpolyadenylated RNAs, termed EBER1 and EBER2 (42), and a set of transcripts, derived from the Bam A fragment of the genome, called BARF0 or BART (43). [EBV genes are named from their locations on the *Bam*HI restriction map of the viral genome, where the largest fragment is A. Thus, BZLF1 is the ***Bam*HI**, **Z** fragment, **L**eftward reading **F**rame number **1**. Commonly studied genes also are identified by colloquial names (e.g., BZLF1 is referred to as ZEBRA or Zta).] The function of these genes is unclear. The EBER transcripts are important because they are by far the most abundant RNAs found in latently infected cells and are routinely used as targets for *in situ* hybridization studies to identify EBV-infected cells in normal tissues and tumors.

The latent genes are transcribed from the viral genome, which exists as a covalently closed episomal circle. The linear genome forms this circle when the infected cell begins proliferating (44). The status of the viral genome in a tissue provides a considerable amount of useful information. Linear genomes indicate viral replication. Circularized genomes indicate latently infected cells that have proliferated at some time in their history (45,46). The number of discrete joint fragments (TR, or terminal repeat analysis) is a measure of how many independent clones are present in the tissue. This latter technique is used to establish the clonal origin of the virus in tumors (46).

EBV-infected, proliferating lymphoblasts resemble, morphologically (47) and phenotypically (48,49), normal B cells that have been activated to become B blasts by antigen. Normal B cells need antigen, T-cell help, and lymphokines to achieve this activated, proliferating state; however, EBV is able to perform the task alone. Therefore the complex machinery of viral latent gene expression found in the growth program is specifically designed to induce a normal, activated phenotype.

It is apparent then that EBV has evolved a transcriptional program that ensures that EBV will efficiently and predictably establish latency and initiate cell growth as soon as it encounters a resting naive B cell in the lymphoepithelium of the nasopharynx. This growth-promoting transcription program puts the host, in which the virus intends to persist, at risk for developing neoplastic disease. Therefore the virus must use this program because it is essential. We now believe that the virus must drive the latently infected naive B cells to proliferate so that they are in the correct differentiation state, activated B blasts, to differentiate into resting, latently infected memory cells where the virus can persist for life.

The Making of a Memory B Cell

To understand how latently infected, naive B lymphoblasts expressing the growth program can become resting memory B cells, with no viral gene expression, it is first necessary to describe how a normal naive B cell becomes a memory cell. EBV infection occurs in the mucosal lymphoepithelium of the pharynx; therefore the biology of mucosal B cells is most relevant to our discussion.

Environmental antigens are sampled directly through the epithelium (28). Naive B cells extravasate through high endothelial vesicles (HEVs) (Fig. 75.1) in the lymphoepithelium and migrate to the mantle zone surrounding the follicles (50). If they encounter antigen, they become activated B blasts that migrate into the follicles and proliferate to form germinal centers (Fig. 75.1 and Fig. 75.2A) (51,52). During this time, the B cells switch the isotype of the immunoglobulin (Ig) they produce and randomly mutate the immunoglobulin genes. For these cells to survive, they need to receive two signals, one from antigen-specific T helper (Th) cells, and one through the B-cell receptor (BCR) for antigen. If Th cells are not available or the mutated immunoglobulin no longer binds antigen, the cell will die (53). Thus B cells expressing the highest-affinity BCR are selected. The cells that survive ultimately have two fates, depending on the length and type of exposure to Th cells and specific lymphokines (54). They can either terminally differentiate into antibody-secreting plasma cells that migrate into the epithelium (55) or become long-lived memory B cells. Memory B cells, as the name implies, carry immunologic memory and are responsible for a heightened secondary response on reexposure to the specific antigen. The maintenance of B-cell memory is not passive. Memory B cells rarely divide (56) but need frequent signaling through the BCR (57,58) and by Th cells (59) to survive.

Establishing a Persistent Infection: Access to the Memory Compartment

From the preceding discussion, we know that two events must happen for EBV-infected naive B lymphoblasts, expressing the growth program, to become memory cells. First, the latent genes that drive proliferation must be turned off, and second, the cells must receive the requisite survival signals. How the EBNA2-driven growth program is turned off is unknown; however, it is clear (Fig. 75.1 and Fig. 75.2B) that germinal center cells, carrying latent EBV, express only three of the nine latent proteins, EBNA1, LMP1, and LMP2a (60). This form of latency is referred to as the default program (61) or latency II (32). LMP1 is a membrane protein that acts as a ligand-independent, constitutively activated receptor (62) delivering T-cell help. It does this by engaging the TRAF and TRADD molecules (63,64), which normally transmit signals for members of the CD40/TNFR family of surface molecules (65). The interaction of CD40 ligand on Th cells with CD40 on B cells is the mechanism by which the Th cell delivers its rescue signal to the B cell. Thus LMP1 is able to deliver a Th-cell signal in the absence of either antigen or Th cells.

LMP2a also is a membrane protein, and it delivers a constitutive ligand-independent BCR signal (66). LMP2a contains the same signaling motifs (ITAMs) (67), as the a and b chains of the BCR. These motifs allow it to engage members of the src family of nonreceptor protein tyrosine kinases usually used by the BCR when it signals after binding cognate antigen. The BCR produces two types of signals. One type is required simply to ensure the survival of resting B cells (57). The other leads to cellular activation, proliferation, and ultimately differentiation into immunoglobulin-secreting plasma cells (51,52). The two signals, therefore, may be referred to as survival and activating, respectively. LMP2a is able to provide the survival but not the activating signal.

By expressing LMP1 and LMP2a, the virus ensures that the B cell will get the BCR (LMP2a) and Th (LMP1) signals necessary for it to survive a germinal center and exit as a resting memory cell into the peripheral circulation (Fig. 75.1 and Fig. 75.2B) (22). EBNA1 also is expressed in the default program because it is essential for retaining the viral genome by tethering it to cellular DNA so it can be replicated (68). For EBNA1 to be expressed without the other EBNAs, the Q promoter is used (69). EBNA1 transcription from Qp, in the absence of EBNA2, is diagnostic for the default program.

EBV Persistence in the Peripheral Memory B Cell Compartment

EBV, in the peripheral blood, is found only in resting cells (23) that have undergone germinal center differentiation (i.e., classic memory B cells) (22,70). The virus is distributed evenly among memory B cells expressing the different switched immunoglobulin isotypes. The frequency of infected cells for any individual is very stable for years. In a population of healthy carriers, it ranges from 5 to 500 for every 10^7 B cells, with a mean of 50 per 10^7 (71). This is about one infected cell per milliliter of blood, for a total of 2.5×10^6 infected cells in the entire body of an average individual. The stability in the number of EBV-infected cells and their random distribution in the memory compartment suggest that the host may not recognize these cells as infected. The number may be maintained through the normal homeostatic mechanisms that keep the levels of memory B and other lymphoid subsets stable. This stability will continue unless something perturbs the system.

Careful quantitative analysis suggests that most of the infected cells in the peripheral blood do not transcribe any of the genes for the known latent proteins (23; D. Hockberg and D.A. Thorley-Lawson, unpublished observations. However, the messenger RNA (mRNA) for LMP2a (72) is often present, probably due to long-lived transcripts. Therefore the final resting place of persistent EBV is a resting memory B cell that is not subject to immunosurveillance because the immunogenic latent genes are not expressed (Fig. 75.2B and Fig. 75.2C). This cell is not a threat to the host because the growth-promoting latent genes are not expressed. This type of latency is referred to as the true latency program.

Maintenance of the Persistent Infection: Using Long-Term Memory

Latently infected, resting, memory B cells in the periphery need to receive the Th and BCR signals necessary to maintain their survival as memory cells. To do this, it is believed that they transit back through the nasopharyngeal lymphoepithelium where they become activated and express the default program (Fig. 75.1 and Fig. 75.2B) (61). Again LMP1 and LMP2 give the necessary survival signals.

The last component of persistent infection is the continuous shedding of infectious virus into the saliva. Memory B cells, transiting the nasopharyngeal lymphoid tissue, must occasionally initiate virus replication and release the virus back into the crypts. B cells replicating the virus in the lymphoepithelium have plasma cell morphology (73,74). This would suggest that, in some circumstances, the activated, latently infected, memory B cells differentiate into plasma cells and release virus (Fig. 75.2B).

The growth program allows EBV to gain access to the memory compartment. Once the persistent infection is established, cells expressing the growth program are no longer essential, but continue to pose a threat to the host. As the immune response attempts to clear most viruses, it tends to select for strains with altered epitope sequences that can no longer be recognized. The opposite is true for EBV. It has conserved the sequences of growth program latent proteins that are recognized by cytotoxic T lymphocytes (CTLs) (75). As discussed in the ensuing sections, the virus has done this to ensure that aberrant cells expressing the growth program are rapidly killed and do not threaten the health of the host.

IMMUNOLOGIC RESPONSES TO THE INFECTION

Immunologic responses to EBV infection can be divided into those that arise during acute infection and those that continue during persistent infection. Responses to acute infection have been studied only in IM. It is assumed, but not known, that these events typify all primary infections.

Humoral Response

HISTORY

Early work on EBV exploited the observation that infected individuals produce antibodies against discrete components of the virus and virus-infected cells. These antibodies define the EBNA, EA-R, EA-D, MA, and VCA antigens (4). EBNA is detected in the nucleus of latently infected cell lines (76). It consists of six discrete components (Table 75.1). EA-R (early antigen-restricted) and EA-D (early antigen-diffuse) define antigens present in cells that are producing virus. R and D describe the pattern of staining seen within the cells. EA consists of a large number of proteins, many of which are involved in initiating replication of the viral genome. Their expression is independent of viral genome replication (4). MA (membrane antigen) and VCA (viral capsid antigen) also are present in cells producing virus, but their expression is dependent on replication of the viral genome (4). MA is present on the cell membrane and the viral envelope and is a complex composed mostly of glycoproteins that play key roles in virus binding and infection. The major components are two heavily glycosylated proteins of 350,000 and 220,000 molecular weight (gp350/220), which are the major targets of neutralizing antibodies to the virus (77). VCA is expressed in the cytoplasm and is a complex of structural proteins that includes components of the capsid and MA present in the cytoplasm of the cells. Measurement of anti-VCA antibodies is the most common, virus-specific, serologic test for the presence of EBV.

ACUTE INFECTION

IM is characterized by a unique profile of antibody responses (4). These include antibodies to EA, VCA, MA, and the EBNAs, with the exception of EBNA1. A definitive diagnosis of acute EBV infection requires the detection of IgM antibodies to VCA in the absence of anti-EBNA1 antibodies. Why anti-EBNA1 antibodies do not appear during acute infection is a mystery.

PERSISTENT INFECTION

Once the infection is established, the serologic profile stabilizes with significant IgG antibody titers to EBNA1, VCA, and MA, including neutralizing antibodies. Antibodies to EA and the other EBNAs decline but remain detectable. The definitive diagnostic serology for a healthy EBV carrier is IgG antibodies to VCA and EBNA1 (4).

Cellular Responses

ACUTE INFECTION

IM is characterized by the appearance of large numbers of virus-specific CTLs, most of which are CD8 positive (78,79) and recognize proteins of the lytic cycle (80). The CTL response rapidly becomes focused on single epitopes (81). In one case of IM, 44% of all $CD8^+$ T cells were specific for a single peptide of the BZLF1 protein (82) that initiates lytic replication of the virus. Only 1% to 2% of the $CD8^+$ T cells were specific for latent proteins.

PERSISTENT INFECTION

As with the humoral response, the CTL response declines and then stabilizes as the acute infection recedes. The response remains highly focused, however, with the dominant epitopes being from lytic proteins (83), particularly the immediate early transcriptional transactivating proteins such as BZLF1 (84,85). Typically 1% to 5% of all $CD8^+$ T cells are specific for a single epitope of a lytic protein, whereas <1% respond to the latent proteins (86). The peptide used is dependent on the presenting MHC class I molecule. For example, individuals who are human leukocyte antigen (HLA)-B8 focus on single peptides from the BZLF1 and EBNA3a proteins (87).

The CTL response to the latent proteins is particularly strong against the EBNA3 family of proteins (87), and most infected individuals mount responses to these proteins. Responses to EBNA2 and the LMPs are detected much less frequently, and responses to EBNA1 are essentially undetectable. The strong focus on the EBNA3s confirms the notion that the key role of the CTL response to latently infected cells is to destroy those expressing the potentially pathogenic growth program, the only program in which the EBNA3s are expressed (Fig. 75.2C). One consequence may be that cells expressing the default latency program, in which only EBNA1 and the LMPs are expressed (Table 75.2), may not be under aggressive immunosurveillance. This includes latently infected germinal center and memory cells (60). The latent proteins of the default program may have evolved to be less immunogenic to protect these critical intermediates of persistent infection from destruction by CTLs. The reduced immunosurveillance of cells expressing the default program may be important in understanding why tumors that express this program, such as HD and NPC (see later), can escape immunosurveillance.

Form of Latency	Tissue/Tumor	Genes Expressed*
Growth program (Latency III)	Normal tissue	EBNA1-6
	MLN naive activated B cells	LMP1-2
	Tumors	EBER 1-2
	Immunoblastic lymphoma	BARF0
	PTLD	
	XLP	
	AIDS	
Default program (Latency II)	Normal tissue	EBNA1(Gly)*
	MLN: germinal center	LMP1-2
	MLN: activated memory B cells	EBER 1-2
	Tumors	BARF0
	Hodgkin disease	
	Burkitt lymphoma†	
	T/NK lymphoma	
	Nasopharyngeal carcinoma	
Latency program	Normal tissue	EBER1-2
	PBL: Resting memory B cells	BARF0
	MLN: Resting memory B cells	

MLN, mucosal lymph nodes; PBL, peripheral blood lymphocytes.
* The expression of EBER and BARF0 transcripts has not been systematically studied in lymphoid tissues.
† In this form of latency, EBNA1 is expressed from a promoter referred to as Qp, which is different for the promoter used in the growth program (Cp) and Wp).
‡ BL is listed, although only EBNA1 is expressed. It is our assumption that BL is derived from a cell that originally expressed the default program.

TABLE 75.2. The Viral Genes Expressed in the Different Forms of Latency and the Infected Tissues Where They Occur

One striking aspect of the CTL reactivity profile is the absence of clones recognizing EBNA1 (87). EBNA1 contains a large repetitive domain, consisting of repeated glycine and alanine residues, that prevents the protein from being processed for presentation on MHC class I molecules (88). It is has been suggested that this represents a mechanism of immune avoidance by the virus and has led to proposals that the site of persistence expresses only EBNA1 (24,89,90). As discussed earlier, however, the site of persistence is the memory B cell (22,25,26) that, in the blood, does not express the EBNA1 gene (91) and, in the lymph nodes, does expresses EBNA1, but always with LMP1 and LMP2 (60), both of which can be processed for presentation to CTLs. Therefore the default transcription program for persistence is EBNA1 + LMP1 + LMP2, not EBNA1 alone. A more mundane explanation for the stability of EBNA1 may be its tethering function (68), which is essential for genome replication. The stability of EBNA1 may simply reflect the importance of having sufficient EBNA1 protein to ensure retention of the viral genome.

Role of the Immune Response

Although antibodies are produced to a wide variety of latent and lytic proteins, it is certain that most play no role in curtailing acute infection or regulating persistent infection. Only neutralizing antibodies are likely to play a role by preventing the dissemination of infectious virus (Fig. 75.2C). Even this response appears inadequate, however, to prevent the continuous production and shedding of infectious virus into the saliva of persistently infected individuals (21).

CTLs against lytic antigens are probably focused on immediate early proteins to ensure destruction of lytically infected cells before they produce infectious virus (Fig. 75.2C). The importance of this response is demonstrated when the CTL response is suppressed. Immunosuppressed patients shed increased levels of virus in their saliva (92) and have cells replicating virus in their blood (23), an event never seen in healthy carriers.

Because the CTL response to latent proteins focuses so aggressively on the EBNA3s, cells expressing the growth program must be their major target. This conclusion is again borne out by studies of the immunosuppressed who are at risk for developing B-cell lymphomas that express the growth program (93,94), not the default program (see later).

THE EPSTEIN-BARR VIRUS–ASSOCIATED DISEASES

So far we have discussed the current ideas of how and where EBV establishes and maintains a persistent infection and the key immunologic responses that regulate it. The following sections discuss the major EBV-associated diseases. Because the etiology of these diseases is not well understood, we attempt to place each of them in the context of the preceding discussion.

Infectious Mononucleosis

Most people in the world become infected with EBV at a very early age, usually in the first years of life (4). Primary infection of children is rarely recognized because it is usually asymptomatic (95). In Western and economically advanced countries, infection is frequently delayed into adolescence or adulthood, when it can cause IM (96). Reported incidence rates range from two to 2,000 per 100,000 (97), but these estimates are inaccurate because they do not take into account the age-related occurrence of the disease and the socioeconomic dependence of the age of primary EBV exposure. IM is exclusively a disease of EBV-negative adolescents and adults, and in this group, 20% to 50% of primary EBV infections result in IM (97). The intensity of the disease varies but can last for weeks or months before finally resolving (98).

IM is characterized clinically by the insidious onset of malaise, sore throat, fever, and lymphadenopathy. There is a characteristic lymphocytosis (99) (>4,500/mm^2 with >51% lymphocytes on differential count) and the production of IgM heterophile and autoantibodies with no specificity for EBV. A subset of the heterophile antibodies recognizes sheep and horse erythrocytes (100). This is the basis of the Paul-Bunnell-Davidsohn agglutination or monospot test, the most widespread diagnostic test for acute IM. Taken together, these three indicators constitute a diagnosis for IM (98).

The lymphocytosis is characterized by the appearance of large numbers of “atypical” lymphocytes (99). These are not infected B cells but predominantly $CD8^+$ T cells, with a minor $CD4^+$ component, and account for the inversion of the CD4/CD8 ratio seen in IM patients compared with healthy individuals (78). The atypical T cells express HLA class II MHC, CD38, and CD45RO (82), all markers of activated T cells, and die rapidly in culture (101). Originally they were thought to represent a nonspecific response that paralleled the production of heterophile antibody; however, it is now known that they represent a vigorous CTL response to the virus (79)

(see later). This response can, during the height of the disease, involve up to half of all the T cells in the body (82). Presumably this explosion of T cells is responsible for resolving the infection, but the ensuing destruction of infected tissue leads to the fever and malaise characteristic of IM.

The normal course of EBV infection involves the precisely regulated expression of transcription programs that allow latent EBV to gain access to and persist within the long-lived memory compartment (Fig. 75.1 and Fig. 75.2B) (60). This process is normally asymptomatic, as observed in the primary infection of young children, which is not associated with lymphocytosis or heterophile antibody (95). By contrast, IM is a chaotic mixture of inappropriate infection. IM patients shed very high levels of infectious virus in their saliva. B cells that are latently infected or replicating the virus (73,74) are present abundantly throughout the tonsils of IM patients, at levels 100- to 1,000-fold higher than those in healthy carriers (102). Many of the latently infected cells express the growth program (103), and most of them represent expanded clones of memory cells (104). Memory cells expressing the growth program are never found in the tonsils of healthy carriers (33,60). Their presence in IM indicates that the tight regulation of the growth program has been contravened, allowing infectious virus to spread beyond the usual boundaries that restrict it to only infecting naive B cells. The result is the inappropriate infection of memory B cells. Directly infected naive B cells can differentiate through a germinal center into memory and therefore do not accumulate as proliferating clones in the tonsil. Directly infected memory cells, however, cannot undergo this differentiation program and therefore accumulate as clones driven to proliferate by the viral growth program. The process is so disrupted that cells expressing the growth program are even found in the peripheral blood (105), an event that is never seen in healthy carriers (72) and is very rare even in the immunosuppressed (23). The viremia is so systemic that B cells in the periphery are being directly infected to become proliferating lymphoblasts. These cells cannot escape the cell cycle through differentiation and continue to expand until as many as 0.1% to 10% of the B cells in the peripheral blood are EBV positive (106). It has been speculated that these cells may be responsible for the production of the low-affinity IgM heterophile antibody characteristic of IM.

It is often incorrectly assumed that IM is an amplified version of typical asymptomatic infection (24). It is now clear that IM is actually a pathologic condition, demonstrating atypical infection, and is not representative of the well-ordered set of host/virus interactions that characterize normal acute infection. It is likely that IM is immunopathologic in nature, meaning the disease symptoms are caused by the inflammatory response of the immune system rather than by the virus itself. The age dependence of symptoms has led to the suggestion that IM is a disease of a mature immune response. What this means mechanistically is less clear. Another explanation has been suggested, based on animal studies on the memory CTL response (107). Early in life, the CTL response to new infections is by naive T cells. These produce high-affinity CTLs that efficiently clear the virus infection and then become memory CTLs. As the organism ages, memory CTLs accumulate to a variety of pathogens. Exposure to a novel infection later in life is more likely to reactivate cross-reacting memory CTLs than naive CTLs. These CTLs are of lower affinity and may produce an ineffectual response, allowing extensive viral replication and spread, resulting in a massive inflammatory response that lasts until the virus is finally brought under control.

As time passes and the symptoms of IM regress, less virus is shed into the saliva, and infected B cells expressing the growth program disappear from the peripheral blood. The ensuing events, however, are strikingly different from infection with most other viruses. Shedding of EBV does not stop, but stabilizes and continues for life at a lower, steady rate (21). B cells expressing the growth and default programs persist in the nasopharyngeal lymph nodes (60), and latently infected memory cells, expressing the latency program, remain in the blood for life at levels that stay remarkably stable (22,71). At the same time, levels of neutralizing antibodies (4) and CTLs also stabilize (81) and continue at significant levels for the lifetime of the host. It is apparent therefore that the immune response ameliorates but never clears the infection. An equilibrium is established between the immune response and the various states of viral latency, allowing the virus to persist and be shed at stable levels, without causing significant impairment to the host.

Immunoblastic Lymphoma in the Immunosuppressed

TRANSPLANTATION

Lymphoproliferative disorders are a relatively common consequence of iatrogenic immunosuppression associated with organ transplantation. They are collectively defined as posttransplantation lymphoproliferative diseases (PTLDs). The use of this common rubric hides the heterogeneity of these disorders. PTLDs are of B-cell origins and almost always are EBV positive, but no clearly identified marker defines PTLDs as a discrete entity.

PTLDs have been known since the advent of immunosuppression in organ transplantation, but became noticed only with the introduction of the powerful immunosuppressant cyclosporin A (108). A large number of factors influence the incidence and onset of PTLDs, including the types, combinations, dosing levels, and lengths of immunosuppressants used (109), the virologic status of the recipient, the nature of the organ, and the setting of the transplant (110). For example, rates tend to be high in intestinal and heart/lung transplant recipients (15% to 20%) and low in bone marrow transplants (<1%) (111) receiving comparably high doses of cyclosporin A. Rates of PTLDs in bone marrow recipients jump from <1% to 10% to 20% if the bone marrow is depleted of T cells, suggesting that donor T cells play a critical role in controlling the EBV-driven lymphoproliferation (112). PTLD is particularly problematic in EBV-negative recipients (113) who develop a primary infection, frequently with organ-derived virus. This makes children a particularly high-risk group for PTLD. Onset of PTLD is typically less than 1 year after transplant for EBV-negative recipients and all bone marrow recipients. For EBV-positive recipients, receiving solid organs, onset time is several years (114).

PTLD lesions are heterogeneous in terms of locality, morphology, and responsiveness to therapy (115). They range from atypical polyclonal B-cell expansions, which more precisely resemble polymorphic B-lymphocyte hyperplasia, to aggressive non-Hodgkin lymphomas that are classified histologically as high-grade immunoblastic lymphomas. The tumors are frequently multifocal, with the hyperplasia tending to be limited to lymph nodes, whereas the high-grade lymphomas frequently localize in the gastrointestinal tract, the central nervous system, or the grafted organ itself (110). Individual foci may be clonal or multiclonal, and separate lesions within the same patient may be of different clonal origin. PTLDs usually express the growth program (116). Consequently, the tumors will frequently regress if immunosuppression is reduced or withdrawn completely (117) or when EBV-specific CTLs of donor origin are infused into the patient (118). Cells expressing more restricted forms of latency also may be present (119), and a subset of cells within the tumor are replicating the virus (120).

The virus found in the tumors is usually of recipient origin, in the case of solid organ transplants, but of donor origin for bone marrow transplants. This suggests that replacement of the hematopoietic system during bone marrow transplantation includes transfer of the capacity to maintain a persistent EBV infection (121). This phenomenon has been interpreted as evidence that the B-cell system is solely responsible for the maintenance of persistent EBV infection.

It is general believed that PTLDs represent the uncontrolled expansion of B cells expressing the growth program because of reduced immunosurveillance by virus-specific CTLs. This is consistent with the oligoclonal origin of the tumors, the increased risk of tumor development with increasing immunosuppression, and the responsiveness of the tumors to reduced immunosuppression. This unchecked growth provides a proliferating population of cells at risk for secondary genetic alterations that cause the cells to become more malignant (122). This type of progression is consistent with the observation that later-arising variants of the tumors become resistant to reductions in immunosuppression and tend to be more malignant as they move from oligoclonal to clonal (115). At this point, the virus is probably no longer the driving force behind the cellular proliferation, and the tumors are more likely to spread to extranodal sites, suggesting that the B cells are highly abnormal and no longer subject to the restraints that keep proliferating B cells within lymph nodes.

The cellular and molecular mechanisms underling these processes are not well understood. The obvious explanation is that immunosuppression causes more infectious virus to be produced. This leads to an increased number of directly infected B cells expressing the growth program, as seen in IM. Unlike IM, however, the CTL response is suppressed so these cells grow out of control, resulting in PTLD. However, it cannot be that simple. Immunosuppression does lead to a dramatic increase in the levels of shed virus, yet prophylaxis with inhibitors of viral replication, such as acyclovir, have little or no effect in preventing PTLD (111,123). Even though the levels of virus-infected cells, in the blood of the immunosuppressed, increase on average about 50-fold (23), the overall regulation of the system of persistence remains intact, such that all of the infected cells in the blood are still resting, memory cells (23). Unlike IM, cells expressing the growth program are rare or absent. Furthermore, PTLDs are often oligoclonal, implying only a few independent events, not the wholesale infection and expansion of EBV-infected B cells seen in IM. Taken together, this suggests that PTLD arises from the rare aberrant behavior of extant latently infected cells rather than from the new infection of B cells.

PTLD does not arise from the latently infected naive B cells expressing the growth program in the nasopharyngeal lymphoepithelium. A limited study of PTLDs revealed that none expressed surface IgD, and most had switched immunoglobulin isotypes, indicating that they are memory cells (124). Because memory cells expressing the growth program are not derived by direct infection, they must come from the pool of latently infected memory cells expressing either the default or latency programs. Some event(s) must occur that pushes latently infected memory cells to turn on the growth program and proliferate. Normally such cells would be destroyed by CTLs, but they would be free to expand in the immunosuppressed. Presumably this transition is an atypical and therefore rare event, because it is not part of the normal biology of EBV persistence. This would explain why PTLD involves a limited number of clones, not wholesale expansion of EBV-infected B cells, and why the early lesions of PTLD are so susceptible to reduced immunosuppression.

The nature of the signal that causes this transition is unknown, but hints come from animal models. It has been observed that xenotransplantation of peripheral lymphocytes from EBV-positive donors into SCID mice results in a high incidence of EBV tumors that bear resemblance to PTLDs (10). This occurs in part because the memory CTLs do not become activated in the transplanted animal. Interestingly, it has been reported that tumor development requires the presence of Th cells (125). PTLDs are known to be heavily infiltrated with such T cells (126). The EBV-associated superantigen (SAG) (127) may be important here. SAGs are able to recruit and activate Th cells in an antigen-independent fashion. PTLDs and the SCID tumors may arise because Th cells interact with latently infected memory cells expressing the SAG. The Th cells may then deliver signals that cause the latently infected memory B cells to switch on the growth program inappropriately. These cells are free to

grow because the environment lacks the CTLs that usually destroy them.

Another feature that the SCID tumors have in common with PTLDs is the presence, within the tumor, of a heterogeneous mixture of B-cell types. This includes actively proliferating lymphoblastoid cells and more differentiated plasmacytoid cells that have stopped proliferating and may support viral replication (128).

Once oligoclonal expansion of cells expressing the growth program has been initiated, the more aggressive clones take over. More malignant, clonal PTLD has been associated with mutations of p53, c-*myc*, and bcl-6. Chromosomal abnormalities also have been reported, including rare cases in which the specific translocations associated with BL (see later) are observed. However, no consistent pattern of mutations or translocations has been found, suggesting either that a wide range of events can result in the malignant progression or that the key events await identification.

ACQUIRED IMMUNODEFICIENCY SYNDROME

Two types of EBV-associated lymphomas have been observed in human immunodeficiency virus (HIV)-infected individuals (93,129). The first tends to occur relatively early in the course of the disease and resembles BL (see later). From 30% to 40% of these tumors are EBV positive (130). The second type of lymphoma closely resembles those found in allograft patients and arises late in the disease, around 4 to 5 years after HIV infection. They are immunoblastic lymphomas and express the growth program (93). They probably arise by a mechanism similar to that of PTLDs, involving aberrant expression of the growth program in the absence of effective CTL immunosurveillance. Like PTLDs, they are morphologically heterogeneous. Based on histology and EBV TR analysis, they can be either polyclonal or monoclonal lesions, although clonal lesions are more frequent (129). An unusual feature of this disease is that the primary tumor often arises in the central nervous system (CNS) (131). This may be due to neurologic damage, caused by HIV infection, allowing EBV-infected cells access to the immunologically privileged CNS, where they can grow unchecked. B-cell CNS lymphoma in HIV-positive patients is invariably EBV positive and has a poor prognosis.

EBV-positive immunoblastic lymphoma in acquired immunodeficiency syndrome (AIDS) patients began to emerge as a major cause of death after successful prophylaxis for pneumocystis pneumonia was developed. This trend has subsequently been halted with the advent of the new HIV therapies (highly active antiretroviral therapy, HAART).

X-LINKED LYMPHOPROLIFERATIVE SYNDROME

X-linked immunoproliferative syndrome (XLP) is a rare X-linked immunodeficiency that results in lymphoma or fulminating IM, culminating in the death of afflicted boys within 1 month of primary EBV infection (11,132). On infection with EBV, the boys typically mount what has been described as a “dysregulated exuberant response” to the virus (133). EBV-infected B cells, $CD8^+$ T cells, and macrophages enter tissues throughout the body, causing massive tissue damage, particularly in the liver and the bone marrow, ultimately resulting in hepatitis and hypoplasia. A common feature of XLP is a pronounced virus-associated hemophagocytic syndrome (VAHS) (133), characterized by widely disseminated histiocytes filled with erythrocyte and nuclear debris. Death is usually associated early on with the accumulation of EBV-infected B cells expressing the growth program in tissues such as the liver (134) or subsequently by the widespread tissue damage of the VAHS. XLP has been fatal in 75% of cases. Surviving boys typically have severely disrupted immune systems, resulting in varying degrees of hypogammaglobulinemia. It has been reported that XLP-associated VAHS has been successfully treated with etoposide and cyclosporin A and cured with allogeneic stem cell transplantation (135).

The XLP gene itself [SH2D1A (136) or SAP (137)], encodes for a small signaling molecule of 128 amino acids that consists essentially of a single SH2 domain with a small C-terminal extension. Before the onset of IM, boys carrying mutations in the XLP gene are not obviously immunodeficient, although several boys have manifested XLP without detectable EBV infection (138). This implies that EBV precipitates an incipient predisposition. What this predisposition may be and how mutations in the XLP gene cause it are currently the focus of intense study. SAP (the XLP gene product) associates with the receptors SLAM (CDw150) (137) and 2B4 (139). SLAM is a self-ligand expressed on T and B lymphocytes and is an important regulator of lymphocyte interactions and activation. 2B4 is a NK-cell recognition receptor. SAP acts as an inhibitory molecule by binding to the cytoplasmic tails of these receptors and blocking their interaction with downstream signaling molecules. This has encouraged speculation that the XLP defect may interfere with NK activity or result in a failure to dampen T-cell responses, resulting in extensive tissue damage. Why these effects would be so specifically precipitated by EBV remains to be elucidated.

Hodgkin Disease

HD is characterized by the presence of unique mononuclear Hodgkin and large multinuclear Reed-Sternberg (HRS) tumor cells infiltrated and surrounded by large numbers of nontumor cells. The infiltrating cells are a mixture of T lymphocytes, plasma cells, and granulocyte that constitute the majority of the tumor mass, such that sometimes less than 1% of the tumor is actually HRS cells. HD is divided into four histologically distinct types based on the relative abundance of infiltrating cells: nodular-lymphocyte predominant (lp), nodular sclerosis (ns), mixed cellularity (mc), and lymphocyte depleted (ld) (140,141). Together HDns, HDmc, and HDly are referred to as classic HD, whereas HDlp is believed to be a distinct entity. HDns is the most common, followed by HDmc. HDld and HDlp are relatively rare. The incidence of HD peaks at around age 25 to 30 years, with HDns being the predominant form. The incidence then decreases till around age 45 years, when it again begins to increase, with HDmc contributing more significantly to the incidence (142).

HRS cells frequently express CD30, CD70, and CD25, but the cell-surface phenotype is, in general, quite variable and does not fit any particular lineage. T (CD2, CD4), B (CD19, CD20), and myeloid (CD15) lineage markers may all be expressed on occasion (143,144). However, the immunoglobulin genes from the tumor cells are hypermutated (145), suggesting that HD arises from a germinal center or postgerminal center (i.e., memory) B cell. Classic HD tumor cells have fixed mutations that often cripple the immunoglobulin gene and usually do not express the germinal center marker BCL-6 (146), consistent with a post–germinal center origin. The tumor cells of HDlp, however, show evidence of ongoing hypermutation (145) and express BCL-6 (146), suggesting that this subset of HD may arise directly from an active germinal center cell.

The consistent defect found in HD is the deregulation of the NF-kB system (147). NF-kB is a multipotent transcription factor that exists as a cytoplasmic trimer including the inhibitory molecule I-kB (148). NF-kB activity is tightly regulated through the expression and degradation of its I-kB component. Appropriate cell-surface signaling leads to the degradation of the IkB moiety and activation of the resulting NF-kB dimer. The active dimer translocates to the nucleus, where it is responsible for the activation of many lymphocyte genes associated with function and growth. HD has defects that lead to the accumulation of active NF-kB in the nucleus. In several cases, mutations within the I-kB itself have been observed, leading to loss of inhibitory function. Of the known targets of NF-kB, cytokine genes are common. This could explain the well-documented association of HD with the production of cytokines, including interleukin (IL)-1, IL-6, IL-9, IL-10, IL-13, and tumor necrosis factor (TNF), all of which modulate T- and B-cell functions in ways that could be critically important in tumor development (149). This could take the form of autocrine growth stimulation (IL-6), recruitment of Th cells (IL-13), or suppression of the immune response (IL-10).

HRS cells are not found in healthy lymphoid tissue, and it is unclear how they are formed. The only other condition in which these anomalous cells are consistently found is in the lymph nodes of acute IM patients (74). Patients with acute IM are at increased risk for developing HD later in life, and elevated antibody titers to EBV are a risk factor for HD development (4). Up to 50% of HD tumors are EBV positive, increasing to 100% in developing countries and pediatric cases (149). The virus is clonal by TR analysis. It is present in approximately 90% of HDmc and HDld, 30% of HDns, but rarely in HDlp. The tumor cells express the EBNA1, LMP1, and LMP2 latent proteins and the EBERs and BARTs. EBNA2 and the EBNA3s have not been detected (150,151,152,153 and 154). EBNA1 is transcribed from the Q promoter therefore HD displays the default transcription program found in normal germinal center and memory cells (Table 75.2). Although some level of systemic immunosuppression is a feature of HD, there is no evidence of immune dysfunction playing a role in the development of the tumor. It remains unclear, therefore, why the LMP1 and LMP2 molecules do not serve as targets of immunosurveillance during the early stages of tumor development.

A likely scenario for EBV-positive HD is that the severe disturbance in the regulation of EBV infection during IM (see later) leads to the massive expansion and inappropriate infection of cells with EBV. Cells expressing the growth program are destroyed by the immune system. Latently infected, activated memory cells and germinal center cells expressing the default program of EBNA1, LMP1, and LMP2 are not under such aggressive immunosurveillance, but normally exit the cell cycle and become resting memory cells. The increased numbers of these cells during IM, however, make them potential targets for cellular mutations. Any mutation causing constitutive expression of NF-kB in the nucleus would block differentiation and prevent the cells from exiting the cell cycle. As a result, the cells would continue to express the viral latent genes LMP1 and LMP2. Both deliver survival signals, and LMP1, in particular, activates NF-kB (155) and is oncogenic when constitutively expressed (41). Continued expression of these molecules would favor the survival and development of the tumor. However, a significant fraction of HD tumors are EBV negative, and no obvious differences have been observed between EBV positive and negative HDs to indicate that they might be distinct entities. Therefore HD can arise with or without the virus. It is likely that the virus is a predisposing but not essential event in HD development.

Burkitt Lymphoma

As discussed in the introduction, the association of EBV with BL is as old as the discovery of the virus itself (3). BL has been subdivided into two distinct but related entities, endemic (eBL) and sporadic (sBL). eBL is usually associated with EBV and is better characterized because it occurs at high incidence in certain geographic areas such as equatorial Africa and coastal New Guinea. sBL, as the name implies, is not geographically restricted, is less common than eBL, and is usually EBV

negative.

eBL has a strikingly early age at onset, with a peak median age of 6 to 8 years (156). It has a very high incidence of five to 10 per 10^5/year for the first 15 years of life in the high-risk areas. In these regions, it is not only the most common tumor of young children, but is more common than all other tumors combined (156). sBL appears somewhat later, median age 10 to 12 years (157), and occurs world wide at a much lower frequency, one to two per 10^6/year.

BL most frequently occurs in the jaw and abdomen (156,157). eBL tends to occur more frequently in the jaw, and sBL, in the abdomen. Jaw involvement is more common in younger patients, and abdominal involvement in the older. It is striking that the jaw-associated tumors arise during the time of peak dental development (158). These tumors frequently extend from the mandible to the orbit of the eye. BL rarely spreads to the peripheral lymph nodes, and it has been described as a tumor of mucosa-associated lymphoid tissue (158). This concept is consistent with the biology of EBV, which colonizes the mucosal lymphoid system. All BL tumors have a characteristic morphology consisting of a homogeneous population of “noncleaved” lymphoid cells with amphophilic cytoplasm and clear vacuoles. Interspersed throughout the tumor are histiocyte/macrophages, containing engulfed lymphoid cells, debris, and nuclei, that give rise to the typical “starry sky” appearance of the tumor (157). The morphology of the malignant cells bears a close resemblance to the cells found in the germinal centers of secondary lymphoid tissue. Another indication that the tumor arises from a germinal center is the surface phenotype of the cells. This includes the characteristic lineage markers of B cells (CD19, CD20, and surface Ig) together with specific markers of germinal center cells (CD10 and CD77) (32). Interestingly the immunoglobulin isotype is predominantly limited to IgM. IgD, a marker of naive B cells, is never found, and switched isotypes, such as IgG and IgA, also are uncommon.

As with HD, there is a consistent genetic lesion that defines BL and supersedes the presence or absence of EBV and the distinction between eBL and sBL (159,160). This lesion is the reciprocal translocation of the c-*myc* oncogene on chromosome 8 with one of the immunoglobulin gene loci, resulting in the deregulated activation of c-*myc* (161). In about 80% of BL, c-*myc* is translocated into the heavy-chain locus on chromosome 14 (the common translocation). Less frequently, the translocation is into the k or l light-chain loci (the variant translocations).

The contribution of EBV to BL remains enigmatic. The most compelling evidence for a role remains the relatively high frequency of tumors carrying the virus. Virtually every eBL (>90%) in the high incidence areas contains EBV, which is clonal by TR analysis (162). The frequency of EBV-positive sBL is lower, but still ranges from 15% to 25% in Europe and North America to as high as 50% in South America and 85% in North Africa. In healthy carriers of the virus, the level of virally infected cells varies from one to 100 per 10^6 B cells in all infected subsets, including germinal center cells (60). In IM and the heavily immunosuppressed, the number is higher, but never exceeds 1%. It is apparent therefore that a strong selection pressure favors EBV-positive over EBV-negative B cells becoming BL tumor cells.

The viral gene expression pattern in BL is unique, being limited to EBNA1 from the Q promoter, EBERs, and BARF0. Therefore the only latent protein expressed is EBNA1 (32). Unconfirmed reports have suggested roles for EBNA1 (163) and the EBERs (164) in BL development.

No latently infected normal cellular counterpart has been found that expresses the very restricted pattern of latent genes found in BL. Unlike PTLD and HD, however, the tumor cells of BL will survive and grow in tissue culture. Within 24 hours, a burst of viral replication is observed (3), followed by stable outgrowth of the tumor cells. The tumor origin of the cell lines is confirmed by retention of the diagnostic translocation. With time, the viral phenotype of the cells shifts, such that eventually all of the latent genes associated with the growth program are turned on (32,165). At the same time, the cellular phenotype changes from a germinal center cell to that of an activated lymphoblast. Conversely, if activated lymphoblasts expressing the growth program are molecularly manipulated to turn off EBNA2 while retaining c-*myc* expression, they express the BL/germinal-center phenotype (166). Taken together, these observations suggest that cells expressing the growth program and BL tumor cells are very closely related to each other, despite the apparently stark differences in cellular and viral phenotypes. Based on the model of EBV persistence presented earlier in this chapter (Fig. 75.2B), we must conclude that BL is a tumor of latently infected germinal-center cells that have switched from the growth program of the B-cell blast to the default program.

If EBV-positive BL is derived from latently infected germinal-center cells, what happens to the expression of LMP1 and LMP2? The lack of LMP1 and LMP2 may be characteristic of a specific state of germinal-center differentiation that has yet to be identified, or LMP1 and LMP2 expression may not be consistent with the long-term survival of the tumor cells, or immunosurveillance may select for their downregulation. These possibilities have been discussed at length previously because the only viral protein expressed, EBNA1, cannot be processed for presentation by MHC class I and therefore cannot be recognized by CTLs (88). This has led to a popular model of BL as a tumor derived from a normal counterpart that is the site of long-term persistence and that avoids immunosurveillance by only expressing EBNA1 (24,89,90). As described earlier, it is now clear that this model cannot be correct. EBV persistence involves the complex interplay of different latent transcription programs with stages of B-cell differentiation. Immunosurveillance does not select for a particular program, it just regulates the absolute level of persistent infection.

The cellular phenotype of the BL tumor also has been cited as evidence that the tumor has escaped immunosurveillance against EBV (167). BL tumor cells lack most of the cell-surface molecules, adhesion and accessory, necessary for cognate activation of CTLs. They have also severely downregulated peptide-processing systems and have a low turnover of HLA class I molecules, which makes them inefficient at presenting peptides for CTL recognition. However, it has been pointed out (24) that the same phenotype is seen with EBV-negative BL, which cannot have been under immunosurveillance for EBV expression. This phenotype is typical of a germinal center cell. Last, the immunosurveillance/selection hypothesis for loss of LMP1 and LMP2 is unlikely because all of the other EBV-associated tumors that arise in the immunocompetent express LMP1 and LMP2 (Table 75.2 and Table 75.3), yet manage to grow in the face of the immune response.

Tumor	Origin
Immunoblastic lymphoma—PTLD/AIDS	Peripheral memory B cell expressing the latency program switches to the growth program and grows because of immunosuppression
Hodgkin disease	Upregulated expression of NFκB in a latently infected activated memory or germinal center cell causes a differentiation block and constitutive expression of the default program
Burkitt lymphoma	Upregulated expression of c-myc in a latently infected germinal center cell causes a differentiation block and constitutive expression of the default program with subsequent downregulation of LMP1 and LMP2
Nasopharyngeal and gastric carcinomas	Fortuitous infection of nasopharyngeal mucosal epithelium by locally replicating EBV leads to expression of the default program that cannot be turned off by differentiation because the virus is in the wrong cell type
T/NK lymphoma	Fortuitous infection of T/NK cells in the mucosal lymphoepithelium by locally replicating EBV leads to expression of the default program that cannot be turned off by cellular differentiation because the virus is in the wrong cell type

EBV, Epstein-Barr virus; NK, natural killer.

TABLE 75.3. The Major EBV-Associated Tumors and Their Suggested Origins

Other risk factors for BL are not well understood. There is a remarkable concordance between the distribution of eBL and regions of hyper- and holoendemic *Plasmodium falciparum* malaria (2), although the reason for this is unknown. The immunosuppressive nature of malaria has been cited (165,168), but immunosuppressed individuals develop IL not BL (see earlier). An alternate explanation is that the high antigenic load of malaria results in chronic stimulation of the B-cell system, increasing the risk of genetic abnormalities (169). Support for this theory comes from mouse models in which prolonged stimulation with malarial antigen can generate tumors that carry the characteristic genetic translocation of BL (170). The true explanation may be a combination of the two, as suggested by the emergence of a third group at risk for BL, AIDS patients in the early stages of the disease (129). This may suggest that the predisposing factor for BL is heavy antigenic load, in this case from opportunistic infections, in the presence of limited immunosuppression.

In summary, BL is likely to be a tumor derived from a B cell (after prolonged stimulation with antigen) that becomes arrested in the germinal center because of deregulated expression of c-*myc*. As with HD, EBV is likely to be a predisposing but not essential component in the development of the tumor. Unlike HD, in which expression of LMP1 and LMP2 provides a possible role for EBV in tumor growth, there is no obvious candidate viral gene or genes to account for the role of EBV in the development of BL.

Epstein-Barr Virus and Epithelial Cells

THE ASSOCIATION OF EPSTEIN-BARR VIRUS WITH EPITHELIAL CELLS

It is broadly accepted that EBV is associated with epithelial cell diseases. EBV was found in NPC (4) soon after the discovery of the virus. With the advent of AIDS, an additional EBV-associated epithelial disease, oral hairy leukoplakia (171), was described, in which EBV replicates aggressively in epithelial cells of the tongue. It is important to stress, however, that there is currently no established body of evidence to show that EBV normally infects epithelial cells. Such an association was originally proposed based on a series of studies claiming to detect EBV in normal epithelial cells *in vivo* (172,173 and 174). As a result, EBV infection of lymphoepithelium became part of the accepted view of EBV persistence and is frequently cited in review articles and chapters on EBV [see, for example (24,38)]. Recent attempts to reproduce these findings, by several independent groups, have failed (73,175,176). It is now established that EBV is present in lymphoepithelium

and does replicate there; however, the cells harboring and replicating the virus are B cells, and no evidence of epithelial infection has been found.

NASOPHARYNGEAL CARCINOMA

NPC arises in the posterior nasopharynx. It is a tumor of the mucosal lymphoepithelium, where EBV replicates to be shed into saliva or infect B cells as part of ongoing viral persistence and spreading (Fig. 75.1; Table 75.2). How this normally B-lymphotropic virus gains access to the epithelial cells is not fully understood, but recent studies suggest that a subset of epithelial cell lines can be infected with the virus because of low-level expression of the viral receptor CD21 (177).

NPC is one of the most common tumors in the world, with incidence rates that vary markedly with geographic location (178,179,180 and 181). High-incidence areas congregate around southern China, but the disease also is common among Inuit Eskimos and certain ethnic groups in North and East Africa. In these areas, the incidence rates can be as high as one per 10^3 per year. Age-adjusted incidence rates are about 100 times lower in the rest of the world, although NPC remains the most common carcinoma of young adults. There is an extremely long latency period between the time of primary infection and tumor onset. Most individuals in high-risk areas become infected in the first year of life, but the tumor has a peak incidence only in middle age (40 to 60) years.

In the high-incidence areas, the tumors lack markers of keratinization. They consist of poorly differentiated or undifferentiated epithelial cells [World Health Organization (WHO) types II and III] and are often referred to as lymphoepitheliomas because of the extensive infiltration of the tumor by lymphocytes. EBV is present in virtually 100% of all undifferentiated or poorly differentiated NPCs irrespective of geographic location (182). In the low-incidence areas, the tumors are more often well differentiated (WHO type I), and EBV association is less consistent.

Undifferentiated NPC is the only EBV-associated tumor found in immunocompetent hosts in which the virus association is virtually absolute. Therefore, NPC is a prime candidate for EBV playing a requisite causative role in tumor development. The viral genomes in the tumors are clonal (46), and the tumor cells express the default program (183,184 and 185). EBNA1 from the Q promoter has been detected in all cases, whereas LMP1 is present in about 65% of cases. Currently LMP2 has only been detected by reverse transcription/polymerase chain reaction (RT-PCR). The EBER and BART (186) transcripts also are present. Considerable effort has been expended in trying to establish the existence of NPC-associated strains of the virus, but currently the strains identified represent geographic rather than pathogenic variants (187). NPC has a unique virologic aspect, which is its association with the appearance in the serum of IgA antibodies against antigens of the viral lytic cycle (EA and VCA; see earlier) (4). IgA antibodies are typically associated with secretory immunity at mucosal surfaces. The appearance of these antibodies is curious because it suggests ongoing replication of the virus in the mucosa-associated tumor, yet analysis of tumor biopsies reveals them to be tightly latent, with little evidence of viral replication (188). These antibodies arise well before the tumor is detectable. Screening for these antibodies has been extremely effective as a way to detect individuals at risk for tumor development.

NPC tumor cells have an unusual cellular phenotype. They express a number of B-cell lineage markers not typically associated with epithelial cells, including CD40, CD70, CD80, MHC class II, and bcl-2 (189). This phenotype, together with the marked T-cell infiltration of the tumors, raises the possibility that tumor growth is a consequence of an interaction between the T cells and the epithelial cells, with the epithelial cells behaving in some sense like B cells. The presence of LMP1 and LMP2 in the tumor cells could then be seen as providing rescue/survival signals, similar to the model for HD. Expression of LMP1 in immortalized epithelial cell lines is sufficient to convert them to a fully malignant phenotype (190); however, it is insufficient alone to cause malignant transformation of primary epithelial cells. Thus EBV is only one step in a multistep progression. Patients at risk for developing NPC do not have any known immunodeficiency; therefore the tumor, like HD, is able to develop while expressing LMP1 and LMP2 in the face of an intact immune response.

Currently very little is known about the other risk factors for the development of NPC. A genetic risk factor is strongly suggested by the close linkage of the disease to specific racial types, such as the southern Chinese population This association is sustained, although somewhat diminished, when they move to the United States, and decreases in the offspring of mixed marriages (180). Potential environmental risk factors also have been proposed, most prominently eating salted fish during infancy (191).

In summary, NPC is likely to be a tumor that arises through the fortuitous infection of epithelial cells of mucosal lymphoepithelium due to the continuous presence of infectious virus produced at that site. On entry into the epithelial cell, the virus switches on the default program. Normally the default program would eventually be turned off in a latently infected germinal center or memory B cell when they exit the cell cycle to become resting memory cells. Because the epithelial cell cannot do this, the default program will stay on, constitutively delivering survival signals through LMP1 and LMP2 that may allow further genetic lesions to be tolerated. Therefore, EBV is probably an essential step in a multistep process leading to the carcinoma.

OTHER CARCINOMAS

EBV is associated with rare NPC-like carcinomas at other anatomic sites such as salivary gland carcinoma (192) and gastric carcinoma (193). Like NPCs, these tumors are undifferentiated and consistently EBV positive. Recently EBV has been associated with a subset of metastatic breast cancer (16) and hepatocellular carcinoma (17). In both cases, the tumor cells were negative for the EBER transcripts. Detection of EBERs is routinely used for screening biologic material for the presence of EBV because of the sensitivity of the test. These associations remain to be established, but if true, raise the possibility that association of EBV with carcinoma will need to be reassessed with probes other than the EBERs.

T-Cell Lymphoma

EBV is a B-lymphotropic virus; however, there is compelling evidence that under certain conditions, the virus can gain access to T lymphocytes. Rare EBV-positive T cells have been observed in the tonsils of patients with acute IM (74), and EBV is occasionally found in rare T-cell malignancies (194). These malignancies include T/NK cell lymphomas and VAHS-associated T-cell lymphoma.

The T/NK lymphomas are extranodal of angiocentric type and are also known as lethal midline granulomas. The cellular phenotype of the tumors varies considerably, suggesting that they may arise from different stages of T-cell development. The NK-cell marker CD56 is frequently expressed, suggesting NK cells as a possible precursor of the tumor (195). The tumor cells are universally EBV positive and express the default program of latent genes: EBNA1 from the Q promoter, LMP1 and LMP2 with no EBNA2 expression (15). It is significant that these tumors arise in the region of the body where EBV replication and infection are ongoing, the nasopharyngeal lymphoepithelium. This makes it likely that fortuitous infection of T/NK cells may be the initiating event in the development of these tumors.

VAHS-associated T-cell lymphoma has already been discussed in the context of XLP. T-cell involvement can be of both CD4 and CD8 subsets. The disease can range from polyclonal lymphocytosis to full-blown malignant clonal lymphoma (196). The disease onset is associated with primary EBV infection, suggesting a relationship to the VAHS seen in XLP.

IMMUNOLOGIC INTERVENTION

Three kinds of immunologic intervention to EBV have been proposed and are in various stages of application.

Subunit Vaccine

A subunit vaccine has been developed based on the major viral glycoprotein gp350/220, the major target for neutralizing antibodies (77). Preparations of this glycoprotein in various formats have proved successful in preventing lymphoma development in cotton-top tamarins (197). However, these trials were essentially meaningless because the animals were not infected orally, the known route of virus transmission. Thus they were essentially *in vivo* tests of the already well-established *in vitro* observation that antibodies to these glycoproteins will neutralize virus infection (77). Several major hurdles exist to the successful development of such a vaccine:

1. It is unclear whether a vaccine can provide sufficient protection to prevent the entry of the virus through the mucosa to establish infection of B cells in the lymphoepithelium.
2. NPC develops despite the lifetime presence of strong neutralizing antibodies.
3. The lag period between EBV infection and NPC is four to six decades; therefore, each vaccine trial will take a lifetime to be completed.
4. EBV infection in the developing world occurs within the first year of life, and it may be difficult to establish protective immunity at such an early age.

Peptide Vaccines

The use of peptide vaccines has been proposed to boost cellular immunity (87). The difficulties of this are manyfold.

1. It is unclear how peptide-based immunization for CTL immunity might protect against primary infection. All forms of latency, including cells expressing the growth program, the major CTL target, are manifestly present during persistent infection (60) despite continued CTL surveillance.
2. Most of the tumors arise in immunocompetent hosts, where they grow despite an intact CTL response. It is unclear, therefore, whether boosting this response will make any difference.
3. Each epitope is restricted to a particular MHC class I allele; therefore the epitopes used must be different for each individual.

Adoptive Transfer

Adoptive transfer of cultured, EBV-specific CTLs has been successful in the treatment and prophylaxis of PTLD (118). This is an ideal setting for this type of treatment because the role of CTLs in controlling PTLD is well established, and it allows the control of the disease without risking loss of the graft. However, it should be regarded as a special case (i.e., a virus-driven tumor in an immunologically suppressed environment). It should not be interpreted as a proof of concept for the treatment of other tumors with adoptively transferred CTLs because most tumors arise in the immunocompetent and do not express strong viral antigens.

EBV AND DISEASE: A SUMMARY

From the preceding discussion, it is apparent that some generalizations can be made about the association of EBV with neoplastic disease.

1. The diseases fall into two groups, those that arise from normal infected counterparts (IL, HD, and BL) and those that arise from aberrant infection of cells that reside in or around the mucosal lymphoepithelium, the site of EBV replication, shedding, and infection (NPC and T/NK lymphoma) (Table 75.3).
2. All the tumors that arise in the immunocompetent express the default program (EBNA1 from Q, LMP1, and LMP2) (Table 75.2 and Table 75.3). This program is expressed in normal infected germinal center and activated memory cells in mucosal lymphoepithelium. Taken together, it is apparent that the virus always uses the default program when it needs to ensure the survival of a latently infected cell. This survival/rescue function is the virus-associated risk factor for tumor growth in the immunocompetent. These tumors are frequently heavily infiltrated with T lymphocytes, suggesting that tumor cell/T-cell interactions may be a recurring theme in their pathogenesis.
3. The default program is expressed by both normal and malignant cells in immunocompetent hosts but does not appear to be under aggressive or successful immunosurveillance. This program is important because of its role in viral persistence. Therefore it may have evolved to be poorly recognized by the immune response. When expressed in the appropriate B-cell subsets, it is not a pathogenic threat because the cells switch off the program when they exit the cell cycle. The inability to switch off the program because of differentiation block or expression in inappropriate cell types would support tumor progression while not exposing the tumor to aggressive immunosurveillance.
4. The tumors that arise in the immunosuppressed express the growth program, but they are not tumors of lymphoepithelial naive B cells. The growth program is a potentially life-threatening state of infection, but not when it occurs in the biologically relevant tissue, the lymphoepithelial naive B cells. These cells can exit the cell cycle by differentiation into resting memory cells. Expression of the growth program by other B cells probably occurs aberrantly at some low level. These cells are destroyed by CTLs.

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76 TUBERCULOSIS AND LEPROSY

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TUBERCULOSIS

John D. McKinney and Barry R. Bloom

Origins of Tuberculosis and Its Impact on Human Health

Mycobacterium tuberculosis, the bacterium that causes human tuberculosis, is one of humanity's most ancient and formidable microbial adversaries. *M. tuberculosis* likely evolved from a closely related species, *M. bovis*, which is today a common cause of tuberculosis in wild and domestic mammals and a significant cause of zoonotic infection in humans (1). The tubercle bacillus resembles gram-positive organisms, but has a unique, lipid-rich cell wall that renders it stainable only after acid treatment, hence the term “acid-fast bacillus” (AFB) (2). This same impermeable waxy cell wall is one of the properties that renders the organism resistant to antimicrobial therapy and to drying in the environment, and probably to the microbicidal activities of phagocytes. In contrast to most bacterial diseases, infection by *M. tuberculosis* is persistent, even lifelong, and previous infection does not prevent reinfection. Persistent infection can be latent (inactive) or chronic (active). Latently infected individuals do not transmit the disease, but they have a 5% to 10% lifetime risk of reactivating and progressing to full-blown, infectious tuberculosis (3,4). In the words of one epidemiologist, “Following infection, the incubation period of tuberculosis ranges from a few weeks to a lifetime” (5). Immune suppression [e.g., by human immunodeficiency virus (HIV) infection] increases the risk of reactivation (6), but reactivation also is common in individuals who are not obviously immune suppressed. For tuberculin skin test–positive individuals, the lifetime risk of developing disease is about 10%, but the risk increases to nearly 10% *per year* for individuals who are coinfected with HIV. In a clinical study of 294 consecutive autopsies of HIV-positive cadavers in Abidjan, Côte d'Ivoire, tuberculosis was considered the prime cause of death (and the single commonest cause) in 32% and a major contributor in another 15% to 25% (7). In sum, there are currently about 16 million individuals with tuberculosis worldwide, 8 million new cases each year, and if one combines deaths from tuberculosis and acquired immunodeficiency syndrome (AIDS) as a result of tuberculosis coinfection, about 3 million deaths each year (8). By far the greatest burden of tuberculosis is felt in developing regions—particularly sub-Saharan Africa, South-East Asia, and eastern Europe—where infection rates are highest, access to medical care is limited or nonexistent, and HIV coinfection is increasingly common.

On current estimates, the global reservoir of latent tuberculosis is nearly 2 billion infected individuals, approximately a third of the earth's human population (8). Given time, approximately 100 to 200 million of these latent infections will develop into active tuberculosis, yet access to effective treatment to prevent reactivation is limited. In persons who are otherwise healthy, reactivation tuberculosis tends to run an indolent, chronic course. In the years before chemotherapy, it was common for chronically infected individuals to be sputum positive (infectious) for years or even decades (9). Even today, it is not uncommon for an individual with chronic tuberculosis to transmit the infection to scores of contacts without even being aware of the disease (10,11). The insidious and persistent nature of tuberculosis poses special challenges to efforts to control and eliminate the disease in communities, and largely accounts for the overwhelming disease burden caused by tuberculosis today.

Vaccination

Vaccination is the most cost-effective intervention available for the prevention of many infectious diseases. Indeed, it is thanks only to concerted vaccination efforts that smallpox has been eliminated as a cause of human morbidity and mortality (12), soon to be followed by poliomyelitis (13). All the more tragic that despite the introduction of the bacille Calmette-Guérin (BCG) vaccine into global public health use in 1928, a truly effective vaccine against tuberculosis remains elusive. Because nine of ten infected individuals never develop tuberculosis, there is ample evidence for at least partial acquired immunity in tuberculosis. It should be possible, in theory, to develop an effective vaccine to protect everyone. Tuberculosis, however, poses special problems for the design and evaluation of vaccine candidates. The first problem is the inadequacy of acquired immunity in natural infection. Most microorganisms elicit protective responses that lead to the rapid and complete elimination of the pathogen, and the establishment of immunologic memory that protects against reinfection. Neither is true in tuberculosis: long-term persistence of *M. tuberculosis* in the tissues is the rule, relapse and reactivation are common, and first exposure does not prevent reinfection. Thus a vaccine against tuberculosis would have to engender immune responses that are qualitatively or quantitatively more effective than those engendered by natural infection. How to identify the necessary and sufficient immune responses to assure protection is a major scientific challenge. A second problem arises from the unusual difficulty that the persistent nature of tuberculosis infection poses to vaccine evaluation. Most adult cases of tuberculosis are thought to arise from the latent residuals of an infection acquired in childhood. Thus, to be effective, a vaccine given at birth would have to protect against reactivation of disease in adulthood. This lengthy time frame poses daunting operational challenges for the creation and evaluation of vaccine candidates.

The only vaccine against tuberculosis in use today is the live-attenuated BCG vaccine introduced by Calmette and Guérin in 1921. It is currently the most widely used vaccine in the world, with 104 million children receiving it each year. The BCG strain was originally derived from virulent *M. bovis* and attenuated by extensive serial passage *in vitro* between 1908 and 1919 (14). The genetic basis of BCG attenuation remains unclear (15). BCG possesses many of the attributes of an ideal vaccine (16,17). It has a long and excellent record of safety: the case fatality rate is just 60 per billion, which is much lower than that of many other live attenuated vaccines, notably the smallpox vaccine. It is inexpensive to manufacture, as low as U.S. $0.10 per immunization. It can be given at birth, or any time thereafter, and a single inoculation elicits long-lived specific immunity. It is relatively heat resistant and is therefore suitable for even the poorest countries not reached by the “cold chain” that many vaccines require. It generates a characteristic scar, which is useful for epidemiologic surveillance. It provides well-demonstrated protection against the most severe forms of childhood disease, including meningitis and disseminated miliary tuberculosis. But unfortunately, these major advantages are overshadowed by the most important consideration of all: despite its widespread application for decades, the efficacy of BCG vaccination against adult tuberculosis remains controversial. In large case-controlled and randomized placebo-controlled trials, the protection conferred by BCG has varied enormously in different areas, ranging from nil to nearly 80% in the British MRC trial (16,17). Most disturbingly, in the largest field trial yet conducted, involving more than 350,000 individuals in the Chingleput district of India, BCG vaccination was not protective in any adult age group (18). Many theories have been forwarded to explain the extreme variability of BCG efficacy, but convincing evidence is lacking. The fact that BCG is still so widely used, despite doubts about its efficacy, has ethical implications for the evaluation of any new vaccine candidate. To gain acceptance, a new vaccine will have to be compared not just with a placebo control, but with the current BCG vaccines. This requirement will obviously complicate clinical trials of vaccine candidates, as well as increasing costs substantially. Given the solid evidence that BCG protects against severe forms of childhood tuberculosis, it could be argued that a placebo control would be unethical. Addressing these operational and ethical issues will require every bit as much ingenuity as the actual design of a vaccine candidate.

Chemotherapy

The lack of a suitable vaccine has shifted the emphasis to chemotherapy as an important agent in tuberculosis control. Effective multidrug therapy for tuberculosis has been available since the introduction of isoniazid in 1952 (19,20). Unlike many bacterial infections, tuberculosis is always treated with multiple drugs, because monotherapy almost invariably leads to treatment failure and bacterial drug resistance. First-line drugs against tuberculosis are isoniazid, rifampicin, pyrazinamide, and ethambutol. Second-line drugs are streptomycin, kanamycin, ciprofloxacin, cycloserine, capreomycin, and *p*-aminosalicylate. Currently, the U.S. Centers for Disease Control in Atlanta recommends a “short-course” regimen administered in two stages (21). The first, “induction” phase consists of isoniazid, rifampin, pyrazinamide, and ethambutol or streptomycin, given every day for 2 months. This is followed by a “continuation” phase of isoniazid and rifampin given twice weekly for 4 to 7 months.

Second-line drugs are typically given only in cases of drug resistance, which requires much longer treatment—typically 2 years, if there is resistance to isoniazid and rifampin. Short-course chemotherapy can provide a lasting cure for 90% or more of patients who complete therapy (22), despite a significant risk of reinfection in high-incidence areas (23), but 90% cure rates are seldom achieved in practice. The problem is the human element. Without close supervision, most patients will fail to complete the 6 to 9 months of therapy needed to ensure a lasting cure (24,25,26,27 and 28). Patients who default on therapy put themselves at greatly increased risk of treatment failure, relapse, and evolution of bacterial drug resistance. Thus, largely because of treatment nonadherence, resistance to every one of the first- and second-line antituberculosis drugs has emerged in clinical settings. Although the exact numbers remain uncertain, the World Health Organization (WHO) estimates that perhaps 50 million individuals worldwide have been infected with multidrug-resistant (MDR) strains of *M. tuberculosis* resistant to both rifampin and isoniazid (29). Some of these MDR strains are untreatable except with second-line drug regimens, and these are often unavailable in developing countries.

Because treatment failure and emergent drug resistance are the result of incomplete chemotherapy, attention has focused on strategies to improve patient adherence. The WHO advocates a strategy of directly observed therapy short-course (DOTS), in which each dose of medications is directly administered by a health care or social worker (29,30). Although DOTS, administered properly, can improve cure rates substantially, there is a growing realization that DOTS alone, which has been implemented in only 20% of developing regions, is not going to rein in the global epidemic of tuberculosis (31). Given the treatment duration required and its impact on patient adherence, development of faster-acting regimens is a high priority. Here, as in so many other facets of tuberculosis, persistence is the problem. Conventional drugs are rapidly effective against tubercle bacilli growing *in vitro*, but they take much longer to kill bacilli *in vivo* (17,32). Why? Penetration of drugs into tuberculous lesions seems to be adequate (33), so it is unsurprising that *in vivo* killing is not accelerated by simply adding more drugs or increasing drug dosage (34,35), What, then, is the explanation for the observed tolerance of *in vivo* tubercle bacilli to antimicrobials? This area of tuberculosis research has received surprisingly little attention, considering its manifest importance. The answer presumably lies in the physiology and growth state of persistent bacteria *in vivo*, which differ from *in vitro* grown bacteria in many respects (36). Indeed, even the basic carbon metabolism of persistent tubercle bacilli is profoundly altered *in vivo* (37), and these variables could influence the effectiveness of antimicrobials.

Pathogenesis: The Natural History of a Tuberculosis Infection

Tuberculosis is transmitted when an individual with active pulmonary disease coughs, sneezes, expectorates, or speaks, releasing tremendous numbers of very small droplets containing tubercle bacilli. These droplets may remain suspended in the air for many hours before they are inhaled and enter the lung. Infection initiates with the implantation of an inspired particle in a pulmonary alveolus, deep within the terminal ramifications of the respiratory tree. Particles that settle higher in the tree are presumably removed by the mucociliary elevator. To reach the alveoli, particles must be very small, no more than ~5 µm in diameter; a particle of this size could not accommodate more than ten tubercle bacilli. Autopsy studies indicate that most cases of human tuberculosis originate from a single respired particle (38), so the infectious inoculum in tuberculosis is presumably quite small. Bacteria that reach the alveoli are immediately phagocytosed by resident alveolar macrophages (39), professional phagocytes that roam the lung, scavenging and destroying foreign invaders. The tubercle bacillus has evolved to parasitize this otherwise inhospitable cell, and replicates intracellularly within membrane-bound vacuoles. Eventually, the bacterial load overwhelms the parasitized macrophage, which lyses and releases the bacteria. These are ingested by fresh alveolar macrophages and monocyte-derived macrophages that emigrate from the bloodstream, and the cycle begins anew. As the bacillary population grows, a nascent lesion or “tubercle” forms, which remains focalized for perhaps 2 weeks, until the expanding tubercle breaches the parenchyma. Bacteria then escape from the primary lesion and are carried to the regional lymph nodes. There, bacterial replication continues, resulting in pronounced lymphadenopathy, which is characteristic of primary tuberculosis (Fig. 76.1A) but uncommon in postprimary (reactivation or reinfection) tuberculosis (Fig. 76.1C). The parenchymal lesion and associated lymph nodes are known as the “primary complex” or “Ghon complex” (40). From the draining nodes, tubercle bacilli are carried via the thoracic duct to the bloodstream, whence they are disseminated to the extrapulmonary organs and to previously unaffected regions of the lung. Within these metastatic foci, bacterial replication continues until a specific T-cell response emerges at 3 or more weeks after infection. Activation of infected macrophages by T-cell–derived cytokines, such as interferon-g (IFN-g) and tumor necrosis factor-a (TNF-a), curtails further replication of the bacteria. A mature granuloma forms, consisting of a central area of necrotic tissue with large numbers of extracellular bacilli, encompassed by a mantle of macrophages harboring intracellular bacilli (which typically include multinucleate Langhans giant cells), surrounded in turn by a sheath of lymphocytes, consisting mainly of $CD4^+$ and $CD8^+$ T cells (Fig. 76.2). The emergence of *Mycobacterium*-specific T-cell populations is signaled by the development of a delayed-type hypersensitivity (DTH) response to intradermally injected antigens of the tubercle bacillus (tuberculin).

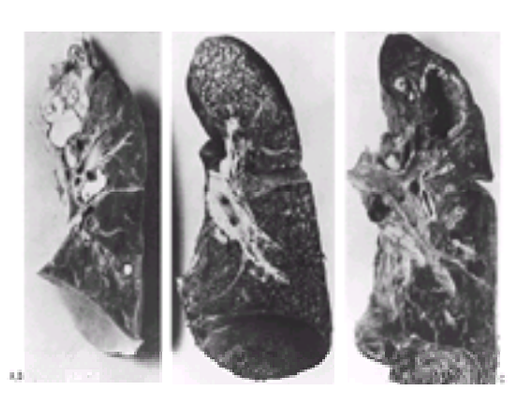

Figure 76.1. A: Primary tuberculosis in a 2-year-old child. A small primary lesion is located in the lower lobe of the left lung. The hilar lymph nodes are greatly enlarged and caseated. **B:** Miliary tuberculosis in an 8-year-old child. Numerous small “millet seed” lesions are evenly distributed throughout the lung. **C:** Postprimary tuberculosis in a 44-year-old woman. A large cavitary lesion is located in the upper lobe of the left lung. The hilar nodes show no appreciable involvement. (Reprinted from Rich AR.*The pathogenesis of tuberculosis.* Springfield, IL: Charles C Thomas, 1944:145 **(C)**, 467 **(A)**, and 775 **(B)**, with permission.)

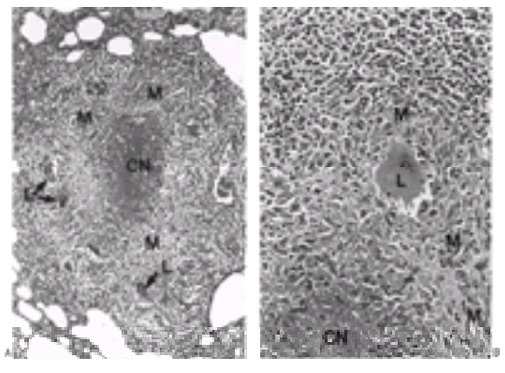

Figure 76.2. Histopathology of a tubercle at low **(A)** and high **(B)** magnification. A central region of caseous necrosis (*CN*) is surrounded by a zone of epithelioid macrophages (*M*), some of which have fused to form multinucleate Langhans giant cells (*L*). The macrophage zone is encompassed by a mantle of lymphocytes, primarily $CD4^+$ and $CD8^+$ T cells. (Reprinted from Wheater PR, Burkitt HG, Stevens A, Lowe JS. *Basic histopathology: a colour atlas and text*. 2nd ed. Edinburgh: Churchill Livingstone, 1991:28, with permission.)

What happens next depends on the immune status of the host. Most (~90%) healthy individuals will hold the infection in check indefinitely. Indeed, autopsy studies suggest that most tuberculous lesions in humans are completely sterilized over time (41,42,43 and 44). Sterilization of lesions correlates with calcification of the necrotic tissue and walling off of the diseased tissue within a fibrous capsule. This is an important and frequently overlooked point: self-sterilization of pulmonary lesions is common in humans, but it has not been observed in animal models of infection, including mice, rabbits, guinea pigs, and monkeys. The explanation for this species difference is unknown, but it is a critical issue for those studying host mechanisms of defense against tuberculosis. A minority of individuals, ~ 5% of all infected persons, fail to control the infection and develop “primary progressive” tuberculosis within a year or 2 of infection (Fig. 76.1A). It is currently impossible to predict who will or will not develop primary progressive tuberculosis, but those at particular risk include infants, the elderly, and individuals with compromised immunity due to HIV infection, malnourishment, stress, steroid therapy, and genetic deficiencies affecting the immune system. In extreme cases, unrestricted dissemination and multiplication of bacilli can lead to “miliary” tuberculosis affecting the lungs and extrapulmonary organs (Fig. 76.1B). Miliary tuberculosis usually progresses rapidly to a fatal conclusion unless treated promptly. Another ~ 5% of all infected individuals will hold the infection in check initially, only to reactivate after years or even decades of clinical latency. Reactivation gives rise to “postprimary” tuberculosis, which tends to remain focalized at the site of reactivation, most commonly the apex of the lung (Fig. 76.1C). Erosion of a tuberculous lesion into an airway promotes bronchogenic spread of tubercle bacilli within the lungs and expulsion of bacilli from the lungs when the infected person coughs or expectorates. The appearance of bacilli in the sputum is correlated with infectivity, so the identification and aggressive treatment of

“sputum positive” patients is currently the most effective means of preventing transmission.

The pathogenesis of primary versus postprimary tuberculosis differs in several respects. In primary tuberculosis, lymphadenopathy is pronounced, the disease process tends to become generalized by lymphatic and hematogenous dissemination, and caseating necrosis of infected tissue is extensive, with rapid expansion of lesions and little reparative fibrosis (Fig. 76.1A). In postprimary tuberculosis, lymphadenopathy is slight or absent, the disease process tends to remain focalized at the site of reactivation, and lesions typically develop slowly, with extensive fibrosis (Fig. 76.1C). Cavitation, one of the most severe forms of pathology in tuberculosis, is common in postprimary tuberculosis, but rarer in primary tuberculosis. Tuberculous cavities are excavated by the expulsion of liquified necrotic tissue into an airway (Fig. 76.1C). Liquefaction of necrotic tissue, leading to cavity formation, has been linked to an excessive DTH response of the host (45). Cavitation is thus an important example of immunopathology in tuberculosis (46). One of the most striking differences between primary and postprimary tuberculosis is the localization of the disease process. Primary tuberculosis occurs anywhere in the lungs (Fig. 76.3A). In contrast, postprimary tuberculosis is largely restricted to the upper lung fields (Fig. 76.3B); cavities, in particular, are located almost exclusively in the upper lungs (Fig. 76.3C). These clinical observations suggest that acquired immunity is effective in the lower lung but not in the upper lung. Why is the upper lung so vulnerable to tuberculosis? Theories abound, but the problem remains mysterious and little studied (17,47). This neglect is perplexing: “The importance of the problem becomes evident when it is realized that, were it not for the failure of the apices to share equally in the increased resistance of the infra-apical portions, there would be almost no pulmonary tuberculosis in white adults” (48).

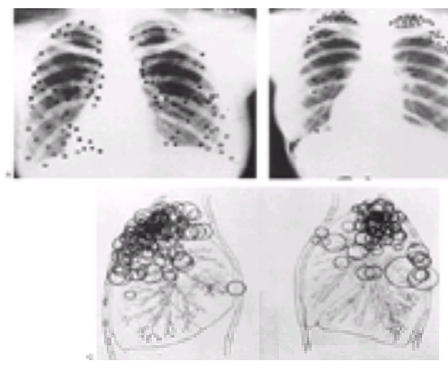

Figure 76.3. A: Location of calcified parenchymal foci (inactive) in 105 individuals with a single primary complex. Foci are randomly distributed throughout the lungs. **B:** Location of parenchymal lesions (active) in 55 individuals with postprimary tuberculosis. Lesions are largely restricted to the upper lung fields. (Reprinted from Medlar EM. The pathogenesis of minimal pulmonary tuberculosis: a study of 1,225 necropsies in cases of unexpected and sudden death. *Am Rev Tuberc* 1948;58:583–611, with permission.) **C:** Location of 268 cavities in 204 patients with postprimary tuberculosis. Cavities occur almost exclusively in the upper lungs. (Reprinted from Sweany HC, Cook CE, Kegerreis R. A study of the position of primary cavities in pulmonary tuberculosis. *Am Rev Tuberc* 1931;24:558–582, with permission.)

It must be emphasized that tuberculosis is a persistent infection. Latent infection can give rise to active disease years or decades after exposure. Established lesions may oscillate between periods of activity and dormancy. Chronic or sporadic disease is the rule, and chronically ill patients may transmit infection for many years, even in cases in which antimicrobials are readily available (Fig. 76.4). The unusual ability of *M. tuberculosis* to maintain this long-term relationship with the host, in which the host survives but continues to shed bacilli over long intervals, is the key to the unparalleled success of this tenacious pathogen.

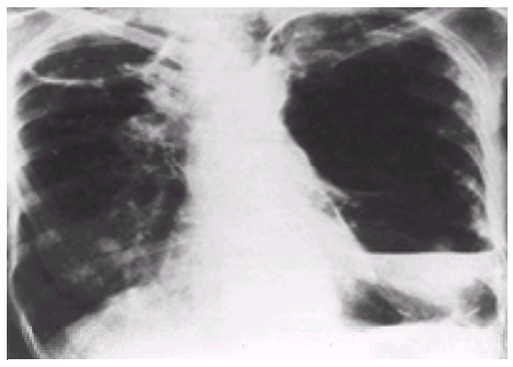

Figure 76.4. Chest radiograph of a 70-year-old woman obtained shortly before her death. “A white woman had cavitary tuberculosis of 41 years' duration. She had bilateral bronchiectasis, cavitation of the right upper lobe, a collapsed left lung, pleural effusion, and an induced left pneumothorax of 36 years' duration. She had received no antituberculous drugs for 28 years from the onset of her illness, and these drugs, when finally prescribed, were taken inadequately. Pleural effusion present for 28 years required aspiration. *Mycobacterium tuberculosis* recovered by culture of sputum was resistant to all antituberculous drugs except streptomycin [which the patient had never received due to allergy].” (Reprinted from Edwards WM, Cox RS Jr, Cooney JP, et al. Active pulmonary tuberculosis with cavitation of forty-one years' duration. *Am Rev Respir Dis* 1970;102:448–455, with permission.)

Animal Models of Tuberculosis and Genetics of Resistance

Among his many “firsts” in the realm of tuberculosis, Robert Koch (49) showed that *M. tuberculosis* will infect, replicate, and cause disease in an astonishing menagerie of mammalian species, including dogs, cats, guinea pigs, rabbits, and mice. Many of these species, with the addition of monkeys, are still used today as animal models of tuberculosis. There is no single ideal animal model of tuberculosis: each model offers unique advantages and shortcomings.

Guinea pigs are exquisitely susceptible to tuberculosis, and uniformly die within 6 to 9 months when infected by the respiratory route with even a minute (five to ten bacilli) inoculum (50,51 and 52). Genetic differences in resistance to tuberculosis were recognized in inbred guinea-pig strains long ago (53), but the biologic basis remains unknown. The reason for the extreme susceptibility of guinea pigs to tuberculosis also is not known, but it is not because of a failure of these animals to control bacillary replication. Indeed, a bacterial load that is fatal to a guinea pig is tolerated by a mouse, notwithstanding the fact that the latter animal has one tenth the body mass of the former (51,52,54). It is tempting to speculate that the difference may reflect the relative contribution of immunopathology to disease in different species, but this has not been proven. Whatever the reason, infected guinea pigs develop a fulminating form of disease that resembles primary tuberculosis in susceptible humans. This has made the guinea pig a species of choice for the evaluation of vaccines against tuberculosis (51). The underlying belief is that a vaccine capable of protecting the highly susceptible guinea pig would also stand the best chance of protecting susceptible humans (i.e., the relevant target for vaccination).

Of the nonprimate animal models of tuberculosis, the rabbit develops pathology that is most faithful to the disease in humans. The classic work of Max Lurie (55) with inbred rabbit lines that he created is the best-studied example of genetic variation in susceptibility to tuberculosis. This difference is manifest at the level of innate resistance to infection, in two ways. First, resistant rabbits require a much larger inoculum of tubercle bacilli to generate a single pulmonary lesion (Fig. 76.5A). Second, growth of the inhaled bacilli in the lungs is delayed by ~4 days in resistant as compared with susceptible animals (Fig. 76.5B). Although the rate of bacillary growth that ensues is similar in both strains, the bacterial load in resistant rabbits never closes the gap (Fig. 76.5B). The rabbit is relatively resistant to tuberculosis and develops a chronic form of the disease that resembles postprimary tuberculosis in humans, although without an intervening period of latency. The rabbit also is the only nonprimate species that routinely develops cavitary lesions, which makes it uniquely valuable for the study of this important pathologic process (46,56,57). Potentially as important is the observation that cavitary lesions are localized in the rabbit in a manner that is reminiscent of apical localization of cavities in humans. In the rabbit, however, cavities are localized to the dorsal/caudal region of the lung, which is the most elevated region of the lung in rabbits because of their four-footed posture and oversized haunches (58). The same localization of cavities to the dorsal/caudal lung fields also is seen in bovine tuberculosis (59). In a daring study that would no longer be ethically acceptable, it was shown that the localization of disease could be shifted by 180 degrees, to the apex of the lung, simply by forcing infected rabbits to stand upright (58). These observations suggest that the rabbit could be developed as a model to study the apical localization of postprimary tuberculosis, an important and sorely neglected problem in the field of tuberculosis research.

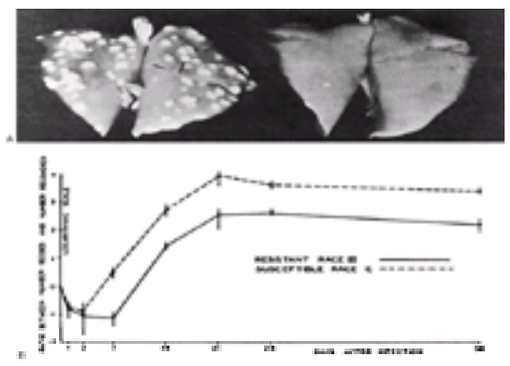

Figure 76.5. A: Lungs of Lurie's genetically susceptible (**left**) and resistant (**right**) rabbits 33 days after inhalation of ~10^4 colony-forming units of *Mycobacterium tuberculosis*. Formation of a single macroscopic tubercle required inhalation of 36 bacilli in susceptible rabbits versus 932 bacilli in resistant rabbits. **B:** Bacterial loads in the lungs of genetically susceptible and resistant rabbits after quantitative airborne infection with *M. tuberculosis*. Bacterial growth is delayed in the lungs of resistant rabbits as compared with susceptible rabbits. *(Reprinted from Lurie MB, Resistance to tuberculosis: experimental studies in native and acquired defensive mechanisms. Cambridge, MA: Harvard University Press, 1964:196* ***(A)****, 201* ***(B)****, with permission.)*

Although the guinea pig and rabbit offer unique advantages for the study of tuberculosis, their utility has been limited by the high cost of purchasing and maintaining these animals, the unavailability of inbred strains, the paucity of species-specific immunologic reagents, and the absence of technologies for generating transgenic and gene knock-out animals. All of these considerations have made the laboratory mouse by far the most intensively studied model of tuberculosis. On the debit side, the pathogenesis of tuberculosis in the mouse bears little resemblance to the disease in humans (60,61). Mice do not generally develop caseating necrosis or liquefaction, lesions do not calcify or encapsulate, and cavitation does not occur, even at late stages of disease. Indeed, mice are relatively resistant to tuberculosis and survive to nearly their normal life span after infection with a small number of bacilli by the respiratory route (62,63). Notwithstanding these shortcomings, the mouse has proven extremely useful for the elucidation of host defense mechanisms against tuberculosis, and many of the lessons first gleaned in the mouse model have been verified subsequently in clinical studies of human patients. A prominent example is the role of reactive nitrogen intermediates (RNI) in resistance to tuberculosis (64,65). It has been known for some time that inbred mouse strains display significant variability in their resistance to tuberculosis (66). In recent years, efforts have been initiated to identify the underlying genetic (67,68).

Monkeys and other nonhuman primates are potentially valuable models of tuberculosis, but their utility has been limited by high cost, ethical considerations, and the difficulty of maintaining colonies of these large animals over the long periods that studies of tuberculosis demand. Rhesus macaques are highly susceptible to tuberculosis and develop a fulminating form of disease (69), but recent studies suggest that cynomolgus monkeys develop a chronic disease that more closely resembles adult-type tuberculosis in humans (70). Importantly, anecdotal evidence suggests that some species of monkeys may provide a model of latent tuberculosis that closely resembles latency, reactivation, and postprimary tuberculosis in humans. This is an exciting possibility, because there is currently no small animal model of tuberculosis latency, and little prospect for the development of one. Over the years, several murine models of latent tuberculosis have been proposed, but these bear little resemblance to the clinical features of latent tuberculosis in humans (62,71,72,73,74,75,76 and 77). Given the importance of latent infection to the epidemiology and pathogenesis of tuberculosis in humans, the primate model is one avenue that deserves further exploration.

Among mammals, humans are uniquely resistant to tuberculosis. This presumably reflects the long history of coevolution of *Homo sapiens* and *M. tuberculosis*. As discussed earlier, ~90% of infected humans never progress to active disease, and humans are the only mammal known to be capable of self-sterilizing tuberculosis lesions. The mechanism(s) responsible for sterilizing immunity is not known, but the species restriction of this phenomenon suggests that animal models might not yield the answer. The native resistance of *H. sapiens* to tuberculosis is illustrated by a tragic accident, the Lubeck disaster of 1929/1930, in which 251 infants were accidentally inoculated with a virulent strain of *M. tuberculosis* rather than the BCG vaccine that was intended (78,79). Of the 251 vaccinees, 72 died of tuberculosis during the first year of life. Of the remainder, 175 infants who were still alive after 4 years of observation showed arrested macroscopic lesions by radiograph. Only four infants escaped infection or suppressed infection so efficiently that macroscopic pathology was not visible. Understandably, the appalling mortality suffered by the Lübeck victims riveted the public eye and cast a pall over the acceptance of the BCG vaccine that persisted for many years. It should not be overlooked, however, that the great majority (175 of 247) of the exposed infants overcame massive infection with highly virulent *M. tuberculosis*, despite having no previously acquired resistance. From this perspective, the Lübeck disaster provides a striking illustration of the native resistance of the human species to tuberculosis.

The question arises why some infected individuals develop disease while others do not. Although environmental factors undoubtedly influence susceptibility to tuberculosis (17), accumulating evidence also points to an important role for genetic factors in resistance to infectious diseases (80). Considering what a powerful selective force *M. tuberculosis* has exerted on certain human populations since antiquity, it seems plausible that races with a longer history of contact would have evolved greater resistance. Although definitive studies are lacking, a wealth of clinical observations suggest that individuals of African origin might be more susceptible than individuals of European origin (10,78,81,82,83,84,85 and 86). This partitioning of resistance along racial lines is historically plausible, because tuberculosis was not introduced to sub-Saharan Africa until the late nineteenth or early twentieth century. Similarly, tuberculosis did not reach remote regions of South America until the mid-twentieth century, but it is now devastating Amerindian populations there. Suggestively, a field study of the native Yanomami of the Brazilian Amazon basin found that in exposed adults, a fulminating form of the disease develops, resembling tuberculosis in children or immunocompromised patients, accompanied by unusually high rates of tuberculin anergy (87).

A role for heredity in human resistance also is supported by twin studies, which point to a greater concordance of risk for tuberculosis among monozygotic than dizygotic twins (88,89,90 and 91). Native resistance may be determined, in part, by the ability to present mycobacterial antigens to T cells. For example: several studies show disproportionate representation of certain human leukocyte antigen (HLA) haplotypes, particularly HLA-DR2, in severe cases of tuberculosis (92,93). In a recent study, mutations at the human *NRAMPI* locus were associated with increased risk of tuberculosis in West African populations (94), although this association was not seen elsewhere (95,96). This is an intriguing observation, because *NRAMPI* allelism in the mouse strongly influences resistance to a number of intracellular pathogens (97), albeit not including *M. tuberculosis* (98,99). Although *NRAMPI* itself might not influence human resistance to tuberculosis, it is possible that naturally occurring polymorphisms at *NRAMPI* are in linkage disequilibrium with a distinct but contiguous resistance locus (68). As discussed later, null mutations in specific cytokine receptors are associated with increased susceptibility to infection with *Mycobacterium* spp. (100,101,102 and 103).

Unraveling the genetic basis of resistance or susceptibility to tuberculosis is an important goal. Despite exposure to a potentially lethal microorganism, 90% of *M. tuberculosis*-infected individuals remain healthy indefinitely. Knowledge of the genetic basis of susceptibility might suggest strategies to extend some measure of protection to the less well endowed 10% who would otherwise develop active tuberculosis. Particularly for immunologists and physicians interested in vaccine development, “it is necessary, therefore, to attempt to gain as clear an understanding as possible concerning the nature, the mechanism and the effects of native resistance in this disease” (104).

Immunoprotection and Immunopathology

Tuberculosis is a classic example of an infectious disease in which the signs, symptoms, and pathology of disease are to a large extent attributed to the host response to the pathogen rather than to direct toxicity of the pathogen itself. But it is also clear that host immunity is essential for resistance to tuberculosis: immune deficient hosts (e.g., HIV-infected individuals) are unable to contain the replication of the tubercle bacillus and succumb more often and more rapidly to infection. A critical question that remains unanswered to this day is whether immunopathology is the unavoidable price paid for protection. It is by no means clear that protection and tissue damage are mediated by distinct mechanisms. Many host defense mechanisms (e.g., generation of reactive intermediates of oxygen and nitrogen) are potentially autotoxic. A multicellular organism can sacrifice some tissue in the struggle to oust a foreign invader, but this gambit is dangerous in the case of pathogens that are not rapidly eliminated. A persistent infection like tuberculosis may lead to gradual, progressive, and eventually overwhelming tissue destruction simply because the host, failing to drive the enemy from the field, cannot disengage. Conversely, there are hints that the “protective” and “tissue-damaging” host responses in tuberculosis may be dissociable to some extent (17,46). The rational design of new vaccines and immunotherapies against tuberculosis will hinge on a fuller understanding of the effector mechanisms responsible for protection and damage.

The “dual nature” of the host response to *M. tuberculosis* was first discovered by Robert Koch in the late nineteenth century, ultimately leading, ironically, to his discredit. Koch (49) won enduring fame when he announced his discovery of *M. tuberculosis*, the etiologic agent of tuberculosis, in a historic address to the Berlin Physiological Society on March 24, 1882. His subsequent reports of a remedy for the disease met with less happy results. In a series of seminal experiments, Koch demonstrated that intradermal injection of a crude extract of the tubercle bacillus (“Old Tuberculin”) into a tuberculous guinea pig caused necrosis and sloughing of the tissue both at the site of injection and at the site of previous infection. This rapid necrotic response, known since as the “Koch Phenomenon,” did not occur when Old Tuberculin was injected into uninfected animals. Encouraged by this demonstration of seemingly beneficial immune modulation, Koch announced to the Tenth International Congress of Medicine, meeting in Berlin in 1890, that he had discovered a substance capable of ameliorating tuberculosis (105,106). At that time, tuberculosis alone was responsible for perhaps one in seven of all deaths in Europe, and no effective treatment was available. So it is hardly surprising that Koch's

announcement of a “cure” met with overwhelming public enthusiasm and acclaim—initially. Unfortunately, clinical studies of tuberculin soon proved that it had little value as a remedy, and could even produce a fatal “tuberculo-shock” in severely affected patients. Although the clinical application of tuberculin was quickly abandoned, the insights painfully gleaned during tuberculo-therapy's vogue laid the foundation for the emerging field of cellular immunology. In modern parlance, the Koch Phenomenon is the first recognized example of the DTH response, which is still considered the touchstone for studies in cell-mediated immunity.

Host Defense

THE ROLE OF THE MACROPHAGE

Koch's (49) histopathologic studies of infected tissues from humans and experimental animals demonstrated that tubercle bacilli growing *in vivo* were localized within large mononuclear cells, which we now know to be macrophages. The recognition that resistance to tuberculosis could be expressed at the level of the infected macrophage came many years later, when Lurie (55) demonstrated that tubercle bacilli grew luxuriantly in macrophages from normal rabbits but were inhibited within macrophages from tuberculous animals. Inhibition was not seen in tissue-culture experiments with highly purified macrophages, whether derived from normal or infected animals, suggesting the involvement of some additional, unrecognized factor. Rich (78) noted the abundance of lymphocytes at the perimeter of tuberculous lesions and suggested that they might play a role in acquired immunity, but he lacked the means to determine their function. In a classic experiment, Chase (107) demonstrated that cutaneous hypersensitivity to tuberculin could be transferred to naive animals by cells (but not serum) derived from the lymphoid organs of tuberculous animals. Mackaness and Blanden (108) demonstrated that antigen-stimulated lymphocytes (now known to be T cells) could trigger macrophages to express nonspecific antimicrobial activity. Evidence that T cells could mediate delayed hypersensitivity responses by secreting diffusible factors (cytokines) was reported independently by two groups in 1966 (109,110). Twenty years later, IFN-g and TNF-a were identified as the key cytokines mediating activation of macrophages to inhibit the intracellular growth of *M. tuberculosis* (111,112).

In the tissues of infected animals and humans, tubercle bacilli are found almost exclusively within cells of the mononuclear phagocyte lineage. This cellular tropism is a puzzle, because tubercle bacilli are highly invasive *in vitro* and will productively infect many cell types. A number of different cell-surface molecules have been implicated in the invasion of macrophages by mycobacteria, including complement receptors, mannose receptor, CD14 [the receptor for lipopolysaccharide (LPS)/LPS-binding protein], Fc receptors, and scavenger receptors (113). Tubercle bacilli that have invaded a resting (unactivated) macrophage replicate exponentially within the confines of a tightly apposed vacuolar membrane. The vacuole apparently divides in synchrony with the bacteria, so it is uncommon to find a vacuole containing more than one bacterium, even in a heavily infected cell (114). Normally, vacuoles containing bacteria or other internalized particles undergo maturation, acidification to pH 4.5 to 5.0, and fusion with lysosomes, where the internalized material is digested. *M. tuberculosis* enters early endosomes and somehow inhibits their maturation, perhaps by inhibition of calcium signaling after phagocytosis (115), perhaps by retention of the actin-binding host coronin “TACO” on the *Mycobacterium*-containing phagosome (116), which is correlated with invasion through cholesterol-rich plasma membrane domains (117). Whatever the precise mechanism, vacuoles containing tubercle bacilli fail to mature, acidify only partially (to pH ~6.5), remain in contact with the recycling endosome network, and fail to fuse with lysosomes (118,119 and 120). The block to phagosome maturation is important for the intracellular survival and replication of *M. tuberculosis* (115,121), presumably because it provides the organism with a more hospitable environment. The *Mycobacterium*-induced block to phagosome/lysosome fusion is reversed by activation of the macrophage with IFN-g and LPS (122,123). By subjecting the bacteria to the harsh environment of the fused phagolysosome, “induced maturation” of *Mycobacterium*-containing vacuoles might play an important role in host defense against tuberculosis.

Murine macrophages activated with IFN-g and TNF-a produce superoxide and other reactive oxygen intermediates (ROIs), as well as nitric oxide and other reactive nitrogen intermediates (RNIs), which inhibit intracellular mycobacteria (124). Early in the course of infection, IFN-g may be provided by natural killer (NK) cells and gd T cells. At later stages of infection, *Mycobacterium*-specific T cells are presumably the most important sources of this key cytokine. Activated macrophages are professional antigen-presenting cells that express both major histocompatibility complex (MHC) class I and II for antigen presentation to $CD4^+$ and $CD8^+$ memory T cells, respectively. Unlike dendritic cells, however, macrophages do not express the costimulatory molecules required to activate naive T cells and initiate an adaptive response (125). Activation and maturation of dendritic cells after uptake of *M. tuberculosis* (126) is therefore likely to play a key role in priming the protective response to infection (127,128).

The importance of both $CD4^+$ and $CD8^+$ T-cell subsets for resistance to tuberculosis is underscored by the extreme susceptibility of knock-out mice that are selectively deficient for either class of T cells (129,130 and 131). In humans, an essential role for $CD4^+$ T cells is suggested by the frequency and severity of tuberculosis in HIV-infected individuals (6). A role for $CD8^+$ T cells in human infection also is likely, as discussed later, but direct evidence is lacking.

Infected macrophages secrete the proinflammatory cytokines TNF-a and interleukin-12 (IL-12). Production of TNF-a and IL-12 is triggered by the interaction of mycobacterial cell-wall components with Toll-like receptors (TLRs) (132,133), which are pattern-recognition proteins displayed on the surface of monocytes and macrophages (134). The IL-12 released from infected macrophages triggers IFN-g secretion by NK cells and T cells (135,136 and 137). Conversely, IFN-g and TNF-a enhance IL-12 release by infected macrophages, generating a potential positive-feedback loop for macrophage activation. Infected macrophages themselves are copious secreters of TNF-a, and are likely responsible for much of the accumulation of TNF-a in tuberculosis lesions (138,139 and 140). Chronic overproduction of TNF-a is thought to be responsible for many of the signs and symptoms of advanced tuberculosis, such as tissue necrosis, fever, malaise, wasting, fatigue, and night sweats (141). Thus TNF-a is thought to play a dual role in tuberculosis, mediating both protection and immunopathology. The importance of the cytokine network in resistance to tuberculosis has been demonstrated in the mouse model by infection of gene knock-out mice lacking IFN-g (142,143), TNF-a (144), TNF-a receptor p55 (145), or IL-12 (146). All of these mutations cause exquisite susceptibility to infection with *M. tuberculosis*. In humans, naturally occurring mutations in the genes encoding the IL-12 receptor (102,103) or IFN-g receptor (100,101) are associated with increased susceptibility to infections by *Mycobacterium* spp. (80).

Tubercle bacilli growing *in vitro* and in cultured macrophages are relatively resistant to ROIs but susceptible to RNIs (124). The contribution of these two antimicrobial mechanisms of macrophages to host defense is underscored by studies with gene-disrupted mice (65). Mice defective in the phagocyte oxidase responsible for ROI production ($phox^{-/-}$ mice) show moderately increased replication of *M. tuberculosis* at early stages of infection, but eventually stabilize bacterial numbers (147,148). In contrast, mice defective in the nitric oxide synthase responsible for IFN-g–inducible RNI output ($NOS2^{-/-}$ mice) are unable to curtail replication of *M. tuberculosis* and die rapidly (147,149). The “nonredundancy” of these two macrophage effector mechanisms in host defense is clear when one compares the response to different pathogens (65). For example, although $NOS2^{-/-}$ mice are more susceptible than $phox^{-/-}$ mice to infection with *M. tuberculosis*, the opposite is true of mice infected with another intravacuolar bacterium, *Salmonella typhimurium* (150). Why this difference? One possibility is that tubercle bacilli have evolved effective mechanisms to evade or counterattack by ROIs, but are less adept at dealing with RNIs. Several mechanisms have been identified that could contribute to the ROI resistance of tubercle bacilli: (a) invasion of macrophages via the CR1 or CR3 complement receptors does not trigger the oxidative burst (which might be true of other receptors, as well); (b) mycobacteria shed lipoarabinomannan (LAM) and sulfatides that inhibit ROI production; (c) LAM also is an efficient scavenger of ROIs; (d) mycobacteria produce superoxide dismutase and catalase, enzymes that detoxify ROIs. Mechanisms of resistance to RNIs are less well defined, but are likely to exist, because mycobacteria are capable of persistence *in vivo* despite exposure to potentially lethal RNIs (64,65,151). The role of RNIs in human tuberculosis remains controversial, in part because conditions that will reproducibly trigger NO release from human macrophages have been difficult to establish. Direct quantification of NO production in human lung granulomas also poses a technical challenge. Definitive proof may await the identification of human genetic deficiencies in NOS2 (64,65,152).

A ROLE FOR CYTOTOXIC T CELLS?

RNI and, to a lesser extent, ROI production by activated macrophages is currently the only genetically proven mechanism of defense against tuberculosis. Other mechanisms undoubtedly exist, because IFN-$g^{-/-}$ mice are notably more susceptible to tuberculosis than are $NOS2^{-/-}$, $phox^{-/-}$, or $NOS2^{-/-}$ $phox^{-/-}$ mice (64; J. McKinney, unpublished observations). As suggested earlier, IFN-g–induced maturation and lysosome fusion of bacteria-containing vacuoles might contribute to host defense, although this remains to be proven. All of the mechanisms described so far are mediated by the parasitized macrophage itself. However, recent studies suggest that cytotoxic T lymphocytes (CTLs) also may provide a “lethal weapon” against *M. tuberculosis*. Lysis of infected human macrophages by $CD8^+$ CTLs is correlated with a significant reduction (~50%) in the viability of the intracellular bacteria (153). Antigen recognition by these $CD8^+$ T cells is restricted by CD1, a family of nonclassic MHC I–like molecules that present nonpeptide antigens including mycobacterial lipids (154). Whereas CD1-restricted double-negative T cells ($CD4^-$ $CD8^-$) are capable of lysing infected human macrophages *in vitro*, only those expressing surface CD8 are capable of killing the intracellular tubercle bacilli. Killing by CD1-restricted $CD8^+$ T cells is apparently mediated by granulysin, a component of the cytotoxic granules of T cells and NK cells (155). Granulysin is directly toxic to *M. tuberculosis* and other microbes, including gram-positive and gram-negative bacteria, fungi, and protozoa (156). Access of granulysin to intracellular mycobacteria is dependent on the pore-forming molecule perforin, which also is a component of cytotoxic granules. Tubercle bacilli exposed to granulysin develop striking surface protrusions and blebs, suggesting that disruption of cell-wall integrity is the mechanism of killing. Genetic confirmation of the role of granulysin in host defense against tuberculosis awaits the identification of a mouse homolog and, if possible, specific human genetic deficiencies. In this context, it is noteworthy that perforin knock-out mice show normal resistance to tuberculosis during the early, acute phase of infection (157,158), although they may be less able to contain bacillary growth during later, chronic stages of disease (156,159). It also is relevant that CD1 molecules capable of presenting mycobacterial lipid antigens have not been identified in the mouse (154).

A ROLE FOR B CELLS?

Serum therapy for infectious diseases became the vogue after the discovery by von Behring and Kitasato in 1890 (160) that immune serum could transfer protection against tetanus and diphtheria. Innumerable claims were made that serum from immunized animals or healthy tuberculin-positive humans could transfer protection in

animal models and in human clinical trials. Little of this evidence would stand up to the more critical standards of contemporary science. It is certainly true that infection with *M. tuberculosis* elicits an antibody response, predominantly against lipid and carbohydrate antigens found in the cell wall. However, human genetic deficiencies resulting in agammaglobulinemia have not been associated with increased susceptibility to mycobacterial infections. In the mouse model, μ chain (immunoglobulin M) knock-out mice, which are B-cell deficient and do not produce antibodies of any class, were reported to be more susceptible to tuberculosis (161); others, however, disagreed (162). In another study, opsonization of tubercle bacilli with specific monoclonal antibodies (mAbs) prolonged survival of mice infected by the respiratory route, although there was no effect on bacillary numbers in the lungs (163).

Even if antibodies do not make a significant contribution to naturally acquired immunity to tuberculosis, a question that merits consideration is whether the B-cell response could be harnessed to provide protection (for example, by vaccination) (160). This issue has been largely ignored by immunologists. Because human infection is initiated by the implantation of small numbers of tubercle bacilli in the airways, it is possible that the presence of mucosal antibodies in the respiratory tree could prevent or diminish infection of the lung, or ameliorate the early stages of infection. For example, mucosal antibodies could promote the expulsion of inhaled bacilli from the lungs or alter their trafficking in the bronchoalveolar epithelium or associated lymphoid tissue. The question is not whether humoral immunity is important for the resolution of natural infection: the weight of evidence suggests that it is not. Rather, the question is whether humoral immunity could be manipulated to provide a level of protection beyond that engendered by natural infection. This issue merits greater attention and study, particularly by those interested in development of vaccines against tuberculosis.

Development of New Vaccines

In the past several years, more than 100 tuberculosis vaccine candidates and antigens have been tested in small animal models. The nature of the different vaccine concepts has been discussed elsewhere (164,165 and 166) and is summarized briefly here:

Subunit vaccines, consisting of protein, lipid, and carbohydrate antigens in various formulations, have the potential to be specific, defined, and safe. Their disadvantage is limited persistence *in vivo* and whether they can induce lasting cellular immunity.

DNA vaccines encoding a variety of *M. tuberculosis* antigens have shown significant protection in mice, in some cases rivaling the protection conferred by BCG. Advantages include ease of production, low cost, and the ability to induce long-lasting cellular immune responses. Current approaches to increasing immunogenicity include delivery of the DNA in adjuvants and altering the nucleotide composition of the vectors. Duration of protection, safety, and heterogeneity of responses remain important issues. In a recent study, tuberculosis relapse after chemotherapy of infected mice was prevented by postexposure vaccination with a DNA vaccine encoding the 65-kd antigen; significantly, this effect was not obtained with BCG (167). These observations suggest the exciting possibility that DNA vaccination might be useful in preventing reactivation of latent tuberculosis—an enormous reservoir of contagion for which no effective or practicable intervention currently exists.

Microbial vectors other than mycobacteria, including attenuated strains of *Salmonella* and vaccinia expressing mycobacterial antigens, are being tested in animal models. The former has the potential to induce mucosal immunity, and the latter, to induce CTLs.

Live attenuated mycobacteria vaccines are under development, including nonpathogenic species (*M. vaccae* and *M. habana*) and recombinant BCG expressing immunodominant antigens of *M. tuberculosis*. It is noteworthy that the MRC vaccine trial in the United Kingdom demonstrated *M. microti* to be as protective as BCG (about 80% in both cases), even though tuberculin skin test conversion occurred in only a small fraction of individuals vaccinated with *M. microti* (168). In addition, attenuated strains of *M. tuberculosis*, including auxotrophic mutants and other mutants defective for virulence in mice, are being evaluated for safety and immunogenicity. An important point in the era of widespread AIDS is that auxotrophic mutants of *M. tuberculosis* have been shown to be as protective as BCG in immune-competent mice, yet safe in immune-deficient mice, in which BCG infection is lethal (169). Live-attenuated *M. tuberculosis* vaccines have the advantage of expressing a wide range of relevant antigens, as well as the persistence and adjuvanticity associated with mycobacteria, but their safety in immune-deficient individuals, their potential toxicity, and the persistence of the responses they engender have yet to be evaluated.

Future Priorities

The massive burden of tuberculosis has not been addressed by appropriate mobilization of scientific or political resources, although encouraging efforts in this arena have been initiated recently. Although almost every aspect of the pathogenesis of tuberculosis and the immunologic mechanisms required for protection versus tissue damage will require further study, we outline several priorities that we believe to be of greatest urgency.

Development of new and faster-acting drugs, particularly targeting persistent infection, whether active or latent. With the complete genome of two isolates of *M. tuberculosis* now complete (170), and with genome sequencing of nonpathogenic mycobacteria including BCG under way, a range of unique targets for drug development will shortly become available. Identification of potential drug targets will be accelerated by the advent of new technologies for identifying genes that are selectively expressed *in vivo*, such as DNA microarrays (171), “selective capture of transcribed sequences” (SCOTS) (172), and “molecular beacons” (173). Novel drugs that could shorten the effective duration of therapy from the current 6 to 9 months head the list of priorities for drug development, because patient noncompliance with lengthy chemotherapy regimens is currently the most formidable obstacle to successful treatment. New strategies for chemoprophylaxis of latent infections are also urgently needed. Current chemoprophylaxis regimens, although effective, are not used because the duration of treatment required (3 to 6 months) is too difficult and costly to implement on a large scale. Development of a simple, effective, and affordable chemoprophylaxis regimen that could be administered to all individuals who are skin-test positive (i.e., presumably harboring latent infection) would have an enormous impact on future case rates. In the absence of such an intervention, some 200 million new cases of reactivation tuberculosis can be expected to arise among the estimated 2 billion individuals who currently harbor latent infection. Indeed, an effective and widely implemented intervention against latent infection is the only hope for truncating the centuries-long intrinsic epidemic cycle of tuberculosis (Fig. 76.6).

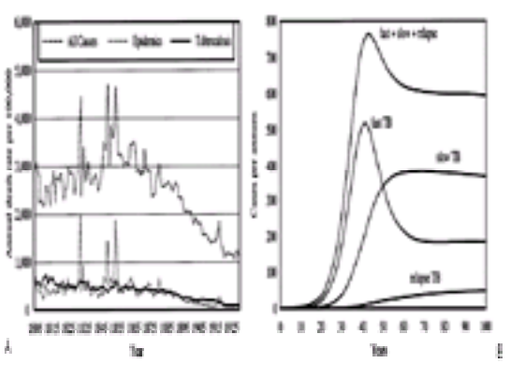

Figure 76.6. Tuberculosis epidemics have slow intrinsic dynamics. **A:** Death rates, New York City, 1804 to 1930, due to all causes, epidemic diseases, and tuberculosis. The urban death rate from tuberculosis was constant at ~500 per 100,000 throughout most of the nineteenth century. Incidence began to decline around 1885, concomitant with a steep decrease in overall deaths rates attributed to advances in public health. (Redrawn from Condran GA. Changing patterns of epidemic disease in New York City. In: Rosner D, ed. *Hives of sickness: public health and epidemics in New York City.* New Brunswick, NJ: Rutgers University Press, 1995:27–41, with permission.) **B:** Numeric simulation of a tuberculosis epidemic initiated by entering one infectious case at time zero into a susceptible population of 200,000. The simulation illustrates the relative contribution of three types of disease to the overall incidence: fast (progressive primary), slow (postprimary), and relapse tuberculosis. A fourth type, exogenous reinfection, is not included in the model, but would presumably increase the peak level and duration of the epidemic. (Redrawn from Blower SM, McLean AR, Porco TC, et al. The intrinsic transmission dynamics of tuberculosis epidemics. *Nature Med* 1995;1:815–821, with permission.)

Development of immunologic correlates and surrogate markers of protection will be essential for the evaluation of vaccine candidates. It is no longer conceivable that multiple vaccine candidates will be evaluated in large prospective, randomized phase III clinical trials, with disease prevention as the sole end point. If the immunologic responses required for protection were known, multiple vaccine candidates and immunization protocols could be evaluated and optimized in relatively small phase II clinical trials. The validity of these immunologic correlates as “surrogate markers” for protection could then be evaluated in phase III efficacy trials. The use of validated surrogate markers as end points of immunization would permit optimization and ranking of multiple vaccine candidates in small, short-term clinical studies without the necessity of carrying out large, multiyear efficacy trials.

Development of a rapid, sensitive, specific, and inexpensive diagnostic test would be of enormous value in permitting early treatment and prevention of transmission. Currently, diagnosis of pulmonary tuberculosis in developing countries is by clinical presentation and microscopy of sputum stained for AFB. These methods are neither sensitive nor specific, nor do they detect bacterial drug resistance, which is increasingly prevalent. In industrialized countries, sputum cultures can

increase sensitivity and specificity, and permit the identification of drug resistance, but diagnosis can be delayed by many weeks and requires relatively specialized equipment and technical skills. A simple, rapid, and inexpensive serologic or molecular test for tuberculosis would do much to ensure that everyone received prompt and appropriate treatment; a diagnostic that detected drug resistance also would contribute enormously to preventing the spread and further evolution of drug-resistant strains.

Development of international collaborations for research and clinical trials would ensure that progress is made in the most efficient and equitable manner possible. The vast majority of tuberculosis patients reside in the developing world, whereas the vast majority of tuberculosis research is carried out in the industrialized world. Evaluation of new drugs and vaccines will require international collaborative partnerships aimed at building infrastructure for basic and clinical research in the countries that are hardest hit, as well as establishing epidemiologic field sites where phase I to IV clinical trials can be carried out effectively. This will require investments in capacity building, international travel, workshops, and conferences, and a commitment to long-term international collaborations. Most important, these collaborations must involve technology transfer and appropriate training of visiting scientists to ensure that the necessary skills are represented "on site."

Addressing market barriers to development of drugs and vaccines. The fact that tuberculosis has become predominantly a Third World disease is a powerful disincentive to the industrialized world to invest in development of drugs and vaccines against tuberculosis. Without collaborations between the public and private sectors, it is difficult to see how new interventions will be developed. Two models have been suggested. The first, a "pull" mechanism, would provide assurance to companies that there would be public-sector financing for purchase and distribution of an effective drug or vaccine, if one were developed. Assurance of a market with the opportunity for at least a small profit is critical for a company to invest in research and development for a new intervention, because the cost of bringing a new drug to market is estimated to be US$ 350 to 500 million. The second mechanism would "push" the development of new interventions by public sector investment in research and development; this approach is of particular relevance for small biotechnology companies that might otherwise not have sufficient resources for such an effort. Either approach would require close collaboration between government research and overseas development agencies, pharmaceutical and vaccine companies, and other private sector concerns, such as foundations and nongovernmental organizations (NGOs). An inspiring recent example of this model is the Global Alliance for Vaccines and Immunization (GAVI), which was established as a result of a major donation from the Bill and Melinda Gates Foundation. The success of this and similar enterprises will hinge on the development of true and equitable partnerships between the industrialized world and the scientists and leaders of the developing world.

LEPROSY

Robert L. Modlin, M.D.

Leprosy provides an extraordinary model for investigating the regulation of immune responses to microbial pathogens. First, the disease still poses a significant health and economic burden on developing countries. Second, because leprosy affects primarily the skin, sampling of the site of disease activity is readily feasible. Finally, and perhaps most important, leprosy presents a clinical/immunologic spectrum, providing an opportunity to study resistance versus susceptibility to widespread infection. By studying the immune response in leprosy, it is possible to gain insights into mechanisms of immunoregulation in humans.

At one end of the leprosy spectrum, patients with tuberculoid leprosy typify the resistant response that restricts the growth of the pathogen (174). The number of lesions is few and bacilli rare, although tissue and nerve damage are frequent. At the opposite end of this spectrum, patients with lepromatous leprosy represent susceptibility to disseminated infection. Skin lesions are numerous, and growth of the pathogen is unabated. These clinical presentations correlate with the level of cell-mediated immunity against *Mycobacterium leprae*. The standard measure of cell-mediated immunity to the pathogen is the Mitsuda reaction or lepromin skin test, a 3-week response to intradermal challenge with *M. leprae*. The test is positive in tuberculoid patients but negative in lepromatous patients (175). Most contacts of lepromatous patients develop protective immunity, as the majority have positive lepromin skin tests but do not develop overt disease.

Perhaps more than any other human disease, leprosy exemplifies the fascinating paradox of the inverse relationship between cell-mediated and humoral responses at the two poles of the spectrum. Cell-mediated immune responses are strong in tuberculoid but not in lepromatous patients; humoral responses including anti–*M. leprae* antibodies are most elevated in patients with the lepromatous form of the disease. This dichotomy can best be explained by the pattern of cytokines produced by T cells at the site of infection.

The Th1/Th2 Paradigm

Studies of murine T-cell cytokine patterns have revealed a paradigm of cellular immunology, the existence of two subsets that differentially regulate immune responses (176). T cells that produce IL-2 and IFN-g, termed Th1 cells, could be envisioned as contributing to cell-mediated immunity; whereas T cells that produce IL-4 and IL-5, termed Th2 cells, augmented humoral responses. In murine models of intracellular infection, resistant versus susceptible immune responses appear to be regulated by these two T-cell subpopulations (177,178 and 179). Th1 cells preferentially activate macrophages to kill or inhibit the growth of the pathogen, resulting in mild or self-curing disease. In contrast, Th2 cells facilitate humoral responses and inhibit some cell-mediated immune responses, resulting in progressive infection.

By the study of leprosy lesions, it became apparent that this paradigm has applicability to human disease in general and to skin immune responses in particular. Initially, IL-2 was detected in leprosy lesions by using mAbs and immunoperoxidase techniques (180,181). An order of magnitude greater number of IL-2–containing cells were present in tuberculoid lesions as compared with lepromatous lesions. By *in situ* hybridization, it became readily apparent that IFN-g mRNA also was more strongly expressed in tuberculoid lesions (182). These early studies indicated that the "Th1" cytokines were most prominent in the form of leprosy that is characterized by cell-mediated immunity to the pathogen.

To probe more fully the patterns of cytokines in lesions at the extremes of the spectrum of leprosy, polymerase chain reaction (PCR) was used to analyze the local cytokine pattern (183,184). The following strategy was devised and proved useful: (a) RNA was extracted from biopsy specimens; (b) polyadenylated mRNAs were reverse transcribed to obtain cytokine cDNAs; and (c) those cDNAs were detected with high sensitivity by PCR by using cytokine specific primers.

The striking observation was that clear differences in cytokine PCR profiles were observed between tuberculoid and lepromatous lesions, and those differences were consistent across lesions of all patients: IL-2 and IFN-g mRNA were markedly higher in tuberculoid lesions, whereas IL-4, IL-5, and IL-10 were characteristic of lepromatous lesions. The cytokine patterns that were detected in the leprosy lesions are remarkably similar to the Th1 and Th2 patterns of murine $CD4^+$ cells. Th1-like cytokine mRNAs were abundant in tuberculoid lesions, which are basically self-healing and characterized by resistance to growth of *M. leprae*. In marked contrast, Th2-like cytokine mRNAs were abundant in lepromatous lesions, correlating with immunologic unresponsiveness to *M. leprae*. Therefore, investigation of leprosy permitted the delineation of comparable human Th1 and Th2 responses in response to a single pathogen, which correlate with the clinical course of the infection.

Earlier immunoperoxidase and immunofluorescence studies had indicated differences in the CD4/CD8 ratio at the poles of the leprosy spectrum (180,181,185,186 and 187). The data from these studies indicated that in tuberculoid leprosy lesions, the $CD4^+$ population predominated with a CD4/CD8 ratio of 1.9:1, whereas in the lepromatous lesions, the $CD8^+$ population predominates with a CD4/CD8 ratio of 0.6:1. Furthermore, $CD4^+$ cells in tuberculoid lesions mark as T-memory cells ($CD45R0^+$) (188). The majority of $CD8^+$ cells in lepromatous lesions are $CD28^-$, indicating that they are of the T-suppressor phenotype (188). Of $CD4^+$ cells cultured directly from tuberculoid lesions, one in 60 react to *M. leprae* antigens (188). $CD8^+$ cells from lepromatous lesions fail to proliferate to antigen, but can be activated by *M. leprae* to inhibit proliferative responses by $CD4^+$ cells and are termed "T-suppressor cells" (189,190).

The patterns of cytokine secretion of these clones revealed the existence of subsets of both $CD4^+$ and $CD8^+$ T cells (191). All of the *M. leprae*–specific $CD4^+$ clones obtained from tuberculoid patients produced IL-2, IFN-g, and granulocyte-macrophage colony-stimulating factor (GM-CSF), but little or no IL-4 or IL-5, similar to the pattern of cytokines characteristic of murine Th1 cells. These clones also lacked helper activity for antibody formation; we therefore designated them "type 1" $CD4^+$ T-cells. CD4+ T cells that recognized tetanus toxoid, in comparison, produced low levels of IFN-g and IL-2, but high levels of IL-4 and IL-5, a pattern similar to that of Th2 cells. These clones were found to have B-cell helper activity and were designated "type 2" $CD4^+$ T cells. Thus functionally different $CD4^+$ T cells from strongly antigen-reactive donors can be subtyped into two groups, similar to the murine counterpart, based on cytokine patterns.

Similarly, it was possible to define subpopulations of $CD8^+$ T cells based on their cytokine patterns. The majority of the $CD8^+$ alloreactive T-cytotoxic clones tested secreted IFN-g, but made no detectable IL-4 or IL-5. Of particular interest, the pattern of cytokine production by the $CD8^+$ T-suppressor cells from leprosy lesions was characterized by high levels of IL-4 and low levels of IFN-g. Based on patterns of cytokine secretion, particularly of IL-4, the data suggest that the human CD8 population also can be divided into two functional subsets, that we designate "type 1" and "type 2" $CD8^+$ T cells.

The inverse relationship between cell-mediated immunity and humoral immunity in the spectrum of leprosy can best be explained according to these cytokine patterns. The abundance of IL-2 and IFN-g produced by $CD4^+$ in tuberculoid lesions is likely to contribute to the resistant state of immunity in these patients. IFN-g is well known to enhance production of reactive oxygen and nitrogen intermediates (192) by macrophages and to stimulate them to kill or restrict the growth of mycobacteria (193,194) and other intracellular pathogens (195). IFN-g also augments expression of HLA-DR and intercellular adhesion molecule (ICAM)-1, which facilitates T cell–accessory cell interaction (196). IL-2 may contribute to the host defense by inducing the clonal expansion of immune activated cytokine–producing T cells and

augments the production of IFN-g (197).

In contrast to the set of cytokine mRNAs present in tuberculoid lesions that might be involved in cell-mediated immunity and inflammation, those found to be increased in lepromatous lesions might be expected to contribute to the immune unresponsiveness and failure of macrophage activation in these individuals. In particular, IL-4 was increased in lepromatous lesions and produced by CD8+ T cells. IL-4 likely contributes to the elevated anti–*M. leprae* antibodies in lepromatous patients through its role in differentiation and immunoglobulin class switching of B cells (198), as well as its ability to stimulate Th2 proliferation (199). IL-4 also has a negative immunoregulatory effect on cell-mediated immunity, which could lead to enhanced bacterial growth.

Innate Immunity: The Role of IL-12 and Toll-like Receptors

Of particular interest to immunologists is the delineation of factors that influence the T-cell cytokine pattern. The innate immune response is one important factor involved in determining the type of T-cell cytokine response. Innate immunity pertains to those cells that have been preprogrammed to respond to foreign molecules. The cells of the innate immune response include macrophages, NK cells, and mast cells. Innate immunity can be contrasted to acquired immunity that involves the selection and expansion of immune cells with the development of memory. The acquired immune response involves T and B cells. Cells of the innate immune response release cytokines, which in turn bias the cytokine profile of the acquired T-cell response.

The ability of the innate immune response to induce the development of a Th1 response is mediated by release of IL-12, a 70-kd heterodimeric protein (200,201 and 202). For example, in response to an intracellular pathogen, macrophages release IL-12, which acts on NK cells, and later T cells, to release IFN-g. The presence of IL-12, IL-2, and IFN-g, with the relative lack of IL-4, facilitates Th1 responses. In contrast, in response to allergens or extracellular pathogen, mast cells or basophils release IL-4, which, in the absence of IFN-g, leads to differentiation of T cells along the Th2 pathway. It is intriguing to speculate that keratinocytes may also influence the nature of the T-cell cytokine response. Keratinocytes can produce IL-10, particularly after exposure to UVB (203). The released IL-10 can specifically downregulate Th1 responses, thus facilitating the development of Th2 responses.

The role of innate immunity in human host defense is perhaps best exemplified in leprosy. The local production of IL-12 in lesions correlated with cell-mediated immunity, being strongest in type 1 cytokine–containing tuberculoid lesions, and weaker in the type 2 cytokine–expressing lepromatous lesions (204). One characteristic of IL-12 is its potent T-cell growth factor activity. T-cell proliferation to *M. leprae* was dependent on the endogenous production of IL-12, in that proliferation could be blocked by the addition of neutralizing IL-12 antibodies. IL-12 induced proliferation of the $CD4^+$ type 1 cells from tuberculoid lesions but not the $CD8^+$ type 2 cells from lepromatous lesions. Therefore, a mechanism by which IL-12 may contribute to cell-mediated immunity in skin is through the preferential expansion of differentiated T cells committed to the type 1 cytokine pattern.

An exciting breakthrough in our understanding of innate immunity to microbial pathogens has been the identification of a human homolog of a *Drosophila* gene product known as Toll, which is responsible for protecting flies from bacterial and fungal pathogens (205,206). Activation of *Drosophila* Toll leads to activation of NF-kB–like kinases, and induction of a series of antifungal peptides, including metchnikowan, defensins, cecropins, and drosomycin. Mammalian TLR family members also are transmembrane proteins containing repeated leucine-rich motifs in their extracellular portions, similar to other pattern-recognition proteins, and a cytoplasmic portion that is homologous to the IL-1 receptor, and hence could trigger intracellular signaling pathways.

Human TLR4 mRNA expression was detected on cells of the immune system, monocytes, macrophages, dendritic cells, gd T cells, Th1 and Th2 ab T cells, and B cells (205). Expression of a dominant active form of TLR4 in monocytes and T cells led to induction of cytokine genes including IL-1, IL-6, and IL-8, as well as activation of NF-kB.

Perhaps the most interesting finding regarding mammalian TLRs is their ability to mediate responses to microbial ligands, lipids, and glycolipids including LPS (133,207,208,209,210,211,212,213,214,215,216,217 and 218). Experiments have led to the exciting finding that microbial lipoproteins trigger host responses through TLRs (133,215). By studying the bacterial cell-wall components that stimulate IL-12, microbial lipoproteins, specifically the *Mycobacterium tuberculosis* 19-kd lipoprotein, the *Borrelia burgdorferi* OspA lipoprotein, and the 47-kd lipoprotein of *Treponema. pallidum* were found to induce IL-12 in macrophages and that induction was dependent on the lipid moiety because deacylated lipoproteins had no activity. The induction of IL-12 was dependent on TLR2, because an mAb to TLR2 completely blocked IL-12 release, and transfection of a dominant negative TLR2 construct inhibited IL-12 promoter activity. Transfection of TLR2 into 293 cells conferred reactivity to microbial lipoproteins. Furthermore, lipoproteins induced iNOS activity and NO production through TLRs, indicating activation of a direct and powerful microbicidal activity. This discovery is relevant to the skin immune response, given that the lipoproteins were derived from two important cutaneous pathogens, *B. burgdorferi* and *T. pallidum*, as well as mycobacterial species that also are a significant cause of skin disease. Other microbial products that activate via TLRs include bacterial peptidoglycan (214,219), *S. aureus* lipoteichoic acid (219), and lipoarabinomannan from rapidly growing mycobacteria (220). Thus Toll proteins represent a host defense mechanism that has been conserved over hundreds of millions of years of evolution.

CD1 and the Universe of Nonpeptide T-cell Antigens

The model of antigen presentation to T cells has centered on the ability of T cells to recognize foreign or self-peptides in the context of MHC class I and II molecules. A new advance in our understanding of T-cell biology has been the demonstration of T-cell recognition of nonpeptide antigens. In one system, gd T cells were shown to recognize isopentenyl pyrophosphate and related structures in the isoprenoid family (221). The ability of gd T cells to recognize these antigens may occur in a manner independent of antigen-presenting molecules. In a second system, ab T cells have been shown to recognize lipid and lipoglycan antigen in the context of CD1 molecules (222,223 and 224).

The human cluster of differentiation I (CD1) gene family consists of five nonpolymorphic genes, CD1*A*, –*B*, –*C*, –*D*, and –*E*, mapped to a cluster on chromosome 1 (225). Human CD1 has a unique tissue distribution, with CD1a, -b, and -c present on thymocytes but not peripheral blood T cells. Human CD1 are also present on professional antigen-presenting cells including dendritic cells, Langerhans cells, and mantle zone B cells. The structural homology of CD1 with class I and class II molecules suggests an antigen-presenting function; however, the amino acids encoded in the predicted antigen-binding site, the a1 and a2 domains, are extremely hydrophobic.

A striking finding was that a unique function of human CD1 was the ability to present nonpeptide antigen to T cells. Specifically, human CD1b and CD1c have been shown to present lipid and lipoglycan antigens to T cells. To date, all the CD1-restricted T cells that have been derived recognize nonpeptide antigens of mycobacteria, specifically *M. tuberculosis* and *M. leprae* (222,223 and 224,226,227). Three major antigens have been elucidated, derived from the cell wall of mycobacteria: mycolic acids, lipoarabinomannan and glycosylated mycolates (GMMs). Mycolic acids are branched long-chain fatty acids that are specifically found in mycobacteria. Lipoarabinomannan contains an arabinose head, branched mannose core, and phosphatidylinositol, which contains two fatty acids: tuberculostearic acid and palmitic acid. For lipoarabinomannan, the data suggested that T-cell recognition was at the level of the branched mannose core.

Investigation of human leprosy has provided direct evidence for the involvement of the CD1-restricted T cells in host response to infection. CD1a, -b, and -c were expressed on dendritic cells in the granulomas within the skin lesions of leprosy patients. Furthermore, the frequency of $CD1^+$ cells correlated with the level of cell-mediated immunity to *M. leprae*, being tenfold more abundant in the granulomas of patients with the immunologically responsive tuberculoid form of the disease, as compared with the unresponsive lepromatous form (228). It is likely that these differences are related to the level of GM-CSF in the lesions, being greater in the tuberculoid lesions. In addition, IL-10, which is specifically found at the site of infection in the lepromatous form, has been shown to downregulate CD1 expression (229). Further evidence for a role of CD1 presentation of antigens in immunity to mycobacterial infection *in vivo* includes the isolation of CD1-restricted T cells from patients with mycobacterial infection. Initially, a CD1b-restricted, *M. leprae*–specific T-cell line was derived from a cutaneous leprosy lesion (224), and more recently, *M. tuberculosis*–reactive CD1-restricted T-cell lines from the peripheral blood of patients with tuberculosis (153).

The study of CD1-restricted T cells from patients with mycobacterial infection as well as normal healthy donors has revealed certain shared functional features. First, all the CD1-restricted T-cell lines isolated produce high levels of IFN-g, but little or no IL-4 on stimulation with mycobacterial antigen. This Th1 cytokine pattern can directly contribute to cell-mediated immunity against intracellular infection by enhancing T-cell proliferation and macrophage activation. Second, CD1-restricted T cells also show a high degree of cytolytic activity against antigen-pulsed macrophages. Furthermore, these T cells can lyse macrophages infected with virulent *M. tuberculosis*. The induction of cytotoxicity is dependent on antigen presentation and T-cell recognition, because lysis was blocked by antibodies to CD1b. Lysis of highly infected macrophages can contribute to host defense either directly by killing the bacteria or indirectly by dispersing the pathogen and thereby allowing freshly recruited macrophages to take up and more effectively dispose of the bacteria (230).

Two mechanisms of cell-mediated cytotoxicity differentially contribute to the outcome of infection with intracellular pathogens. $CD4^-CD8^-$ (DN) CD1-restricted T cells lyse targets through the Fas/Fas-ligand pathway; whereas the lysis of targets by $CD8^+$ cells depends on the release of granules containing perforin and granzymes. However, only the $CD8^+$ subset of CD1-restricted T cells kills the intracellular *M. tuberculosis* during the lysis of the target cell. Although the DN T cells may have an immunoregulatory role by reducing the local cellular infiltration and thereby limiting tissue damage, the $CD8^+$ T cells may have the greatest impact in combating intracellular pathogens (153).

There is an intriguing linkage between the direct antimicrobial effect of $CD8^+$ CTLs (including those that are either CD1 or MHC class I restricted) and their expression of granulysin (231), a 9-kd protein component of CTL granules (232). Granulysin inhibited the growth of a broad spectrum of pathogenic bacteria, fungi, and parasites *in vitro*. Granulysin directly killed extracellular *M. tuberculosis*, and in combination with perforin, the pore-forming molecule that colocalizes with granulysin in CTL, caused a dramatic decrease in the viability of intracellular *M. tuberculosis*. Scanning electron micrographs of granulysin-treated *M. tuberculosis* revealed lesions in the surface ultrastructure indicating alteration of membrane integrity, leading to the death of the organism. These studies defined a new mechanism by which T cells can directly contribute to immunity against microbial pathogens.

Conclusion

By studying the skin lesions of leprosy, it is possible to gain insight into immunoregulatory mechanisms in humans. The generation of cell-mediated immunity to microbial pathogens involves two arms of the immune system, the innate and the adaptive response. We have learned that microbial ligands activate cells of the innate immune response through TLRs, a family of proteins that have been highly conserved from *Drosophila* to humans. Activation of the innate response can lead to a direct antimicrobial pathway, but also may serve to amplify the adaptive T-cell response. In particular, by the release of IL-12, the innate system can instruct the adaptive T-cell response toward the Th1 cytokine pattern, the pattern of cytokines required for effective cell-mediated immunity. T cells, once activated, also can mediate an antimicrobial activity through the release of granulysin. Our studies provide evidence that both peptide and nonpeptide antigens can stimulate such T cells. In leprosy, the T cells and monocytes cooperate in granulomas to deal with the invading pathogen. It should be possible to use this knowledge to develop new strategies both to prevent and to treat cutaneous infections such as tuberculosis and leprosy, as well as other infectious, malignant, and autoimmune conditions.

The genome sequence of the laboratory strain H37Rv was determined by the Sanger Centre (Cole ST, Brosch R, Parkhill J, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998;393:537–543. Available online at http://www.sanger.ac.uk/Projects/M tuberculosis. The genome sequence of a recent clinical isolate, strain CDC1551, was determined by The Institute for Genomic Research (TIGR), available online at http://www.tigr.org/tdb/CMR/gmt/htmls/Background.html

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77 CRYOPATHIES: CRYOGLOBULINS AND CRYOFIBRINOGENEMIA

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A number of cold-related hypersensitivity syndromes have been grouped together under the term *cryopathies* and include such diverse conditions as Raynaud phenomenon and the paroxysmal cold hemoglobinurias (255). Several of these disease entities are treated in detail in other chapters of this volume. This review focuses on the various forms of cryoglobulinemia and cryofibrinogens, with specific emphasis on molecular mechanisms and associated clinical symptom complexes.

CRYOGLOBULINS

Cryoglobulins are immunoglobulins that undergo reversible precipitation in the cold (Fig. 77.1) (93,141,425). Early studies showed that this phenomenon is a concomitant of immune complex formation (156) and also occurs in patients with multiple myeloma (414). Subsequent clinical series established that cryoglobulinemia can accompany a wide variety of disorders, but can also occur in an apparently “essential” or idiopathic form (42,135,169), which is now known to be significantly associated with hepatitis C virus (HCV) infection.

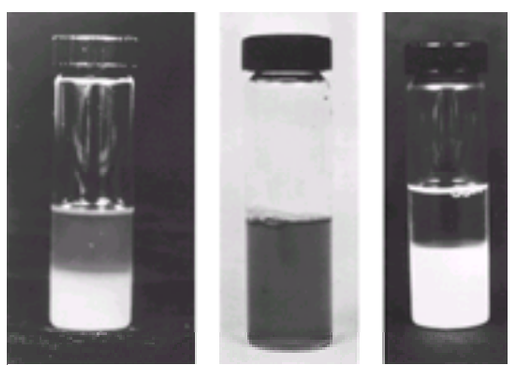

Figure 77.1. Serum of a patient with a 30-year history of clinical cryoglobulinemia. A gelatinous precipitate is apparent when the serum is kept at 4°C overnight (**left**), and dissolves completely when it is rewarmed to 37°C (**center**). The isolated cryoprotein (**right**) is composed of immunoglobulin M (IgM) and IgG.

The immunochemical classification first suggested by Brouet et al. (42) has been adopted in many clinical reports. Type I cryoglobulins consist of a single component, almost invariably a monoclonal immunoglobulin, usually IgM or IgG. Mixed cryoglobulins are cold-precipitable rheumatoid factors (RFs), usually IgM, which form cryoprecipitates by virtue of complex formation with polyclonal IgG at low temperatures (210,231). This RF may be monoclonal (type II) or polyclonal (type III) and, similar to classic RFs seen in rheumatoid arthritis, has specificity for the Fc portion of IgG (325). Generally, type I cryoglobulins are found in diseases in which malignant B-cell transformation has occurred, whereas type III cryoglobulins may be found in a large number of disorders in which hyperglobulinemia, hyperimmunization, and immune complex formation have been documented (Table 77.1) (135). The relative frequency of single-component and mixed cryoglobulins has varied considerably when large numbers of sera have been screened for cryoglobulinemia (Table 77.2) (42,135,169,253,362,368). In recent series, the incidence of type II cryoglobulins has increased dramatically compared with that in older studies (Table 77.2), reflecting the greater use of immunofixation for typing (49), and recognition of the often striking clonality of the humoral immune response in many patients with mixed cryoglobulinemia (MC) associated with chronic HCV infection (338). In addition, the IgG component of type II cryoglobulins also may be found to be oligoclonal when analyzed by IgG subclass or in isofocusing studies (262,263).

TABLE 77.1. Cryoglobulinemia: Clinical and Experimental Associations

	Brouet et al. (1974) (42) (N = 86)	Gorevic et al. (1980) (135) (N = 126)	Invernizzi et al. (1982) (169) (N = 135)	Tzioufas et al. (1992) (362) (N = 116)	Monti et al. (1995) (253) (N = 897)	Tarantino et al. (1999) (368) (N = 2252)
Type I	24.5	38.1	9.6	5.0	5.9	3.4
Type II	25.5	13.5	36.5	72.0	62	62.2
Type III	50.0	48.4	53.9	23.0	32	34.4

TABLE 77.2. Relative Frequency of Different Types of Cryoglobulins

Isolation and Characterization

Methodologic guidelines for the isolation and characterization of cryoglobulins have recently been published (49,137,178). Cryoproteins are isolated from blood that has been allowed to clot at 37°C. The serum is removed and kept for a variable period at 4°C. Most type I cryoglobulins yield copious flocculent, and occasionally crystalline, precipitates that appear within 24 hours of exposure to the cold, whereas type III cryoproteins are usually gelatinous, occur in lower amounts, and may require up to a week to be apparent. These differences reflect the homogeneity or immune complex nature of these proteins, respectively, as well as the relative quantities present in blood. Although considerable overlap may occur, the majority of type III cryoglobulins are present at concentrations of less than 1 mg/mL (i.e., cryocrits <1% volume of serum collected), whereas type I cryoglobulin concentrations are often greater than 5 mg/mL (i.e., cryocrits >1%, and occasionally 20% to 40% or more) (Table 77.3) (42).

Cryoglobulin Type	<1 mg/mL (%)	1-5 mg/mL (%)	>5 mg/mL (%)
Type I	10	30	60
Type II	20	40	40
Type III	80	20	0

Type III cryoglobulins, at concentrations up to 80 μg/mL, may be found in 40% of normal individuals (76).
Source: Modified from Brouet JC et al. Biological and clinical significance of cryoglobulinemia: report of 86 cases. Am J Med 1974;57:775, with permission.

TABLE 77.3. Cryoglobulin Levels

The temperature at which cryoprecipitation starts, as well as the rate at which it occurs, may vary considerably among individuals and even in the same individual when assessed at different times. This appears to be a function of a number of different factors, including heterogeneous mechanisms responsible for cryoprecipitation (Table 77.4) and concentration effects (141,179,194,425). In general, the greater the concentration of cryoprotein in serum, the higher the maximal temperature at which precipitation begins to take place (391). Among cryo-IgMs, 1,000- to 10,000-fold differences of insolubility were found when different proteins were compared (236). Cryoglobulins with high (i.e., greater than room temperature) thermal amplitudes may cause unusual clinical findings (e.g., purpura in atypical locations), be missed because of rapid precipitation during clotting, or cause *ex vivo* laboratory artifacts (137,379,419). Occasionally, type I cryoproteins may gel in the syringe shortly after venipuncture if care is not taken to keep the sample at 37°C. Typically, cryoprecipitation occurs over a temperature range less than 10°C (141,425). Once precipitated, the cryoprecipitate dissolves completely on rewarming to core body temperature, and this process can generally be repeated again and again (Fig.77.1). Cryoglobulins are purified by low-speed centrifugation in the cold, followed by repeated washing in cold physiologic saline solution at neutral pH. The purification may be monitored by assessing the disappearance of contaminating serum proteins from the reprecipitated protein by immunodiffusion at 37°C against, for example, antisera specific for albumin. [In rare instances, albumin may be specifically complexed to the cryoglobulin (see later) (174,244).] Mixed cryoglobulins may lose material on reprecipitation in saline (141,416), a feature that has suggested in turn the importance of serum factors (possibly including fibronectin, low-density lipoprotein, and C1q), which may act as cryoprecipitagogues (151).

Monoclonal cryoglobulins
V region determinants (primary amino acid sequence) (4,60,131,396)
Nucleation reactions (333,383)
Altered protein-solvent interactions at low temperatures (235,236)
Aggregation due to electrostatic and dispersion forces (14,61,186,241)
Extension of normal solubility phenomena (239)
Concentration effects (391)
Deficient carbohydrate side chain (sialic acid residues) (149,427)
Monoclonal immunoglobulin serving as both antibody and antigen (IgRF, IgM-IgM) (142,318,374,402)
Mixed cryoglobulins
Cold-precipitable rheumatoid factors (IgM, IgA) (210,231,408)
V region determinants (133,189,396)
Cryoprecipitagogues in serum (? fibronectin, C1q) (151)
Deficient carbohydrate side chains (425,427)

TABLE 77.4. Mechanisms of Cryoprecipitation

The pH and solute effects on cryoprecipitation are similarly variable (141,425). Loss of precipitability with various solvents and mild denaturing agents has been taken as an indication of the importance of immunologically nonspecific noncovalent interactions in determining cryoprecipitation, especially in regard to type I cryoglobulins. In some instances, occupancy of the antigen-binding site (94) or modification of electrostatic interactions (195) can prevent aggregation. Other factors may include nucleation reactions (333,383), aggregation caused by electrostatic and dispersion forces (14,61,186,241), temperature-induced conformational changes (237,241,318), altered protein/water interactions (235), abnormal stability of disulfide bonds linking heavy chains (332,334), deficient carbohydrate side chains (notably sialic acid) (425,427), nonimmunologic binding to other serum proteins (161,205), and differences in variable (V) region primary amino acid sequence (4,60,131,396). All have been implicated in specific instances (Table 77.4). Most cryoglobulins remain cryoprecipitable in the pH range from 5.0 to 8.5; that is, they are least soluble at physiologic pH, and undergo dissociation and/or dissolution beyond this range (141,425). Rarely strict pH (14,328) or calcium (298) dependence of cryoprecipitation has been demonstrable.

After repeated washing in cold saline solution, purified cryoglobulins may be further characterized by immunoelectrophoresis against antisera specific for immunoglobulin light and heavy chain isotypic determinants. More sensitive techniques are agarose or composite agarose polyacrylamide gel electrophoresis, immunofixation, capillary zone or two-dimensional gel electrophoresis techniques, and immunoblotting with isotype and light chain–specific antisera (49,62,137,262,338,368). These techniques, as well as detailed immunochemical and structural studies in individual patients, have shown that occasional mixed cryoglobulins may be biclonal, oligoclonal, or a mixture of monoclonal and polyclonal components, thus blurring the classic distinction between types II and III (368). Further characterization has included sizing by analytic ultracentrifugation and dissociation on gradients or by gel filtration (210,231). These methods have been widely used for the study of mixed cryoglobulins and have clearly demonstrated that the phenomenon of cryoprecipitation in types II and III cryoglobulinemia is dependent on immunologically specific complexing of the constituents of the cryoproteins (357). Ultracentrifugal analysis has shown that IgM and IgG components may not circulate as 22S complexes, as do classic RFs, but rather as individual 19S and 7S IgM and IgG, with relatively low affinity for each other at core body temperatures. Affinity of cryoIgM RF for IgG may increase significantly as the temperature is lowered, resulting in complex formation and aggregation (41). Cryoprecipitation can be demonstrated only in density-gradient fractions containing both IgM and IgG (Fig. 77.2) and is not a property of the isolated components. Furthermore, pooled normal IgG may be substituted for the IgG component of the mixed cryoglobulin, and cryoprecipitation will occur when the temperature is lowered, whereas this is not seen when the IgM component is substituted for by an unrelated noncryoglobulin monoclonal or polyclonal IgM (231).

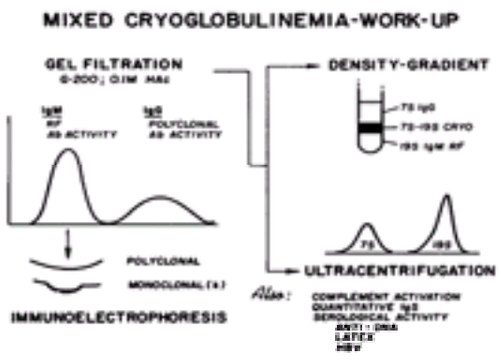

Figure 77.2. Workup of mixed cryoglobulins may include (a) ultracentrifugal analysis (**bottom right**) which generally yields two distinct peaks at 37°C, corresponding to 19S immunoglobulin M (IgM) and 7S IgG, although additional peaks may be evident if complexes form or if IgA or C1q is also present; (b) density-gradient centrifugation (**top right**): 19S IgM and rheumatoid factor are found in the heavier fractions of the gradient, but cryoglobulin formation at a lower temperature occurs only at the interface tubes containing both IgM and IgG; and (c) gel filtration under dissociating conditions (**top left**): IgM and rheumatoid factor activity can be shown in a high-molecular-weight peak on various columns, which can then be tested by immunoelectrophoresis (**bottom left**) against antisera to μ, k, and l determinants to define whether it is monoclonal (type II) or polyclonal (type III); most type II cryoglobulins are IgMk. Additional studies may include quantitation of serum immunoglobulins, complement activation, or a search for specific serologies in dissociated and fractionated material.

Type I cryoglobulinemia may be due to immunologically specific, temperature-sensitive interactions in which the monoclonal immunoglobulin is both antibody and antigen (142,318,374,402). Demonstration of this mechanism is difficult because antibody and antigen may be of the same isotype. Dissociation and fragmentation of the cryoprotein may be required to show that the Fab fragment and not the Fc portion of the molecule retains specific binding in the cold (142,318). Such studies have shown binding to other species of molecule (e.g., albumin, lipoprotein, transferrin) in some instances (161,174,244). Immunologically specific binding to the Fc fragment or light chains of other immunoglobulin molecules of the identical isotype, that is, demonstrating a mechanism analogous to that of IgG RF, has been shown in other cases.

Isolated cryoglobulins may be quantitated by reference to the volume of serum from which they are isolated as a *cryocrit* (analogous to the hematocrit) or as an absolute value, determined by various protein assays (137,142,178).

Simple Cryoglobulins

LIGHT CHAINS

In one series, 3% of Bence Jones proteins (BJPs) were found to be cryoprecipitable (229). Only a small number of these proteins have been studied in any detail, and all were obtained from the urine of patients with multiple myeloma (15,152,207,380). Limited sequence and serologic studies have failed to show clear-cut k/l or V-region subgroup specificity (59,183,229,230). Four of six l cryo-BJPs studied by amino acid sequence analysis had unblocked amino termini, an incidence that contrasts with that of 20% among l BJPs in general (230). One well-characterized k cryo-BJP was found to form crystals at 4°C and to belong to the VIII subgroup by sequence analysis (59,265). However, whereas the VIII subgroup is extremely common among type II cryoglobulins (see later), it does not appear to be particularly enriched among type I cryoproteins.

In one well-studied example, the isolated l light chain of an IgG1 myeloma protein was found to be cryoprecipitable, whereas the parent molecule was not. Only the dimeric form of the light chain underwent reversible temperature-induced conformational changes in the cold, and proteolysis of the intact light chain caused loss of cryoprecipitability (183). Two cases of subacute vasculopathy associated with l light chain crystals have been reported (356).

IMMUNOGLOBULIN G

Approximately 3% to 10% of IgG M components are cryoprecipitable, occurring both in individuals with established multiple myeloma and in others without clear-cut underlying plasma cell dyscrasia (42,135,169). A small percentage of type I IgG cryoproteins also are crystalglobulins (95) and, as such, have provided useful tools for x-ray diffraction analysis of immunoglobulin structure (102).

Among IgG cryoglobulins, several investigators have shown that determinants of cryoprecipitability may reside in the variable region of the molecule and are retained in some instances by peptic $F(ab')_2$ fragments, but not by Fc or Fc-related digestion products (317,318 and 319,383). In other instances, cryoprecipitation cannot be localized to specific enzymatic fragments (208,272). Different series have provided conflicting data regarding abnormal k/l or IgG subclass ratios among IgG cryoglobulins (4,42,385,396). Chemical analyses have shown, however, that most of these monotypic proteins have V_hI heavy chains and unblocked light chains (396). In one series, idiotypic cross-reactivity of several IgG cryoglobulins was shown serologically (5). Temperature-induced conformational changes can occur in the Fab portion of such molecules, which may in turn lead to aggregation and cryoprecipitation. One mechanism for the latter is interaction with the Fc portion of IgG (142,318,319). In other proteins, small amounts of soluble polymers may act as nucleation centers for cold precipitation or crystal formation. Such precipitates also may be obtained with Fab or $F(ab')_2$ fragments but not Fc, indicating the association of the former to be the primary event (334,383).

Structurally abnormal IgG cryoglobulins also have been described. Abnormalities reported include extra disulfide bonds linking heavy chains (333), formation of unusual fragments after enzymatic digestion (279), a lack of sialic acid residues (149,426), and, in one case, a deletion in the hinge region of the molecule (88). Aberrant immunoglobulin and c-*myc* gene rearrangements have been found in some patients (285). In other instances, no structural abnormality or specific antibody activity can be identified, and it must be assumed that cryoglobulinemia is simply due to unfavorable interactions with solvent in the cold, presumably reflecting tertiary structural groupings (208). More than 30 cases of crystal cryoglobulinemia of monotypic proteins have been reported (95,143), again mostly in patients with multiple myeloma (19,387). Clinical associations have included vasculitic purpura (143,244,278), skin ulcers (143,244), glomerulitis (95), pyoderma gangrenosum (407), erosive arthritis (192,278), severe Raynaud phenomenon (143), and corneal deposits (139,187). Crystalglobulins may form tubular structures that can be visualized *in vitro* by electron microscopy (35,290,355,407) and that also can be identified in myeloma and mononuclear cells (198). In one instance, Fab and $F(ab')_2$ fragments cryoprecipitated to form tubular structures similar to the parent molecule on cooling (383); in another IgG3 crystalglobulin, the Fc fragment was necessary for cryoprecipitation (3). Instances of crystalglobulins formed by specific complexing of IgG to albumin have been reported (174,244).

IMMUNOGLOBULIN M

From 10% to 20% of macroglobulins are cryoprecipitable (42,135,169). Detailed studies of a series of monotypic IgM cryoglobulins failed to provide a specific physicochemical basis for cryoprecipitation. No major differences were found between noncryoglobulin IgM molecules by spectroscopy (238), hydrodynamics (241), solute variation (236), or immunochemical analysis (240), and this lack of specific structural determinants has been borne out by limited sequence studies (131). Based on these findings, it has been suggested that cryoprecipitation among many of these molecules may be an extension of a normal solubility phenomenon (239). This observation appears to be corroborated in the marked relation between viscosity and temperature among sera of patients with Waldenström macroglobulinemia (regardless of the presence of clinical cryoglobulinemia) when varying levels of IgM are compared (391).

IMMUNOGLOBULIN A

Rare examples of monotypic IgA cryoglobulins have been identified, some of which caused gelation of serum (295,346), or were found also to be pyroglobulins (i.e., to precipitate at temperatures >56°C) (397,398). Similar to IgG type I cryoglobulins, cryogel formation has been localized to the $F(ab')_2$ fragment in some instances (295).

Mixed Cryoglobulins

The incidence of *type II* cryoglobulins in recent series has increased with greater appreciation of the prevalence of this form of cryoglobulinemia (Table 77.2) (42,135,169,253,362,368). In addition to lymphoproliferative disorders and cryoglobulinemia associated with HCV infection, associations with Sjögren syndrome (371,392) and other forms of chronic hepatitis (347) have been noted in a number of case reports and small clinical series. The large majority (>95%) of type II cryoglobulins contain IgM molecules with k light chains (Table 77.5).

"Essential": cryoglobulinemia
Waldenstrom macroglobulinemia
Sjogren's syndrome (371)
Autoimmune diseases (rheumatoid arthritis, lupus, polyarteritis, polymyositis) (214,215)
Chronic active hepatitis (347)
Angioblastic lymphadenopathy (215,297)
Cold agglutinin disease (299)

[a] More than 95% of typed light chains are κ.

TABLE 77.5. Diseases Associated with "Monoclonal" IgM-polyclonal IgG Mixed Cryoglobulins

IgA/IgG mixed cryoglobulins have been described in association with cold-induced urticaria (219,389) and glomerulonephritis (400).

Serologic, structural, and genetic studies have provided the basis for this striking enrichment of k light chains among type II cryoglobulins. Initial investigations of a small group of monoclonal antiglobulins, some of which were isolated from the blood of patients with lymphoma or macroglobulinemia, indicated that these proteins could be divided into three groups by using antisera developed against individual IgM K proteins and rendered antiidiotypic by rigorous absorption with normal IgM and noncryoglobulin IgM M components (50,188). The Wa and Po groups defined 60% and 20%, respectively, of these proteins by hemagglutination inhibition. About 20% could not be classified with these antibodies (188).

Amino acid sequence studies of the variable sequence region (V_{REGION}) light chain of a number of these monoclonal RFs have shown that proteins belonging to the Wa group invariably have light chains belonging to the V_kIIIb subsubgroup (133,189). The V_kIII subgroup constitutes approximately one third of the normal k light-chain pool, and about half of these proteins can be typed as V_kIIIb by serologic or sequence analysis. The rest are V_kIIIa or untypable (227). Whereas 60% to 70% of monoclonal IgMk autoantibodies are V_kIIIb, only 8% of non-RF IgM paraproteins belong to this subsubgroup (189,291). Furthermore, complete V_{REGION} sequences of a small number of these proteins have established striking homology that extends through the third hypervariable region of the light chain (133,196,291). In contrast, the Po group is less well defined. Although none of these proteins are V_kIIIb, a significant percentage also appear to belong to the V_kIII subgroup (189). Two Po-positive proteins completely studied to date shared four of six heavy- and light-chain hypervariable sequences, although they differed significantly when framework sequences were compared (51,52).

Monoclonal and polyclonal antibodies to mixed cryoglobulin cross-reactive idiotypes (MC CRIs) have been used to analyze relationships between MC and other rheumatic and lymphoproliferative diseases (136). Antibodies have been obtained by generating hybridomas to IgMk isolated from mixed cryoglobulins, or as polyclonal antisera raised to synthetic peptides corresponding to specific hypervariable region or framework sequences. MC CRIs may be light- or heavy-chain specific or may depend on the association of heavy and light chains for expression; light- and heavy-chain CRIs were found frequently to be associated with each other, suggesting that combination is not random. Some CRIs depend on the integrity of the antigen-binding site for expression; binding of others to V-region determinants is only partially or not inhibited by antigen. Whereas two to three MC CRIs account for virtually all type II IgMk cryoproteins, the expression of these CRIs among noncryoglobulin or RF paraproteins and in the normal serum IgM pool is quite low. MC CRIs are particularly prevalent in primary Sjögren syndrome, CD5-positive B-cell chronic lymphocytic leukemia (CLL), some non-Hodgkin lymphomas (NHLs), and monoclonal cold agglutinin disease (136). In these disorders, CRIs may be demonstrable both in the fluid phase and on the surface of B cells, either in peripheral blood or in tissue lesions (e.g., glandular tissue of patients with Sjögren syndrome). In long-standing rheumatoid arthritis, although MC CRIs are prevalent, they constitute only a small proportion of the RF pool quantitatively, which may be skewed away from these idiotypes (326). In contrast, in Sjögren syndrome, the bulk of IgM RF is accounted for by these CRIs, and serial measurements have been used to monitor for the lymphoproliferation that occurs with increased incidence in this disorder (136,339,372). These observations suggest that most type II IgMk RFs resemble "natural" autoantibodies, which are often IgM, low affinity, polyreactive, prevalent in the fetal repertoire, and commonly found to have CRIs; however, unlike other "natural" autoantibodies, MC IgM are not known to be polyreactive, and do not appear to be synthesized specifically by CD5-positive B cells (78,281).

MC CRIs have been correlated with specific heavy- and k light-chain V genes, and the roles played by somatic mutation, diversity and/or joining segment use, and recombination in determining idiotype defined at the molecular level. Most V_kIII light chains are encoded by two genes, designated *Kv328* and *Kv325* (A27 locus or k IIIb gene), accounting for the (a) and (b) subsubgroups and the Wa and Po groupings that were previously defined serologically. Kv325 is significantly associated with expression of a specific V_H1 heavy-chain gene, designated V_H*7*83 (51p1); other MC V_kIII-associated idiotypes (Po, Bla) are linked to increased expression of V_H3 or V_H4 subgroup genes (136). Overall, skewing of the V_H gene repertoire to some subgroups (notably V_H4) has been noted among autoantibodies. Specific genes (e.g., V_H4.21) are highly associated with autoantibodies; this gene accounts for virtually all monoclonal cold agglutinins, as well as some polyreactive RFs and anti-DNA antibodies (280).

Type II IgM RFs manifest a high degree of specificity for human and primate IgG (141). Selectivity also is seen in the pattern of reactivity with different IgG subclasses and fragments, which appear to vary from one cryo-IgM to another (175). The relative contribution of the IgG subclasses to the polyclonal IgG fraction isolated from mixed cryoglobulins also varies from protein to protein (77,175) and is significantly different from the pool of noncryoprecipitable IgG remaining in serum from the same individual; specific increases in IgG1 and IgG3 have been reported in older and more recent studies (77,263,416). Generally, however, reactivity is greatest with intact IgG, less so for Fc fragments, and has been reported to be nonexistent for isolated Fab fragments (175). Two thirds of cryoglobulins react selectively with the staphylococcal protein A-binding site at the interface between C_H2 and C_H3 (325).

Type III cryoglobulins are associated significantly with a variety of disorders in which immune complexes may occur (Table 77.1) (42,135,169). These cryoproteins may be complex and may also have a significant nonimmunoglobulin content. In some cases, this has been correlated with the presence of other proteins or specific antigens enriched in the cryocomplex relative to serum levels. These other proteins include various complement components, notably C1q, which may be especially important in cryoglobulin formation in the sera of patients with systemic lupus erythematosus (SLE) (148,352), and fibronectin, which appears to be a significant constituent of serum cryoprecipitates in some patients with connective tissue disease (24,106,332), as well as those found in high incidence in the joint fluid of patients with rheumatoid arthritis (53). DNA, as well as enrichment of anti-DNA and anti-Ku antigen antibodies, and idiotype/antiidiotype complexes, have been found in type III cryoglobulins associated with SLE (305,411). Hepatitis B surface antigen (HBsAg) occurs in cryoprecipitates isolated from the sera of patients with chronic hepatitis (223,367) or extrahepatic syndromes associated with hepatitis B virus (HBV) infection (110,138,166).

Familial Cryoglobulinemia

Type III cryoglobulinemia may rarely occur as a genetic disorder. IgM/IgG cryoglobulins with RF activity have been reported in several kindreds (32,81,268,345). One family was asymptomatic, and the cryoproteins were detected on routine screening (399). In other families, several members were affected by recurrent purpura or membranoproliferative glomerulonephritis. In one kindred, two brothers were found to have a cold-precipitable RF and depletion of the early components of complement without apparent symptoms (345). Detailed studies of one family provided evidence for an idiotype on the IgM component of the cryoglobulin that was inherited in an autosomal dominant fashion (269,270). An unusual form of cryoglobulinemia has also been described in three siblings affected by dysgammaglobulinemia type I, an immunodeficiency disorder characterized by high levels of IgM and IgD but decreased or absent IgA and IgG. In these individuals, the cryo-IgM, which was polyclonal, was itself found to have anti-IgM activity, leading in turn to the conclusion that cryoglobulin formation was due to self-association of this class of immunoglobulin (413).

Experimental Cryoglobulinemia

A murine model of cryoglobulinemia characterized by severe leukocytoclastic vasculitis of the ears, tail, and footpads, and glomerulonephritis associated with deposition of immune reactants, complement activation, and electron-dense deposits (wire looping) was developed with IgG3-producing hybridomas obtained from MRL-lpr/lpr mice (145,172). This strain spontaneously develops a lupuslike syndrome characterized by high levels of IgG3 cryoprecipitating RFs; hybridomas transfer disease to nonautoimmune mice in 5 to 10 days (145,337). Murine IgG3 has a predilection for cryoprecipitation because of an intrinsic tendency to self-associate by Fc/Fc interactions that appear in part to be glycosylation dependent (276). However, not all IgG3 are cryoglobulins, and this activity appears to depend on V-region electrostatic charge and may be potentiated by IgM RF directed to IgG3 (28,29). RF activity of IgG3 monoclonal cryoglobulins and the formation of IgG3/IgG2a immune-complexes initiates cutaneous vasculitis, whereas cryoprecipitability of the IgG3 is sufficient for nephritogenicity, which can thus be dissociated in this system (173,306). Exact correlations between specific features of renal pathology and V and C domain primary structures were identified in a murine model for

cryocrystalglobulinemia (307). In this model system, disease can be prevented by monoclonal antiidiotypic antibody and is inhibited by noncryoglobulin IgG3; antiadhesins (ICAM-1/LFA-1) selectively attenuate the cutaneous vasculitis, which depends on neutrophil/endothelial cell interactions for expression (172,173,350).

Glomerulonephritis with pathologic and ultrastructural features (hyaline thrombi; mesangial and subendothelial deposits) resembling glomerulonephritis seen in cryoglobulinemia has been induced in mice by short-term injection of human type II cryoglobulins (121)

Antibody Activities in Cryoglobulins

Enrichment of a number of antibody activities and, in several instances, the presence of specific antigens in cryoprecipitates, have been demonstrated in various diseases associated with cryoglobulinemia (Table 77.6). These examples have provided the basis for the supposition that mixed cryoglobulins are pathogenic immune complexes and not just epiphenomena. Elevated levels of antibodies to specific antigens associated with Epstein-Barr virus (EBV) infection, or to HCV (see later), as well as persistence of viral genome demonstrated by molecular techniques in patients with MC, have been taken as evidence for ongoing infection (12,118). In other instances (e.g., cryoprecipitates associated with subacute bacterial endocarditis or poststreptococcal glomerulonephritis), evidence for the presence of specific exogenous antigenic determinants, or even enrichment of antibody activity, remains inconclusive (165,213).

Antibody	Antigen
Antiglobulin (IgM) Fc specific	DNA (34,305,309,411) Ku antigen
Antinuclear (IgM) (34,309,411)	Hepatitis B virus (110,138)
Lymphocytotoxic (IgM) (412)	Epstein-Barr virus (393)
Cold agglutinins (IgM) (132,214)	Renal tubular antigens (181)
Smooth muscle (IgM) (90)	Bacterial products (395)
Mitochondrial (IgM) (120)	Hepatitis C virus
Renal tubular antigens (IgM) (181)	
Pemphigus (IgM) (249)	
Albumin (IgM) (344)	
Lipoprotein (low density) (IgM) (165,204,291)	
α-Fetoprotein (IgM) (179)	
Intestinal microorganisms (IgM) (395)	
Streptolysin O (IgM) (334)	
VDRL (IgM) (378)	
Epstein-Barr virus (IgM) (393)	
Cytomegalovirus (IgM) (335), (IgM) (390)	
Hepatitis B surface antigen (IgM) (110,120,138,200,223,304,367)	
Toxoplasma (IgM) (75)	
Coccidioidin (IgM) (126)	
Candida (turnwald) (IgM)	
Hepatitis C virus (see Table 77.11)	

TABLE 77.6. Antibody Activity and Antigens Detected in Cryoglobulins

The simultaneous occurrence of cryomacroglobulinemia and cold agglutinin disease has been reported in chronic cold agglutinin disease, CLL, and primary macroglobulinemia (216,296). About 5% of Waldenström macroglobulins are also cold agglutinins. Cryoglobulins with cold agglutinin activity are most commonly IgMk and have specificity for the I-i antigen system; non–I-i or Pr (protease-sensitive)-specific proteins also have been described (64,417). Monoclonal cold agglutinins may occur in lymphomas, Kaposi sarcoma, severe combined immunodeficiency disease with B-cell proliferation, and rarely, mycoplasmal pneumonia (296). Cold agglutinins with anti-I specificity are usually k, whereas those specific for i are commonly l; one third of anti-i cold agglutinins are cryoprecipitable (294,296).

Consistent with experience in other types of cryoglobulinemia, different molecular mechanisms for cryoprecipitability have been identified in individual cases of cold agglutinin disease. In two instances, the thermal properties of the protein were found to be dependent on binding to carbohydrate moieties found on the red cell membrane and on the cryoagglutinins themselves (370,401). Fab_{μ} and $Fc_{\mu 5}$ fragments of an IgM l cold agglutinin associated with Waldenström macroglobulinemia were found to undergo self-association in the cold that was inhibitable by sialyllactose or by neuraminidase treatment (401). These observations imply immunologic specificity for sialic acid residues as a necessary mechanism for cryoprecipitability. In another instance, both cryoaggregation and cold agglutinin activity of IgMk macroglobulin was found to depend on binding to *N*-acetylneuraminosyl–containing carbohydrate residues (370). More rarely, IgM/IgG cold agglutinins that are type II mixed cryoglobulins have been described (132); most type II cryoglobulins, however, do not have cold agglutinin activity (135).

Phlogistic Potential of Mixed Cryoglobulins

Clinical symptoms suggestive of immune complex disease, that is, cutaneous vasculitis and glomerulonephritis, have been associated with both single-component (26,68,260) and mixed cryoglobulins (42,68,135,169). Nevertheless, the occurrence of cryoglobulins in normal subjects (76,178), in apparently asymptomatic individuals (286,399), as well as in association with diseases and in individual patients not manifesting immune complex symptoms (345,399), suggests that other factors, analogous to those recognized for immune complexes in general, are necessary for the clinical expression of disease. These may include the level and composition of the cryocomplexes, complement activation, specific antibody activities, efficiency of clearance of immune complexes from the circulation and binding to specific receptors, direct interaction with cell types important in inflammation (including mononuclear cells, basophils, and platelets), and direct effects leading to the generation of other inflammatory mediators (including coagulation factors, kinins, and leukotrienes) (365).

Patients with cryoglobulinemia frequently have a characteristic complement profile in blood, consisting of depletion of early components (C1q, C4) in the presence of relatively normal C3 and factor B levels (Fig. 77.3) (147,360). The presence of C1q in cryoproteins occurring in SLE (148,352) and mixed cryoglobulinemia (147,360) may be responsible for the often undetectable levels of this component in the serum of some patients.

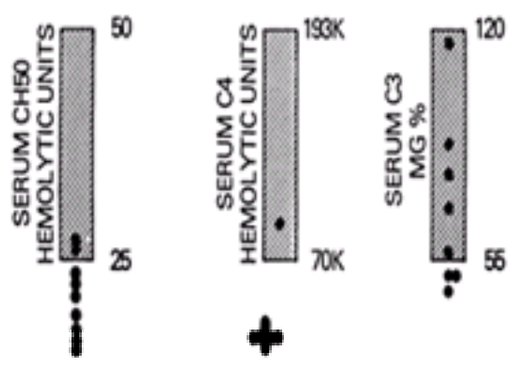

Figure 77.3. The characteristic complement profile of serum in mixed cryoglobulinemia includes depression of C4 and CH (measured here in hemolytic units) and normal or depressed levels of C3 (measured as mg/dL). The *hatched bars* indicate normal range for these determinations, with the upper and lower limits defined numerically (*K*, thousand). (From Hamburger MI, et al. Mixed cryoglobulinemia: association of glomerulonephritis with defective reticuloendothelial Fc receptor function. *Trans Assoc Am Physicians* 1967;93:104, with permission.)

Despite these findings, hypocomplementemia in association with cryoglobulinemia only poorly correlates with clinical symptoms and has been found in asymptomatic “healthy” individuals (399). Temperature-dependent activation of both the classic and alternative pathways of complement *in vitro* has been reported among mixed (IgM/IgG, IgM/lipoprotein) (9,154,206) and monoclonal (IgG) cryoglobulins (74,292). Activation of the alternative pathway has been correlated with the presence of IgA in mixed cryoglobulins (394) and with an IgG3 monoclonal cryoglobulin occurring in a patient with membranoproliferative glomerulonephritis (292). Among IgM/IgG mixed cryoglobulins, fractionation experiments have established that the majority of anticomplementary activity is associated with IgM RF (18). Nevertheless, *in vitro* studies indicated that IgM RF/IgG immune complexes may not fix C3 and C4 efficiently in spite of fluid-phase complement activation, and bind poorly to erythrocyte complement receptor 1 (CR1) (418). *In vivo* turnover studies have indicated that hyposynthesis and increased catabolism of C4 contribute to the low level of this protein in blood (315). *In vitro* studies suggest an alternative explanation: inability to activate C3 due to regulation of classic pathway C3 convertase by C4-binding protein and C3b inactivator (154). Dissociation of the thermal properties of cryoprecipitation and complement activation has been noted in specific instances in which it has been studied (314).

How cryoaggregates generate vasoactive substances and proinflammatory mediators to produce tissue lesions is unclear. That such mechanisms might be operative in some patients is suggested by elevated levels of complement fragments with anaphylatoxin activity (C3a, C5a) in serum (234) as well as the ability of isolated cryoproteins to activate basophils (233) or Fc receptors on neutrophils (164), cause platelet aggregation (71), and interact with kallikrein-kinin (180) *in vitro*. These mechanisms may be most relevant to the pathogenesis of disease in the peripheral vascular beds, where increased protein concentration and skin temperatures

significantly below core levels might facilitate the occurrence of such molecular events and cell interactions (113).

Increased production of immunoglobulin (232) and RF (341) by peripheral blood mononuclear cells correlates generally with elevated levels of cryoglobulin in the fluid phase of blood, although some patients are found to be significantly hypogammaglobulinemic (135). In one case of mixed cryoglobulinemia associated with Sjögren syndrome, *in vivo* turnover studies indicated that hypogammaglobulinemia was due to increased catabolism of IgG complexed with the IgM RF (392). In other studies, turnover of a monoclonal IgG cryoglobulin (2) and the IgG components of type II mixed cryoglobulins (267) were normal. IgG Fc receptor function, assessed by clearance of IgG-sensitized autologous erythrocytes or the kinetics of ingestion of IgG-coated material by circulating monocytes, has been found to be abnormal in some patients, particularly those with active nephritis or neuropathy (267,311). Similarly, CR1 numbers were decreased when peripheral blood erythrocytes were analyzed with a monoclonal antibody to this receptor (72). Defective immune adherence and elimination of immune complexes may lead to trapping in tissues, resulting in clinical vasculitis or nephritis (217,312).

Clinical Features of Cryoglobulinemia

Monotypic cryoglobulins are more likely to be associated with acrocyanosis, retinal hemorrhage, severe Raynaud phenomenon, and arterial thrombosis. By contrast, vascular purpura and joint symptoms are more typically seen in patients found to have type II or III cryoglobulins (Table 77.7) (42).

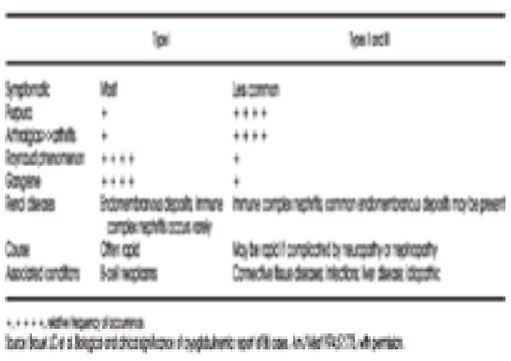

TABLE 77.7. Contrasting Clinical Features of Simple and Mixed Cryoglobulins

A syndrome of purpura, arthralgias, and nephritis associated with mixed cryoglobulins was delineated in 1966 (231), the clinical and laboratory aspects of which have been expanded and corroborated in a number of case reports and series since then (Table 77.8 and ,Table 77.9) (123,134,135,253). These patients constitute from 25% to 62% (42,253) of large series of patients found to have cryoglobulinemia.

TABLE 77.8. Clinical and Laboratory Features of Mixed Cryoglobulinemia

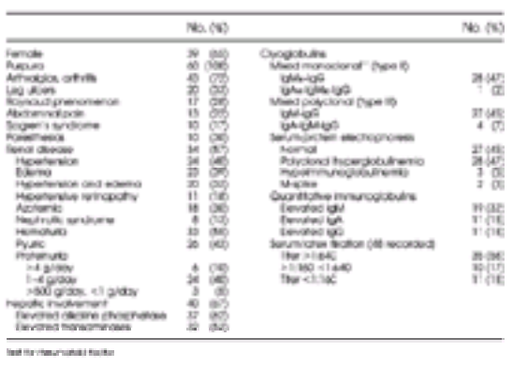

TABLE 77.9. Mixed Cryoglobulinemia (60 Patients)

SKIN

Cutaneous symptoms are especially common in these disorders, most often occurring as vascular purpura (76). The histology of these lesions is characteristically that of a leukocytoclastic angiitis (76,231), although different histologic patterns have been delineated in some instances (68). Occlusion of small vessels by hyaline thrombi may occur in cutaneous lesions of patients with type I disease (17), and in skin ulcerations complicating mixed cryoglobulinemia (27). However, care must be taken in handling biopsy specimens to exclude artifacts caused by cryoprecipitation *ex vivo*. Characteristic cutaneous manifestations of type I cryoglobulinemia are ischemic necrosis of the helix of the ear and other exposed parts on cold exposure, and livedoid vasculitis; hyperkeratotic spicules also have been observed in areas exposed to cold (40). Positive immunofluorescence showing cryocomplex constituents (monotypic or mixed) in vessel walls has been noted in some reports and used as evidence for an etiologic role for the cryoglobulin in the pathogenesis of the skin lesions (68,242,260). Often, however, results of these studies are negative.

Cold-induced urticaria may be a prominent feature in some patients. This symptom has been reported in 15% of patients with type I and in 10% of patients with type III cryoglobulinemia (42), and has been associated particularly with IgA/IgG mixed (219,389) and IgG monoclonal cryoglobulins (73,74,177). In the latter, a direct pathogenic role for the cryoprotein in the production of the skin lesions has been suggested on the basis of (a) cold-dependent complement activation *in vitro* as well as *in vivo* during an episode of cold-induced urticaria (74), (b) passive transfer experiments in which urticarial lesions can be induced by cold at skin sites sensitized directly by the cryoglobulin, and (c) positive immunofluorescence for IgG in urticarial skin lesions showing leukocytoclastic angiitis (73,384). The vast majority of patients affected by cold urticaria, however, do not have cryoproteins in their blood.

JOINTS

Although articular manifestations are common in types II and III cryoglobulinemia, frank arthritis, erosive disease, and progressive joint deformity are distinctly unusual (42,135,169). Nonspecific radiographic findings have been noted in small series (404), and rare arthrocenteses have yielded a noninflammatory joint fluid. More severe arthritis, associated with intraarticular cryocrystalglobulinemia of an IgG paraprotein, also has been reported (192,278).

NERVOUS SYSTEM

In early series, neurologic symptoms were reported to affect about 20% of patients with mixed cryoglobulinemia, most commonly in the form of a mild sensorimotor neuropathy seen as dysesthesias (135). Neuropathy may be subclinical, may result in symptoms that cause the patient to seek medical attention, or may develop later in the disease. More recent series reported prevalence rates up to 90% on the basis of symptoms, electrophysiologic findings, or nerve biopsy results, the latter of which may be positive in some instances even when nerve-conduction velocities are normal (58,115,128,130,266,376,377). Neuropathy may be symmetric and mainly sensory, patchy and asymmetric, or present as a mononeuritis multiplex. In addition to axonal degeneration, vascular changes may be prominent among endoneurial vessels, and can range from chronic vasculopathy, ischemia due to occlusion, to frank necrotizing vasculitis (130,266,376). Antimyelin antibodies have been implicated in some cases (366). In contrast to peripheral involvement, central nervous system disease due to cryoglobulinemia is rare (288). Instances of pseudotumor cerebri (96) and cerebrovascular events due to vasculitis (6) have been described.

RENAL DISEASE

From 30% to 60% of patients with MC initially have or develop renal involvement (42,135,169,253). The typical clinical picture is that of chronic glomerulonephritis and may consist of edema due to nephrotic-range proteinuria and hypertension, which is frequently severe (Table 77.9). Rarer presentations are acute renal failure, renal tubular acidosis, and papillary necrosis. Urinalysis shows proteinuria, hematuria, and red cell casts (70,135,361).

Pathologic findings are in many respects typical of an immune complex glomerulonephritis, but with several distinctive features (Table 77.10). In a large clinical series, about two thirds of patients with renal involvement were found to have membranoproliferative glomerulonephritis, and one third had mesangial proliferative glomerulonephritis (361); focal glomerulitis and purely membranous changes also have been described (42,135). Membranoproliferative glomerulonephritis has also been found in rare cases of monoclonal cryoglobulinemia in patients with chronic lymphocytic leukemia (259) or lymphoma (312), or occurring without apparent underlying disease (26,139,157,171). Here, renal deposition of IgG/anti-IgG complexes has been implicated in some instances (135). About one third of biopsy specimens from patients with MC contain large occlusive eosinophilic thrombi in small blood vessels and/or in a subendothelial or intramembranous distribution in glomeruli (361). The presence of these hyaline thrombi may suggest the diagnosis of cryoglobulinemic nephropathy even before the protein is detected in blood; their presence correlates only poorly with prognosis. From 15% to 30% of biopsy specimens have an associated renal vasculitis, which postmortem studies have indicated to be focal and segmental. Tarantino et al. (361) stressed the presence of a monocytic infiltrate in glomerular capillary loops and suggested an important pathogenic role for these cells, analogous to that indicated from studies of experimental serum sickness.

Membranoproliferative glomerulonephritis, type I (42,70,135,361)
Increased mesangial matrix
Focal glomerulitis
Membranous glomerulonephritis
Endocapillary proliferation (382)
Nodular subendothelial, intramembranous, or intraluminal deposits (hyaline thrombi) (111)
Positive immunofluorescence (IgM > IgG; C') (42,135,361)
Along glomerular basement membranes
Intraluminal thrombi
Monocytic infiltration of glomerular capillary loops (70,361)
Focal fibrinoid renal arteritis (135,361)
Interstitial infiltrate of plasma cells and/or lymphocytes
Fibrillar or tubular structures in deposits (109,111)
Crystalline inclusions in endothelial cells

TABLE 77.10. Renal Pathology

Immunofluorescence studies are positive in almost all cases and provide evidence for localization of cryocomplex components in tissue lesions (i.e., IgM > IgG > C') (42,135,361). In addition, specific MC CRIs have been demonstrated in glomerular immune deposits by using the polyclonal anti-Wa reagent or newer monoclonal antibodies (342). Particularly striking may be positive fluorescence corresponding to intraluminal thrombi in peripheral subendothelial deposits or in vessel walls at sites of vasculitis (382). Ultrastructural studies confirm the presence of electron-dense immune deposits along glomerular basement membranes. Several reports also noted the presence of distinctive fibrillar or tubular structures in deposits (109,111), reminiscent of the ultrastructure of some cryocrystalglobulins that have been examined electronmicrographically (290,355).

HEPATIC INVOLVEMENT

A number of early reports noted an association between clinical cryoglobulinemia and inflammatory liver disease (36,101,202). The latter is often subclinical, recognized by hepatomegaly and/or mildly elevated liver enzyme levels, and occasionally appreciated only at postmortem examination (135,202). Pathologic studies reveal a spectrum of lesions in different patients, ranging from minimal triaditis to chronic active hepatitis and/or cirrhosis. Most commonly, a chronic persistent hepatitis, often with a striking mononuclear cell infiltrate, is seen (36,202,252). Mixed cryoglobulins also may be found in patients with acute and chronic hepatitis and inflammatory bowel disease (ulcerative colitis, Crohn ileocolitis) without other clinical manifestations of cryoglobulinemia (101,120,201,223).

HEPATITIS C VIRUS AND MIXED CRYOGLOBULINEMIA

Since 1990, after the identification of the causative agent of “non-A non-B” hepatitis, a number of well-described case reports and clinical series corroborated an association between HCV infection and cryoglobulinemia (Table 77.11). Most of these studies originated from areas in southern Europe known to have a high incidence of HCV-associated hepatitis (30,55,82,92,114,211,246,283,359,406). Both types II and III mixed cryoglobulins have been found in several series (Table 77.10), whereas small surveys of type I cryoglobulinemia did not reveal a significant incidence of HCV antibody or HCV RNA. Overall, the prevalence of cryoglobulins in HCV infection has ranged from 13% to 54% (44,63,153,204,212,415), and the incidence of HCV associated with MC has ranged from 42% to 98% (93).

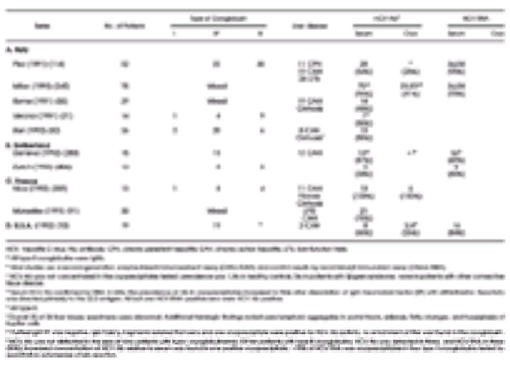

TABLE 77.11. Hepatitis and Cryoglobulinemia

By analogy to previous studies (Table 77.6), an etiologic role for HCV-containing immune complexes has been inferred in reports of concentration of HCV antibodies (HCVAbs) and/or HCV RNA in the cryoprecipitates of patients with MC (10,31,49,114,261). Most HCVAb activity directed to standard linear recombinant antigens used in commercial assays appears to be associated with the IgG fractions of mixed cryoglobulins (329). Of some interest has been the increased prevalence of HCV RNA compared with HCVAb in cryoprecipitates. RNase insensitivity of the HCV RNA in the cryoprecipitates suggested the possibility that they might contain antibodies to conformational determinants on the intact virion that are not recognized by standard assays for nucleocapsid or nonstructural protein antigens (10); alternatively, antibody activity may be directed to HCV quasispecies hypervariable regions that may be complexed in the cryoprecipitates or are not recognized in standard assays (13,190). An etiologic role also is suggested by the presence of HCV viral RNA and viral antigens in skin (11,320) and renal lesions (321) of patients with

cryoglobulinemia.

Several reviews have summarized an increasing spectrum of extrahepatic disease associated with HCV infection (144,191,228,410), including rheumatologic (405), cutaneous (69,146), and renal (85,331,344) manifestations that overlap with and extend descriptions of the clinical and pathologic features of cryoglobulinemia before appreciation of this association (Table 77.12). These series and case reports have expanded the articular (43,197,308), renal (80,254), cutaneous (98), and neurologic (86,364) disease manifestations that may be associated with, or that may occur in HCV infection apparently without cryoglobulinemia. More problematic have been reports associating HCV infection with other rheumatologic disorders, such as Sjögren syndrome and polyarteritis nodosa, where conflicting results have been obtained from different geographic areas (Table 77.12) (191,381); although HCV may be significantly associated with lymphocytic sialoadenitis (289) and sicca syndrome (176), most of these patients do not have other immunologic or clinical features of Sjögren syndrome. Overall, two large surveys from France (one multicenter and one a single monocenter cohort) reported prevalences of 19% to 23%, 15% to 17%, 11% to 12%, and 9% to 17% for joint disease, skin manifestations, sicca, and neuropathy, respectively, among patients with chronic HCV infection (45,47).

TABLE 77.12. HCV: Autoimmunity, Immunologic/Musculoskeletal Diseases, and Lymphoproliferation

Laboratory Findings

Serologic evidence of IgM RF activity (135) and selective depletion of the early components of the classic pathway of complement are characteristic although not invariable (360). For these studies, care is necessary in the processing of whole blood, as exposure to the cold for periods of time may allow cryoprecipitation to occur before the serum is separated, and cause complement activation *in vitro*. Rarer artifacts are pseudoleukocytosis and pseudothrombocytosis on Coulter analysis due to precipitation of cryoprotein in aggregates with the dimensions of white blood cells or platelets (137). Elevation of the erythrocyte sedimentation rate due to rouleaux formation also may be seen (391). In different series, disease activity, presumably due to active vasculitis, has been reflected in blood levels of endothelial cell markers and a fragment of the basement membrane antigen, laminin (125,369); anti–endothelial cell antibodies have been reported among HCV-infected patients with cryoglobulinemia (see also Table 77.8 and ,Table 77.9) (46).

A phenomenon that has been termed cold-dependent activation of complement (CDAC) is prevalent in HCV-infected persons (373). In CDAC, CH50 is low after cooling to 4°C overnight; C4 protein is normal, even though hemolytic C4 is low. CDAC was found in 28 (41%) of 69 HCV-infected sera, and none infected by hepatitis B virus. More than half of CDAC sera show cryoprecipitation, and 35% of samples with cryoprecipitation also showed CDAC. CDAC appears to represent a complement abnormality that may correlate somewhat with liver damage and response to treatment with interferon-a (IFN-a) (273). The mechanism responsible for this phenomenon is not understood, but may be consequent to HCV/antibody/mRF complexes with differing stoichiometry (403).

Similar to other chronic viral infections, in several of which [e.g., EBV, cytomegalovirus (CMV)] cryoglobulins and RF activity may be prevalent (Table 77.1), other autoantibodies have been found to occur with increased frequency in patients chronically infected by HCV (45,47,65,282,375). In general, the prevalence of autoimmune phenomena greatly exceeds that of autoimmune disease. Thus most ANAs are low titer and lack identifiable antigen specificity, most anticardiolipin antibodies are not associated with anti-b_2 glycoprotein 1 or clinical thrombosis (150,200,274), and, although thrombocytopenia secondary to hypersplenism is common in end-stage liver disease, autoimmune thrombocytopenia also may occur (Table 77.12) (23,150,158). The relative contributions of upregulation of specific germ-line genes encoding autoantibodies versus virus, or viral antigen-driven, autoimmune responses to these phenomena remain to be defined (327).

Studies that have addressed the possible contributions of viral versus host factors to the expression of autoimmunity in the setting of chronic HCV infection and hepatitis have yielded inconclusive results. Although skewing to a 2a genotype has been seen in some surveys of cryoglobulinemic patients, most studies have not revealed significant correlations with specific genotypes or subtypes of HCV compared with appropriate controls (79,122,275,343,409,421,422 and 423). A role for HLA restriction of cellular or humoral immune responses in HCV infection is suggested by increased expression of HLA antigens by infected hepatocytes (20); the fact that HLA class I–restricted cytolytic cells specific for HCV core, E1/E2, and NS2 proteins have been identified among liver-infiltrating lymphocytes, and can be expanded from peripheral blood with antigen (155,416); and increased levels of soluble HLA class I antigens in blood (209). Nevertheless, two studies that examined HLA antigens in patients with HCV-associated mixed cryoglobulinemia produced somewhat discordant results (16,199).

Lymphoproliferative Disease

Laboratory evidence of clonal B-cell proliferation in patients with type II cryoglobulinemia includes selective V-region gene use of heavy and light chains of the circulating monoclonal IgM, based on protein sequencing and the prevalence of MC CRI, increased numbers of IgMk-bearing B cells in peripheral blood, increased numbers of cells producing V_kIIIb-positive antiglobulins, evidence for immunoglobulin gene rearrangement among peripheral B cells (221), and increased numbers of clonal or oligoclonal lymphoid or plasmacytoid aggregates in bone marrow, assessed immunohistologically or by fluorescence-activated cell sorter analysis (FACS) (90,136,221,251,293,303). Interestingly, B-cell proliferation in the livers of patients with chronic hepatitis and cryoglobulinemia is distinct from that occurring in the peripheral compartment, or possibly in lymph nodes (84,322), in that hepatic B cells are more often CD5 positive (252), do not appear to be associated with HCV RNA, and do not appear to be significantly skewed to RF or MC RF V-region gene use (218,323). Clonal B-cell proliferations in peripheral blood are common among HCV-infected patients with or without evidence of type II cryoglobulinemia (124), and may regress with effective antiviral therapy (221). Limited V-region sequence analysis of monoclonal B-cells from patients with HCV-associated immunocytomas, or isolated from peripheral blood by using anti-CRI have confirmed skewing to kv325/51p1 gene use and intraclonal diversity consistent with antigen stimulation (78,170).

It has been recognized for many years that patients with type II mixed cryoglobulins may be found to have, or may develop, overt B-cell lymphoproliferative disease (42,135,136,168,293). Thus initial evaluation of such patients may include a computed tomography (CT) scan of the abdomen to look for organomegaly or significant retroperitoneal adenopathy, and a bone marrow examination that might include FACS analysis. An increased prevalence of HCVAb and/or HCV RNA among patients with NHL has been reported from a number of geographic areas in Italy since 1994, confirmed from France, Japan, the United States, Romania, and Turkey, but has not been universally corroborated (103). Limited studies have suggested that NHL in this setting of chronic infection may be analogous to mucosa-associated lymphoid tissue (MALT) tumors that develop after chronic *Helicobacter pylori* infection, and that the development of malignant lymphoid cells may be antigen driven (87,170). HCV-specific mechanisms that may potentiate oncogenesis in this setting include biologic effects of the core protein in modulating apoptosis and cell transformation (226), and direct interactions between envelope protein 2 (E2) and CD81, a cell-surface receptor on B (and other) cells, which may potentiate activation and proliferation (287); a correlation between lymphomagenesis and T(14;18) translocation also has been reported (424).

Clinical Course

Long-term follow-up studies of patients with essential mixed cryoglobulinemia indicated that in the absence of renal disease or progressive neurologic involvement, symptoms may remain static over prolonged periods (Fig. 77.4) (135,168). Patients need to be monitored closely, however, as some progress to clear-cut autoimmune or lymphoproliferative disease (134,284). In one recent series, NHL developed in five of 13 patients (123). The clinical course of patients with renal involvement is highly variable, with an overall 5-year survival rate of more than 60% (250). Fewer than 10% of patients have acute nephritis and have rapidly progressive renal failure with a poor outcome. The more common clinical picture is that of chronic glomerulonephritis, which may be recognized incidentally during evaluation for hypertension or cutaneous vasculitis. In a large clinical series, in 30% of patients with renal disease, acute renal failure developed at some point in their course, 7% progressed to chronic insufficiency, and only one (0.02%) of 44 patients required dialysis (361).

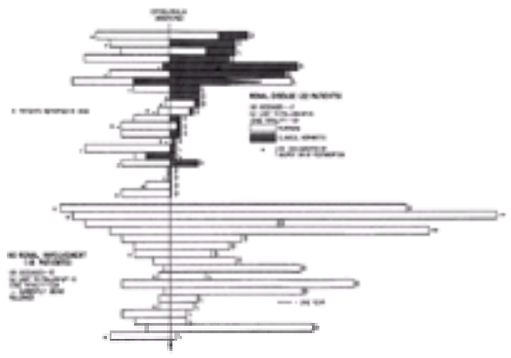

Figure 77.4. Long-term follow-up of 40 patients with “essential'` mixed cryoglobulinemia. *GN*, glomerulonephritis. (Updated from Gorevic PD, et al. Mixed cryoglobulinemia: clinical aspects and long-term follow-up of 40 patients. *Am J Med* 1980;69:287, and Gorevic PD. Mixed cryoglobulinemia: an update of recent clinical experience. In: Ponticelli C, Minetti L, D'Amico G, eds. *Antiglobulins, cryoglobulins, and glomerulonephritis*. Hingham, MA: Martinus Nijhoff, 1986:179–192.)

Treatment

Patients with mild cutaneous symptoms may require no therapy at all or can be managed symptomatically. A low-antigen diet was found to benefit stable purpura and to improve liver function and immune complex clearance function in a study involving 24 patients, nine with type II and 15 with type III cryoglobulinemia (37,117). Some patients may benefit from short courses of high-dose corticosteroids, and/or cytotoxic agents (6,191). High-dose intravenous gamma globulin (IVIG) has been reported to result in renal failure in one case of mixed cryoglobulinemia (22), and therefore should be used with caution; nevertheless, it led to disappearance of cutaneous vasculitis in a separate report (39), and appears to be well tolerated in others.

Immunosuppressive therapy has generally been reserved for patients with renal or progressive neurologic disease or other major organ involvement. Aggressive treatment of renal disease due to cryoglobulinemia may be necessary in the few patients in whom acute renal failure develops early or in those in whom nephrosis or progressive azotemia develops later (80,129,361). Adequate control of hypertension is particularly important in the group of patients with stable chronic renal disease (361). Because early reports indicated little benefit of corticosteroids alone in these patients (248), many have been treated with cytotoxic agents combined with high-dose steroids (129). Monthly pulse methylprednisolone has been used to reverse deteriorating renal function in uncontrolled studies (89). Cytotoxic agents that have been found to be effective include melphalan, chlorambucil, and cyclophosphamide (7,93). Purine analogs (2-chlorodeoxyadenosine; fludarabine) have been reported to be efficacious for refractory disease (105). Patients with end-stage renal disease can be maintained by dialysis (361) or can undergo transplantation; in some patients with mixed cryoglobulinemia, however, disease quickly recurs in renal allografts (160).

The effects of plasma exchange on the laboratory and clinical manifestations of cryoglobulinemia were studied in several series and case reports (129,316). Plasma exchange is rarely effective alone and is usually combined with cytostatic drugs (112) to suppress a rebound phenomenon that may develop about a week after each exchange (1). Treatment schedules and exchange volumes have varied greatly between reports, and controlled trials with sham apheresis have not been attempted. Cryopheresis, in which autologous plasma depleted of cryoprecipitable protein is returned to the patient, or cryofiltration using large-bore membranes, has been advocated by some investigators (1,162,340). Plasma exchange effectively lowers the cryocrit and other immune complex assay parameters, and may lead to improvement in measures of reticuloendothelial clearance, but has a variable effect on antiglobulin activity and complement levels (96). It may be particularly useful in the short-term management of cold-induced symptoms in type I cryoglobulinemia, and may benefit renal disease (107) or neurologic involvement (129) in patients with mixed cryoglobulinemia. Proof of efficacy of IFN-a was shown in an initial study involving seven patients with type II mixed cryoglobulinemia reported 2 years before the description of HCV (38), and has been confirmed in a number of series since 1991 (Table 77.13) (99,184). IFN-a has been used mostly in the setting of stable disease, is active for both types II and III HCV-associated cryoglobulinemia, but has not been shown to be efficacious for acute, progressive, or life-threatening presentations; appropiate therapy for cryoglobulinemia in patients coinfected with HBV or human immunodeficiency virus (HIV), or in the posttransplant (liver, kidney) patient with HCV-associated cryoglobulinemia, has not been established. IFN appears to be most effective for cutaneous vasculitis, and may be useful for glomerulonephritis, even after other therapies have failed (256,324), or combined with apheresis (182). Crossover studies have shown superior efficacy to corticosteroids with regard to virologic, biochemical, and clinical parameters (83,193). Although an antiviral effect has been implicated, it is not clear whether the beneficial responses observed also may be due to the antiproliferative or immunomodulatory effects of this drug; thus regression of clonal B-cell proliferation (221) and gammopathy (330), even in the absence of cryoglobulinemia, has been seen, and IFN-a may be effective therapy for HCV-negative cryoglobulinemia (57). Similar to the general experience in chronic HCV, although many patients respond to IFN-a, some are unable to tolerate the drug (277), relapses are frequent, and sustained responses off treatment are rare (Table 77.13). Ribavirin appears to be an effective modality for the treatment of mixed cryoglobulinemia associated with HCV, either as a monotherapy (100), or combined with IFN-a. Combination therapy has largely replaced the use of IFN-a alone because of higher response rates; similar to IFN-a, the effectiveness of ribavirin may be consequent as much to its immunomodulatory as to its antiviral effects (271). Additional treatment strategies may be expected to result from better understanding of the ways by which HCV succeeds in evading IFN-induced antiviral responses (363).

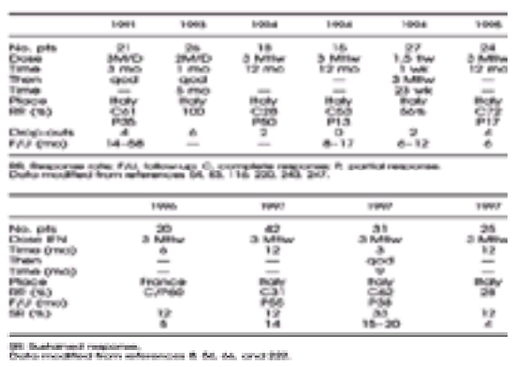

TABLE 77.13. Efficacy of IFN-a Monotherapy for Mixed Cryoglobulinemia Associated with HCV

The goal of specific therapies directed to immune abnormalities characteristic of cryoglobulinemic states has great appeal, stimulated by the introduction of biologics for the treatment of other immunologic and lymphoproliferative diseases. For the cryopathies, these include the therapeutic potential of monoclonal anti-CRI antibodies as generic agents that may downregulate specific antibody production, or help to clear specific immune complexes from the circulation (5,136), other monoclonal antibodies targeted to specific B-cell subsets (420), and agents that may interfere with complex formation between the IgM and IgG components of mixed cryoglobulins at the level of the antigen-binding site (302).

CRYOFIBRINOGENEMIA

The term *cryofibrinogenemia* has traditionally been used to refer to the presence of reversibly cold-precipitable protein in anticoagulated blood that can be shown antigenically or functionally to be fibrinogen (300,349). Cryoglobulinemia is excluded by the absence of a comparable precipitate in a paired sample of serum and the fact that immunoglobulin is not a significant component of the isolated cryofibrinogen. Plasma is collected in citrate, oxalate, or ethylenediaminetetraacetic acid (EDTA) (but not heparin) at 37°C and allowed to stand overnight at 4°C. Blood should be collected rapidly and sufficient anticoagulant added (e.g., 9:1, vol/vol blood/citrate) to avoid *in vitro* induction of fibrin formation. Thrombin inhibitors such as hirudin (1 to 5 units/mL) also may be added to citrated blood during collection to inactivate any formed thrombin (354). Although older reports noted the presence of “trace” amounts of cryofibrinogen in the plasma of “healthy” controls, these precautions were not routinely observed (224). Consequently, the presence of significant amounts of cryofibrinogen in normal plasma should be considered problematic and unlikely. In contrast, normal plasma that is freeze/thawed forms a major cryoprecipitate containing fibrinogen, fibrin, and fibronectin, and that is enriched in factor VIII and von Willebrand factor. This preparation has been used as replacement therapy for patients with von Willebrand disease, hemophilia A, or afibrinogenemia.

Clinical Features

In older and recent screening surveys, cryofibrinogen was reported in about 3% of random blood samples from hospitalized patients or healthy blood donors (33,349).

Prominent clinical associations include neoplasms and thromboembolic disease. Neoplastic states seen in association with cryofibrinogenemia are multiple myeloma, various solid malignancies (notably prostatic carcinoma), and leukemia. Thromboembolic disease may occur as occlusion of major vessels with secondary ischemia and gangrene, myocardial infarction, or stroke. Other associations noted in some large series have included pregnancy-at-term, use of oral contraceptives, diabetes mellitus, thrombotic vasculopathy (25), Raynaud phenomenon, cold-induced urticaria, pseudotumor cerebri, IgA nephropathy (264), and connective tissue diseases (33,224,349). Rare cases of apparently essential or familial cryofibrinogenemia also have been described (25,300,378).

Mechanisms

Fibrinogen-containing precipitates may form in plasma by at least two distinct mechanisms, neither of which requires the presence of a structurally abnormal fibrinogen. Both mechanisms yield cryoprecipitates consisting of fibrinogen, fibrin, and fibronectin in varying ratios (Fig. 77.5) (257).

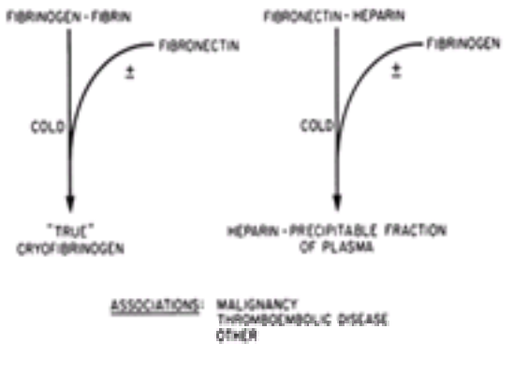

Figure 77.5. Pathogenic mechanisms for the formation of cold-insoluble fibrinogens.

HEPARIN-PRECIPITABLE FRACTION

The phenomenon in which the addition of a sulfated mucopolysaccharide such as heparin to plasma leads to a cryoprecipitate was first described as a concomitant of endotoxemia. It has since been found in a variety of other clinical states (348). The inciting event is the formation of cryoprecipitable complexes between heparin and fibronectin, although with reduced threshold and increased amounts in the presence of fibrinogen. Fibrinogen is incorporated secondarily during cryoprecipitation, accounting for its variable and sometimes minor contribution (353,358). This form of cryofibrinogen may be seen in the evaluation of any patient who is treated with heparin or who may have heparin in the line from which blood is taken.

FIBRIN COLD INSOLUBILITY

In this entity, the primary event appears to be the formation of complexes between fibrin and fibrinogen, with fibrin being the essential component for cold insolubility. Thus the amount of precipitate obtained will be determined by the relative concentrations of fibrin and fibrinogen, and fibrinogen alone will not cold-precipitate to any significant extent (354). The requisite for this molecular event will be any condition in which increased circulating levels of fibrin may be present, such as frank chronic intravascular coagulation (258). Cryofibrinogens also can be induced in normal plasma by addition of thrombin or thrombinlike enzymes in trace amounts sufficient to generate fibrin at low enough levels (15–30:1 *M* ratios of fibrinogen to fibrin) to prevent polymerization and permit the formation of soluble complexes with fibrinogen (257,354).

Fibronectin is not an essential component of this form of cryofibrinogen, but it is frequently incorporated because it may circulate as a complex with fibrin. Fibronectin itself is cold insoluble and, as noted earlier in regard to cryoglobulins, may act as a nucleus for binding of other proteins (e.g., C1q, IgG) or as a cryoprecipitagogue (53,151,313).

Treatment

Therapy is directly primarily to the underlying disease. The evaluation of thromboembolic disease in a young woman should also include a search for a lupus anticoagulant or antibodies to cardiolipin and, as noted earlier, positive results must be carefully interpreted in the setting of chronic HCV infection (200,274). Heparin has been used for the chronic intravascular coagulation syndrome, with the level of cryofibrinogen reflecting to some extent the response to therapy (258), stanozolol has had some success for the treatment of painful leg ulcers (108).

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78 VIRUSES

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VIRAL DISEASES AND THE ORIGINS OF VIROLOGY

Diseases caused by viruses have been described since antiquity, but the identification and description of viruses as infectious agents occurred just over 100 years ago, near the end of the nineteenth century (141). Thus, smallpox and measles made their appearance with the urbanization of cities along the banks of great rivers and the gathering of both people and animals in more restricted areas. Pox marks reminiscent of smallpox are found on the face and neck of the mummy of Ramses V (1158 BC), the earliest illustration of a suspected case of poliomyelitis occurs in an Egyptian stele dating from the eighteenth dynasty (1580–1350 BC), and description of rabid dogs is noted in early Greek and Roman literature. It was not until the late 1890s, however, that viruses were recognized as distinct infectious agents and as dangers to health.

The mid-1800s were the time of discovery of bacteria and the pioneering work of Louis Pasteur, Robert Koch, and their associates. During that period, the laboratory culturing process was developed so that bacteria could be grown, fixed on glass slides, stained, and observed under the microscope. Bacteria were retained on filters with defined pore sizes. After their identification, specific bacteria could be linked with particular disease states. This was the framework in which the first viruses were uncovered. Just before the twentieth century began, Dmitri Iosifovich Ivanovski in Russia and Martinus Beijerinck in the Netherlands demonstrated that the material responsible for a disease of tobacco plants was not retained but was passed through the pores of a Pasteur–Chamberland filter without losing infectivity (reviewed in 141). They found that this soluble residue of filtration could grow on healthy tobacco leaves. Their results constituted the first report of a plant virus, the tobacco mosaic virus. Similarly, Friedrich Loeffler and Paul Frosch in Germany concluded that the agent causing foot-and-mouth disease of cows also passed through porcelain filters and induced symptoms of disease when inoculated into previously healthy cattle. These observations, highly controversial at the time, provided the basis for defining viruses as subcellular entities that could cause distinct forms of tissue destruction, which became marks of specific diseases. Using similar techniques, yellow fever virus was the first virus isolated from humans. This isolation was achieved in 1901; so the twentieth century has seen the isolation, characterization, and understanding of viruses that involve both human and animal populations.

DEFINITION OF VIRAL DISEASES

Many viral infections are recognized as an acute illness. The causative virus enters the body, multiplies in one or more local tissues, and then may spread further through the blood or along nerves. The incubation period of 2 days to 2 or 3 weeks is followed by signs and symptoms of disease and local or widespread tissue damage. Viruses can be isolated from the patient's blood (serum or cells) or secretions for a short time just before and after the appearance of symptoms. The infected host either dies during the acute phase of illness or recovers from the infection and may exhibit life-long immunity to that virus.

Distinct from acute infections are persistent infections in which the immune response fails to completely eliminate the infection from the body, and virus may persist for months or years. Some viruses, for example, human immunodeficiency virus type 1 (HIV-1), persist at high levels and can be recovered throughout the entire course of infection. Although both humoral and cell-mediated immune responses are mounted following HIV-1 infection and play an important role in controlling virus replication, the immune response is not capable of terminating the infection. During the end stages of this infection, T-cell immunity declines, the viral load increases, and death from acquired immunodeficiency syndrome (AIDS) occurs.

A variation of persistent infection is *latent* infection, during which virus may remain in the host or within certain host cells without ongoing productive replication. Activation from latent to productive virus replication can occur, however, to allow virus spread. Latent infection is best exemplified by herpesviruses, for example, herpes simplex virus type 1 (HSV-1), in which reactivation results in cold sores, or varicella zoster virus (VZV), in which reactivation results in shingles. Figure 78.1 illustrates the clinical differences between acute, persistent, and latent infections.

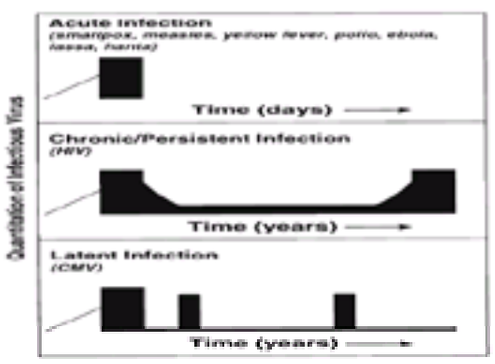

Figure 78.1. Infections caused by viruses differ. Some are acute, with virus elimination occurring in a week or two. Others are chronic, like HIV, which routinely runs a prolonged infectious course, whereas other viruses, like herpes simplex virus, persist in a latent form and infection sporadically reactivates, causing exacerbation of disease. The dark areas indicate the presence of infectious virus. (For more information, see Oldstone MBA. *Viruses, plagues and history*. New York: Oxford Press, 1998.)

Despite attempts to implement the current wealth of information about viral structure, genetics, replication, epidemiology, and pathogenesis in the control of viral diseases, viruses remain the most common cause of acute illness today and are implicated in an ever-increasing number of chronic diseases. How do viruses cause disease? Three mechanisms may be involved. First, the virus or its proteins may be directly toxic to cells, and the virus may kill the host cells it infects. With some viruses, this process serves to release virus particles from within cells to the outside environment. Second, the virus may not kill the cells it infects, but instead it may alter their differentiated functions. By this means, the synthesis of an important product made by a cell may be turned down or turned up; some examples are discussed later. The third way in which injury and disease can follow a viral infection is through the participation of the host's immune response. The antiviral immune response is generated to destroy infected cells or rid them of virus progeny and to remove infectious virus from the host's blood and other body fluids. By destroying virally infected cells or by causing “bystander” damage to surrounding cells, the immune system can damage tissues that are critical for the health of the organism. The immune response thus has potential to cause disease: this is called *immunopathology*. The balance between the protective and destructive processes of the immune system is in large part responsible for the clinical symptoms and signs that accompany virus infections.

The importance of the immune system in regard to viral diseases is thus tripartite. First, it may prevent the establishment and spread of virus infections so that pathologic consequences do not ensue. Prevention occurs when the nonspecific immune defenses are sufficient to block infection or if there is preexisting specific immunity to the virus as a result of vaccination or previous infection. Second, the immune response may combat established virus infections and terminate virus-mediated damage to host tissues. Third, antiviral or autoimmune responses evoked following virus infection may themselves have immunopathologic consequences.

This chapter briefly overviews viruses and their life cycles, giving some examples of their importance in human disease. It goes on to discuss different immune effector mechanisms and what is known from studies in animal models and in humans of their importance in protection against and the pathogenesis of viral diseases. Finally, it outlines strategies viruses have evolved to counter immune defenses.

VIRUS GENOME AND STRUCTURE

Viruses are among the smallest of all microorganisms and have an obligate requirement for intracellular growth because they depend on host cell structural and metabolic components. A virus particle, or *virion*, is composed of the viral genome, which may be single- or double-stranded, segmented or nonsegmented DNA or RNA, complexed with viral (and sometimes host) protein(s) to form the nucleocapsid, which may be filamentous or icosahedral. This nucleic acid and protein assembly may be surrounded by a lipid envelope in which one or more viral proteins are embedded, or it may be a more complex structure (Fig. 78.2). Viruses are classified on the basis of the type of genome they possess and whether the virion is enveloped. Table 78.1 illustrates this point and provides selected examples of human pathogenic viruses in various families. There is great diversity in size and complexity among viruses of different families; whereas poxvirus and herpesvirus genomes encode hundreds of proteins, parvoviruses and arenaviruses encode only four or five proteins. Viruses have relatively few genes, 4 to fewer than 200, compared with other organisms, for example, 5,000 to 10,000 genes for the smallest bacteria and approximately 70,000 to 90,000 genes for a human.

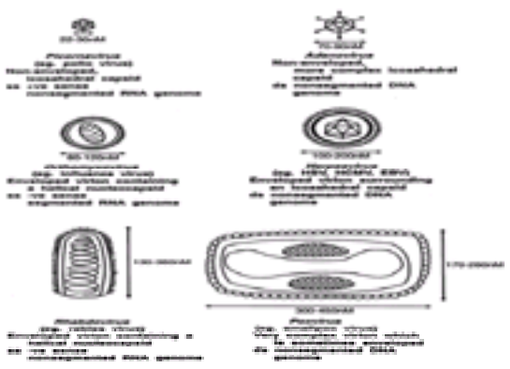

Figure 78.2. Examples of virus structure.

Class/delta	Virus family	Example
DNA viruses		
ds DNA, enveloped	Poxviridae	Smallpox, vaccinia
	Herpesviridae	Herpes simplex types 1 and 2, cytomegalovirus, Epstein-Barr virus, varicella-zoster virus
ds DNA, nonenveloped	Adenoviridae	Human adenoviruses
	Papovaviridae	Human papillomaviruses; JC and BK polyomavirus
ds/ss DNA, nonenveloped	Hepadnaviridae	Hepatitis B virus
ss DNA, nonenveloped	Parvoviridae	Human parvovirus B19 and B19-2
DNA virus with a DNA step in replication		
ss RNA, enveloped	Retroviridae	Human T-lymphotropic viruses I and II; HIV-1 and -2
Other RNA viruses		
ds RNA, nonenveloped	Reoviridae	Colorado tick fever virus, human rotaviruses
ss RNA, positive sense, enveloped	Togaviridae	Rubella virus and many encephalitis-causing alphaviruses
	Coronaviridae	Human coronaviruses
	Flaviviridae	Yellow fever virus, dengue virus, West Nile virus, and other encephalitogenic viruses
ss RNA, positive sense, nonenveloped	Picornaviridae	Poliovirus, coxsackie viruses, human enteroviruses, hepatitis A virus, human rhinoviruses
	Caliciviridae	Norwalk and other gastroenteritis viruses
ss RNA, negative sense, nonsegmented genome, enveloped	Paramyxoviridae	Parainfluenza viruses, measles virus, respiratory syncytial virus
	Rhabdoviridae	Rabies virus, vesicular stomatitis virus
	Filoviridae	Marburg and Ebola viruses
ss RNA, negative sense, segmented genome, enveloped	Orthomyxoviridae	Influenza viruses
ss RNA, ambisense, segmented genome, enveloped	Bunyaviridae	Bunyamwera virus, LaCrosse virus, Rift Valley fever virus
	Arenaviridae	Junin, Lassa, Machupo, LCMV

LCMV, lymphocytic choriomeningitis.

TABLE 78.1. Families Containing Viruses of Pathogenic Importance to Humans

It has been argued that the nucleic acid of viruses may have evolved from normal cell genes. Via gradual mutation, recombination, and reassortment, viruses could have evolved their own genetic structure. Mechanisms by which viruses can mutate are diagrammed in Fig. 78.3. Some viruses may have stayed within the parental host from which they evolved and display symbiotic or near-symbiotic relationships; but, as viruses moved from one host species to another or mutated to form new genetic mixtures, these formerly nonpathogenic viruses may have achieved a high level of virulence. A recent example is that of a primate virus that causes little or no disease in its natural host, the chimpanzee: Its transmission to humans (either through bites, contact with contaminated blood, or consumption of infected animals) is thought to have been the cause of the HIV-1 epidemic (59). Other examples include arenaviruses that produce asymptomatic or almost asymptomatic infections in their natural rodent hosts but on transmission to humans cause Lassa fever or Argentinian hemorrhagic fever and influenza viruses that cause asymptomatic infections in aquatic birds but cause disease in humans.

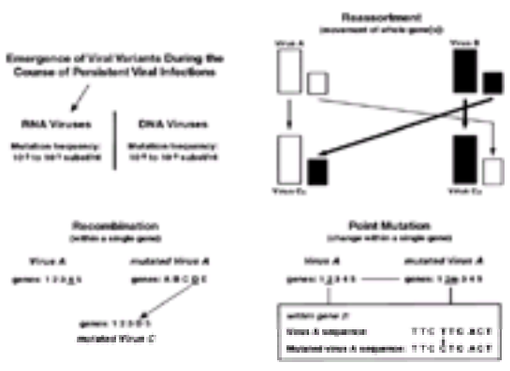

Figure 78.3. Cartoon of the constantly evolving landscape of viruses. **A:** The high mutational rate of RNA viruses. Because the polymerases of RNA viruses lack a proofreading mechanism to correct errors that occur during genome replication (DNA virus polymerases have such a proofreading mechanism), swarms of mutant viruses, making up a quasispecies, are constantly generated, with the fittest viruses selected over time by evolution. Hence, the progeny of a cloned RNA virus is not a clonal population (subst/nt = substitutions per nucleotide). B–D schematically show the three primary ways genetic changes occur in viruses. **B:** Reassortment (i.e., the movement of entire segments of genetic information). Each box represents a separate piece of nucleic acid that may encode one or several genes. **C:** The process of recombination, illustrated here as causing change in a single gene. In this example, gene D from mutated virus A replaces gene 4 of virus A to form new virus C. **D:** Changes introduced within a single gene by point mutation. Alteration of a single nucleotide within a gene can give rise to a new virus with distinct biological properties. Nucleotides depicted: (T) thymine; (C) cystosine; (A) adenine. The example shown is real data from a study using LCMV. Virus A (which represents part of the sequence from Armstrong strain) undergoes a T C mutation at base pair 855. The TTC triplet codes for the amino acid phenylalanine; the mutated *C*TC codes for the amino acid leucine. This single amino acid change allows the mutant virus to cause immunosuppression and establish a persistent infection in adult mice; by contrast, the parental virus does not cause immunosuppression and is readily cleared from adult animals.

VIRUS INFECTION AT THE CELLULAR LEVEL

Virus replication in host cells involves a series of steps. First, the virus contacts the cell to be infected and then attaches to its surface. Second, the virus conveys its genetic material and accessory proteins across the plasma membrane barrier into the cell cytosol. Further uncoating then may need to occur, and the virus may traffic to a particular site in the cell (e.g., the nucleus). Viral gene expression and genome replication then take place. Finally, virion assembly and release of mature virions from the host cell occur.

The first steps in virus infection of a host cell, attachment, and entry are dependent on interactions between virion proteins and specific components of the host cell surface. As illustrated in Table 78.2, different viruses may use cell membrane lipids, carbohydrates, or proteins as binding and entry receptors on host cells. Of the

viruses that bind to protein receptors, a surprisingly large number evolved to use cell membrane proteins that are members of the immunoglobulin superfamily or have immunologic functions as their host cell receptors. One explanation could be that virion binding to these proteins confers some advantage on the virus in terms of immune evasion. For example, in HIV-1, use of the CD4 protein as its primary cellular receptor (46,118) results in this virus infecting $CD4^+$ T cells, which may contribute to impairment of this arm of the antiviral immune response observed in most HIV-1-infected patients (165). In many cases, however, such a link has yet to be demonstrated. In addition to access through classic receptor interactions, viruses sometimes enter cells by other means. For example, infectious virus complexed with antibody can bind to and enter Fc receptor positive cells even though they may not bear specific receptors for the virus.

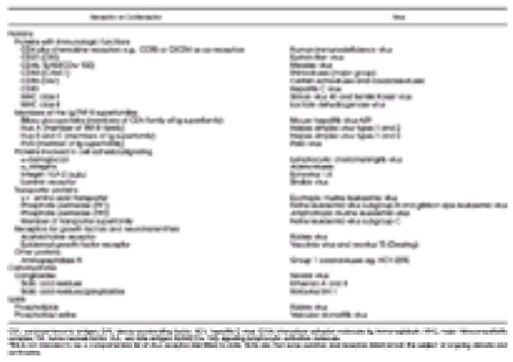

TABLE 78.2. Examples[a] of Host Cell Surface Components Used as Receptors by Viruses

Virion binding to the host cell surface is followed, often within seconds, by virus entry. This is an active, energy-dependent process. For some enveloped viruses, including HIV-1, entry is achieved by a virion protein with fusion function causing the viral envelope to merge with the host cell plasma membrane so that the viral nucleocapsid is released into the cytoplasm. Many other viruses enter the host cell by endocytosis, and then, as the endocytic vesicles move into the host cell and their internal pH drops, virion proteins are triggered to mediate transfer of the virus genome into the cytoplasm. Specific immune response that prevent the establishment and spread of virus infection are thus generally directed against virion structural components, frequently the viral proteins involved in attachment and fusion.

The pattern of expression of specific attachment and entry receptors for viruses on host cells is a key determinant of virus tropism within the host; however, virus replication also may be blocked at subsequent steps in the replication cycle. The virus must be able to traffic within the cell to the site where replication takes place. A major block to HIV-1 replication in resting cells is the inability of the viral replication complex to cross the nuclear membrane into the nucleus, where proviral DNA integration and replication take place. The cell also must provide an appropriate environment for viral transcription, protein production, and genome replication to take place. Viruses may use a variety of strategies for ensuring that their own products are efficiently produced within the host cell. Host cell transcription and translation may be shut off completely, as seen, for example, with poliovirus, or they may be more subtly altered.

Assembly of progeny virions then occurs within the nucleus or the cytoplasm. For enveloped viruses, the virion envelope proteins are inserted into a host cell membrane, in some cases the plasma membrane, and new virions bud from this surface. Virions formed within the cell may be released without cell lysis; in other cases, the host cell is lysed to enable virion release. Virus infection may result in death of the host cell by a number of mechanisms, including shutdown of cellular macromolecular synthesis, toxicity of accumulated viral capsid proteins, syncytium formation, or direct lysis. However not all productive virus infections are lytic, as exemplified by the noncytopathic infections caused by arenaviruses and many retroviruses.

From this brief, generalized account of virus–host cell interactions, it can be seen that immune responses that clear an established virus infection may be targeted against (a) virion structural proteins inserted into the host cell plasma membrane; (b) peptide epitopes of virion structural or nonstructural proteins, which as these proteins are being produced intracellularly, are presented on the cell surface in association with the host's class I major histocompatibility (MHC) molecules; or (c) novel or modified host proteins or epitopes, which start to be expressed as a result of virus-induced alterations in host cell transcription and translation. Immunopathologic damage may result from these responses directed against virus-infected cells or from autoimmune responses induced either to host antigens presented following destruction of host tissues or as a result of virus-induced dysregulation of the immune system.

VIRUS INFECTION, SPREAD, AND PRODUCTION OF DISEASE

After contact with a eukaryotic host, viruses may directly invade deeply into the host, but they frequently undergo an initial round of replication at the site of entry (often the respiratory or gastrointestinal tract). Whereas many respiratory and enteric viruses (e.g., rhinoviruses and rotaviruses) are confined largely to epithelial surfaces, others (e.g., measles) reach subepithelial tissues, where they may infect cells of the immune system, such as macrophages, and be carried to local lymph nodes, where further replication occurs. After primary replication, virus may be disseminated by the blood or lymph, often within infected immune system cells, and cause a systemic infection. Other routes for virus spread are the cerebrospinal fluid and nerves. Viruses show tropisms for distinct host tissues, resulting in characteristic patterns of disease (e.g., skin rashes, pancreatitis or diabetes, orchitis, meningitis).

Disease results from virus effects on host tissues or the immune response to infection. As discussed already, viruses can cause direct damage to cells by a number of mechanisms. Examples of diseases in humans that result from such damage are poliomyelitis, which follows infection of motor neurons in the spinal cord by poliovirus, and some common colds, which follow infection and destruction of nasal epithelial cells by rhinoviruses. Noncytopathic viruses may cause more subtle changes in infected host–cell functions that result in disease (e.g., by affecting cellular “luxury functions”) (reviewed in 47,136). Examples are the murine virus lymphocytic choriomeningitis virus (LCMV), which can cause a persistent infection of growth hormone (GH)-producing cells in the anterior pituitary; in the absence of cytopathologic changes, the virus produces a selective decrease of GH transcription, which results in a failure of the animal to grow because of GH deficiency (96,145,189). Rabies virus is another example; it impairs acetylcholine and other membrane receptor functions in neural cells *in vitro* and *in vivo* without producing a cytopathic effect (100,188). In addition, viruses may transform the cells they infect and may be involved in neoplasia; for example, human papilloma viruses 16 and 18 are associated with more than 90% of human cervical cancers. Because of this association, it has been shown that screening of women to detect papilloma virus in cervical tissue is far more reliable than the conventional Papanicolaou smear. It is estimated that 12% to 20% of all cancers in humans are caused by viruses. Examples of diseases that arise as pathologic consequences of various types of antiviral immune response are discussed later in the chapter as these responses are detailed.

As in an individual cell, virus infections *in vivo* may be acute or persistent. To persist *in vivo*, viruses, in addition to possessing unique component(s) or a strategy of replication that ensures a noncytopathic phenotype, must avoid detection and clearance by the immune system; mechanisms by which viruses achieve this are discussed later.

IMMUNE DEFENSES AGAINST VIRUS INFECTION

Vertebrate hosts possess an armamentarium of complex and interrelated defenses against invasion by viruses and other microbial pathogens. Such defenses may be subdivided into *innate mechanisms* and the *acquired response*. Nonspecific defense mechanisms include physical barriers to virus infection, such as the skin, gastric acid, and the mucociliary system in the respiratory and gastrointestinal tracts, and components of the immune system, including complement, interferons, macrophages, and natural killer (NK) cells, which provide early defenses against virus infection in the nonimmune host. The acquired immune response is characterized by (a) specificity (it is mediated by T- and B-lymphocytes, which have surface receptors capable of distinguishing distinct foreign antigens); and (b) memory (on second contact with a particular antigen, a more rapid immune response of higher avidity is mounted). The effector components of the specific immune response are antibody molecules produced by B cells, cytokines produced by T cells, and cytotoxic T-lymphocytes (CTLs). The specific immune system plays a major role later in the infection and in preventing reinfection. In addition, there is interplay with nonspecific immune effector mechanisms, which are activated during the specific immune response and interact with specific effector mechanisms to mediate virus clearance.

Nonspecific Immune Responses

INTERFERONS

Interferons are the earliest of the known host-induced defenses that operate to control virus infections. Local interferon production occurs within hours of infection, before the development of the inflammatory response. There are three distinct types of interferon (IFN): IFN-a, formed primarily in leukocytes; IFN-b, synthesized predominantly in fibroblasts; and IFN-g, or immune interferon, secreted by stimulated lymphocytes. Normal cells usually do not synthesize detectable amounts of interferons; lymphocytes produce IFN-g in response to stimulation by mitogens or specific antigen, and IFN-a and IFN-b production by leukocytes and fibroblasts is

induced by a variety of agents, including double-stranded RNA, various metabolic activators and inhibitors, and most importantly virus infection. In general, RNA-containing viruses are good interferon inducers, whereas DNA-containing viruses, with the exception of pox viruses, tend to be poor interferon inducers.

Interferons have many biologic effects, including conferring an antiviral effect on host cells, cell multiplication–inhibitory activity, effects on differentiation, and effects on the immune system that include enhancement of cell surface MHC molecule expression, enhancement of NK cell activity, and antibody-dependent cell-mediated cytotoxicity, suppression of antigen- and mitogen-induced leukocyte migration inhibition, and driving the turnover of memory phenotype T cells (which may play a role in the maintenance of immune memory) (186). The various interferons have differential efficiencies in mediating the various effects; for example, IFN-a and IFN-b, are more efficient inducers of an antiviral state in host cells than IFN-g, whereas IFN-g has more pronounced effects on the immune system and potentiates the antiviral activity of IFN-a and IFN-b.

The IFN-induced antiviral state renders infections abortive and in some virus–cell systems protects cells from the effects of virus infection; so more IFN-treated than -untreated cells survive (171). Although the basis of the IFN-induced antiviral state in cells is still not completely understood, there is now some insight into this problem (reviewed in 191). IFNs themselves do not inhibit virus multiplication; IFN binding to cell-surface receptors is followed by induction of the synthesis of proteins, which are the actual effectors of the antiviral state. One of these proteins is the enzyme oligoisoadenylate synthetase, which is activated by the presence of ds RNA to catalyze the formation of a family of oligonucleotides collectively termed 2,5-oligoA. 2,5-OligoA activates RNase L, a ribonuclease that is present in latent form in the cytoplasm of mammalian cells, which cleaves viral and cellular RNA. Although RNase L itself possesses no specificity, it is thought that some specificity may be achieved by the highly localized formation of 2,5-oligoA. A phosphodiesterase also found in IFN-treated cells can regulate this system by cleaving 2,5-oligoA. Another IFN-induced enzyme is a 67-kd protein kinase that, when activated by virus infection or ds RNA, phosphorylates the small subunit of protein synthesis initiation factor eIF2, resulting in inhibition of protein synthesis. Several viruses encode inhibitors of the 67-kd protein kinase (e.g., the VA_1 RNA of adenoviruses) (94,95,146), which may render the virus resistant to IFN. The 2,5-oligoA synthetase and the 67-kd protein kinase system are of different importance in providing the basis for the antiviral activity of interferons against different viruses in different cell types (reviewed in 191). Yet another IFN-inducible gene is the *Mx* gene, which determines resistance to influenza viruses in mice (181). This gene, which is also present in humans, encodes a protein that drastically inhibits primary transcription of influenza virus genes by an unknown mechanism and does not confer resistance against any other virus.

Interferons are thought to be a key component of the *in vivo* antiviral defense mechanisms. Their production is associated with a reduction of virus titers not just locally but throughout the body, suggesting that interferons limit virus production and spread. It is difficult to assess the precise role of IFN in virus infections in humans because primary and secondary immunodeficiency states, where there are defects in IFN production, usually involve multiple immunologic changes. There are some reports of diminished IFN production capacity resulting in repeated or severe infections (e.g., in children with recurrent respiratory infections (79) or patients with acute fulminating viral hepatitis) (109). Moreover, clinical trials have shown that human IFN-a and IFN-b, when inoculated early at high doses, can shorten the course of a number of virus diseases (reviewed in 30,203). In addition, there is strong support from experimental animal models that IFN is a critical antiviral defense mechanism. Treatment of animals with IFN or IFN inducers before or during acute virus infections has protective effects (13), whereas elimination of IFN or IFN receptors increases susceptibility to a number of viruses (16,63,106,128,192).

In addition to their protective role, IFNs may have immunopathologic effects. Clinical trials revealed side effects that included nausea and vomiting, anorexia, fatigue and malaise, and myalgias (157); and it has been suggested that many of the clinical symptoms of influenza virus infections may be caused by IFN. A good example of immunopathologic roles of IFN-g is provided by work with LCMV infection of mice. Anti-IFN treatment of one strain of mice persistently infected from birth with a particular strain of LCMV inhibits liver necrosis, glomerulonephritis, runting, and deaths that normally are seen; and experiments done by Leist et al. (106) showed that treatment of mice with anti-IFN-g antibody would protect them from the lethal, T-cell-mediated choriomeningitis that follows intracerebral inoculation of small doses of LCMV.

NATURAL KILLER CELLS

Natural killer (NK) cells are a subset of non-T, non-B lymphocytes that do not bear antigen-specific receptors generated by gene rearrangement (NK cells develop normally in *RAG* knockout and severe combined immunodeficiency disease, or *SCID* mice). They are a component of the innate immune response and are activated early after infection: They can help to combat virus infection both via cytotoxic activity and via the production of cytokines and chemokines, in particular IFN-g (reviewed in 21). The factors responsible for the activation of NK cell migration, expansion, and effector functions after infection still are not defined completely, but it is thought that soluble mediators such as type 1 IFNs, other cytokines [tumor necrosis factor-a (TNF-a, interleukin (IL)-12, IL-15] and chemokines plus contact with cell surface ligands are involved. In addition to these triggering signals, NK cell activity is tightly controlled by inhibitory signals. These are transmitted by NK cell surface receptors that contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic tails. There are two main classes of receptors on NK cells that can mediate either stimulatory or inhibitory signals: (a) the killer immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LIRs) and (b) C-type lectin (Ly49 and CD94-NKG2) receptors (reviewed in 105,114,206). Human KIRs recognize sets of classic human leukocyte antigen (HLA) class I allotypes, and the human CD94-NKG2 receptors bind to the class Ib molecule HLA-E, which is stably expressed at the cell surface only after binding peptides derived from the signal sequences of other HLA allotypes. These receptors thus appear to play complementary roles in surveying the biosynthesis and expression of class I molecules, which can be hampered during viral infections or in certain tumors.

Natural killer cell activity usually peaks rapidly (within hours to days) after virus infection, paralleling levels of virus-induced interferon, and declines as the virus-specific CTL response is induced (around day 7 after infection). It is therefore tempting to speculate that NK cells represent a rapid, nonspecific first line of defense against virus infections, which is superseded by the virus-specific CTL response. The contribution that NKs cell make to early control of virus replication has been demonstrated both in murine and human virus infections by documentation of increased sensitivity to certain viruses in situations where NK cells are depleted or deficient. Mice depleted of NK cells by treatment with antiserum against NK cell markers show greatly increased replication of some viruses, for example, murine cytomegalovirus (MCMV), mouse hepatitis virus (MHV), and vaccinia but not others (e.g., LCMV) (21). Similarly, adoptive transfer of NK cells protected suckling mice from MCMV but not LCMV (28). Beige mutant mice, which have aberrant large granular lymphocyte morphology and reduced NK cell lytic activity, show increased susceptibility to MCMV compared with control mice (173). Similarly, the Chédiak-Higashi syndrome in humans, in which granule-dependent functions including NK cell lysis are depressed, is associated with increased susceptibility to virus infections (121,149,202), and patients in which NK cells are absent also exhibit poor control of certain virus infections. The best documentation of this is for control of primary herpesvirus infections (20). Additional evidence that NK cells have an important role in this infection in humans derives from the finding that human cytomegalovirus (HCMV) has evolved two proteins that are hypothesized to decrease the susceptibility of infected cells to NK cell lysis: gpUL18, a class I homolog that binds to the NK cell inhib itory receptor LIR-1 (44,51,107,160) and gpUL40, which upregulates HLA-E expression (185).

COMPLEMENT

The human complement system comprises more than 20 proteins present in normal serum, which on activation undergo a cascade reaction, generating products that mediate and amplify immune and inflammatory reactions. The complement cascade (see Chapter 30) can be initiated in three ways: (a) by the classical pathway, which is activated by the binding of the C1q subunit of C1, the first complement component to an immune complex; (b) by the alternative pathway, which does not involve antibody (in the latter process, C3b generated by slow spontaneous hydrolysis of C3, is protected on the surface of an activator, e.g., a virion, from factor H, which initiates a cascade of reactions); (c) by the binding of mannan binding lectin to a suitable acceptor with subsequent activation of specific proteases that ultimately activate C3. The later complement components have various biologic activities; for example, C3a and C5a are anaphylotoxins, C3b can attach to receptors present on macrophages and polymorphs and mediate opsonization, and the C5b-9 complex can insert into the lipid bilayer of cell membranes, producing cylindrical membrane lesions similar to those induced by the perforins of CTL and NK cells.

Following virus infection, the classical complement pathway may be activated by complexes of virus and antiviral antibodies or antiviral antibodies bound to viral proteins expressed on the surface of infected cells. Certain viruses are also capable of triggering the classical pathway directly in the absence of antibody; they include retroviruses such as Moloney murine leukemia virus, in which the viral envelope protein p15E is known to bind the C1q component of complement, causing C1 activation (14); Sindbis virus; and Newcastle disease virus (72,196). Other viruses activate complement directly via the alternate pathway, including Sindbis, vesicular stomatitis, measles, and respiratory syncytial (RSV) viruses (179). The alternative pathway also can be activated by cells infected with viruses such as mumps, parainfluenza, herpes simplex, and measles in the absence of antibody, although measles virus-infected cells are not lysed unless immunoglobulin G (IgG) is present (43,176).

Complement thus may be activated *in vivo* at early times after virus infection before the development of the specific immune response and can potentiate the antiviral effects of antibody, which may be particularly important during the early stages of infection when the antibodies present are only of low affinity. Complement mediates its antiviral effects by at least three mechanisms: (a) It produces an acute inflammatory reaction with leukocyte accumulation. (b) Complement activation on the surface of infected cells may result in lysis of those cells. With measles virus-infected cells, activation of the classical pathway alone is not sufficient for cell lysis; it requires activation of the alternate pathway as well (43). (c) Complement activation (with or without antibody) on the virion surface may result in virolysis (damage to or lysis of the membrane of enveloped virions, leading to leakage or destruction of viral nucleic acid (142,168); enhancement of neutralization of virions by antibody; the complement components, perhaps providing extra coverage of the viral proteins involved in viral attachment (7); or opsonization of the virion, allowing it to be more readily destroyed by phagocytes.

Although complement clearly can mediate a number of antiviral effects, its importance in protection against virus disease *in vivo* is uncertain. Mice that were

C3-depleted by treatment with cobra venom factor showed increased severity of Sindbis and influenza virus infections, and a C5-deficient mouse strain showed increased organ titers of Sindbis virus (70,72). Human genetic deficiencies of complement, although predisposing to bacterial infections, do not seem to predispose to virus infections (102).

MACROPHAGES

Macrophages and related cells play a central role in the immune response to viruses, having an essential function in antigen presentation to and the activation of antiviral T cells as well as possessing virucidal, cytotoxic, virustatic, and monokine-secreting potential. Details of antigen presentation and T-cell activation by macrophages and other presenting cell types are covered elsewhere in this book. Here we focus on the other antiviral roles of macrophages.

It is hypothesized that the activation of macrophages and dendritic cells initially is triggered following infection by both endogenous and exogenous signals. Endogenous signals include the presence of necrotic cells, heat shock proteins, and cytokines, such as type 1 IFNs (58). Exogenous signals are thought to be delivered by the binding of components of pathogens to pattern recognition receptors on the surface of macrophages and related cells (81). The best documented of these is the murine Toll-like receptor Tlr4, a signal-transducing receptor activated by bacterial lipopolysaccharide (19); other pattern-recognition receptors have been shown to bind to yeast mannans (10). To date, no mammalian pattern recognition receptors that bind to viral components have been identified; but plant resistance genes with homology to Toll proteins have been described, for example, the *N* gene of tobacco, which mediates resistance to tobacco mosaic virus (197).

Following activation, macrophages secrete a large number of soluble mediators, termed *monokines*, which include IFN (see preceding discussion) and IL-1, which not only is required for the initiation and amplification of T-cell-dependent immune responses and the inflammatory response but acts as an endogenous pyrogen, generating the fever that commonly accompanies virus infections in large mammals. Fever may well have significance in antiviral defense: *In vitro* immune responses are enhanced when cells are cultured at 39°C rather than 37°C (12); and in experiments where virus-infected animals were maintained at low temperatures, disease severity was markedly increased (115,161). These experiments are difficult to interpret, however, because animals undergo complex physiologic changes under these conditions.

Macrophages are also able to destroy virus-infected cells. There is some evidence that cytotoxicity may be mediated in the absence of antibody (33,108), but more common is macrophage-mediated antibody-dependent cell-mediated cytotoxicity (ADCC), a process whereby macrophages are targeted to virus-infected cells by specific antibody bound to Fc receptors on the macrophage surface, introducing specificity into what otherwise would be a nonspecific effector mechanism. In addition, macrophages are able to block cell-to-cell spread of viruses such as herpes simplex in monolayers by a noncytolytic, IFN-independent mechanism. Wildy et al. (200) suggest that arginase secreted by macrophages deprives the cells of arginine essential for the growth of herpes viruses and has a virostatic effect.

The interaction of viruses with free and tissue-fixed macrophages (e.g., Kupffer cells in liver, alveolar cells in lung, and microglia in brain) at early times after infection can be of key importance to the outcome of virus infection. Many viruses multiply within macrophages, and macrophage susceptibility or resistance may determine host susceptibility or resistance, as is illustrated in infections of mice with MHV-3, ectromelia, and HSV. With MHV, the liver macrophages of susceptible mouse strains (e.g., PRI) are destroyed by the virus (93), whereas the virus is not uncoated following adsorption to and penetration into macrophages of resistant C3H mice. A virulent strain of ectromelia productively infects liver macrophages and thus is able to undergo extensive replication in the liver, whereas, in contrast, an avirulent strain could establish only limited infection of liver macrophages and thus could not invade liver cells on a significant scale and produce disease (162,163). Similarly, the age-dependent susceptibility of mice to HSV appears to be closely correlated with changes in the susceptibility of macrophages to infection (123). In humans, dengue virus virulence also has been correlated with the ability of the virus to grow in monocytes (67).

Antigen-specific Immune Responses

ANTIBODY

Antibody responses are mounted following almost all virus infections, and their presence is used as the classic marker for prior exposure to a virus. Antibodies can exert antiviral effects through several distinct mechanisms, some directed against free virions and others against virus-infected cells. Antibody binding to virions can prevent infection of host cells. The antibody may prevent viral attachment to host cell receptors (deposition of complement proteins on the virion surface may contribute to the block of receptor binding, too); alternatively, it may not affect receptor recognition but may alter the capacity of the virion to undergo conformational changes involved in virus entry. Studies with monoclonal antibodies that bind to different epitopes on the influenza virus Hemagglutinin (HA) molecule, the positions of which have been mapped on the three-dimensional structure of the molecule, suggest that antibodies binding to different regions of the molecule mediate neutralization in different ways. Those binding near the tip of the molecule around the cleft that is involved in receptor recognition perhaps block attachment, whereas those binding near the HA_1/HA_2 cleavage site perhaps block entry and uncoating. Yet another monoclonal antibody against the influenza virus HA does not prevent attachment, penetration, or uncoating, but it inhibits viral RNA transcription (156).

Because antibodies are multivalent, they may cause clumping (agglutination) of virions, which not only may prevent virus entry but reduce the number of infectious units. In addition, the formation of virus–antibody complexes promotes uptake and clearance of virus by Fc receptor-positive phagocytic cells (opsonization); again, this process may be enhanced by complement deposition. As described earlier, antibody also may initiate complement-mediated lysis of virions.

One mechanism by which antibody combats virus-infected cells is by antibody- and complement-mediated lysis. The antibody involved may be directed against the envelope proteins of budding viruses, other viral proteins expressed on the plasma membrane, or even neoantigenic determinants. Cells may express sufficient levels of viral antigen to bind immunoglobulin, leading to lysis, from within a few hours after infection. Cooper and Oldstone (43) studied the lysis of human diploid cells infected 48 hours previously with HSV or measles virus, and they calculated that 5×10^6 antibody molecules must be bound to the infected cells to achieve virtually complete lysis: antibody-mediated lysis of infected cells is thus much less efficient than CTL-mediated lysis or ADCC. IgG antibody also may render infected cells targets for ADCC mediated by Fc receptor-bearing cells, including polymorphs, macrophages, and NK/K cells. ADCC was shown by Shore and Romano (174) to be hundreds of times more efficient per antibody molecule than complement-mediated cytolysis and hence might be more relevant *in vivo*.

Antibody bound to the surface of infected cells also can inhibit virus multiplication by mechanisms that do not involve cell lysis (134). Work with measles virus–infected cells has shown that antibody can also “modulate” (i.e., induce elimination of) viral antigens expressed on the cell surface (reviewed in 144). Such modulation interferes with viral assembly and, in the case of measles virus, may inhibit F protein-mediated cell–cell fusion and virus spread. It also may interfere with cytoplasmic events of viral replication; for example, levels of the viral phosphoprotein in the cytoplasm of measles virus-infected cells can be influenced by antibody to cell surface elements. Again, antigenic modulation can occur with significantly less antibody than is required for antibody-dependent, complement-mediated lysis of infected cells. Similar findings have been shown for antibodies and Sindbis virus *in vivo* and for influenza virus *in vitro*.

Antibodies of different classes are of different importance in antiviral defense in certain situations. IgM and IgG are the major classes of antibody responsible for virus neutralization in serum. They are particularly important for controlling viruses that produce systemic disease characterized by a plasma viremia (e.g., yellow fever or poliovirus). Children with severe hypogammaglobulinemia recover normally from measles or smallpox vaccination but are about 10^4 times more likely than normal children to develop paralytic disease after vaccination with live poliovirus vaccine (204). They are also susceptible to echovirus, meningitis, and VZV infection (42,201). The first antibodies to be produced following infection are of the IgM class. The multivalency of this antibody, which, for example, enables complement to be activated when a single IgM molecule is complexed with antigen (not the case with monomeric IgG), compensates somewhat in terms of protective efficiency for the fact that the early antibody response is usually of low affinity. The higher-affinity serum IgG response that develops subsequently may persist, providing protection against reinfection for many years; in addition, IgG antibodies can cross the placenta and confer passive protection on the fetus. Secretory IgA antibody neutralizes viruses on mucosal surfaces, which is of particular importance for resistance to viruses that normally gain entry through the respiratory or intestinal epithelium. In contrast to IgG, IgA does not recruit effector systems such as phagocytic cells or complement but mediates its protective effects by directly preventing virus binding to mucosal surfaces. In addition, secretory IgA does not provide good long-term protection against reinfection. The importance of IgE in antiviral protection is uncertain. It may be involved in some forms of virus-induced immunopathology, possibly contributing to the rashes that accompany certain virus infections by inducing local inflammation, and the wheezing seen in respiratory infections (e.g., with RSV) by inducing bronchospasm. Both these conditions result from a type 1 hypersensitivity reaction in which mast cells with surface-bound IgE are triggered to degranulate when the antibody contacts its specific antigen, releasing histamine and other mediators.

In a number of other instances, the antibody response to virus infection has detrimental effects in addition to or as a by-product of its protective effects. As reviewed by Oldstone (137), the binding of antibody to viral proteins or free virions may result in the formation of circulating immune complexes, which may deposit in the walls of small blood vessels in the skin, joints, and kidney, giving rise, for example, to the prodromal rashes seen in exanthematous virus infections and hepatitis B and to immune complex glomerulonephritis (Fig. 78.4). With persistent virus infections, where virions and viral antigens are continuously generated and antibody responses are frequently of low affinity or are directed against nonneutralizing epitopes, complexes may be deposited for long periods. A classic example is provided by mice persistently infected from birth with LCMV, where circulating immune complexes are progressively deposited in glomeruli (137,143), resulting in certain mouse strains in glomerulonephritis, uremia, and death. Systemic immune complex reactions also may give rise to disseminated intravascular coagulation, which occasionally is seen with yellow fever and other arbovirus hemorrhagic fevers.

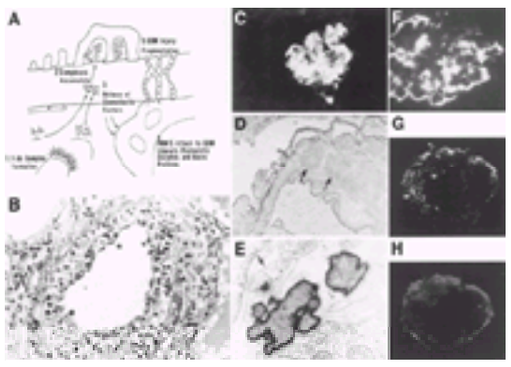

Figure 78.4. Illustration of how disease can be triggered by virus-induced antibody–antigen immune complexes in an experimental small animal model and the subsequent expansion of that information into human disease. **A:** How trapping of virus–antibody (V-Ab) complexes in the kidney can trigger local inflammation resulting in damage to the glomerular basement membrane (GBM). **B–E:** Examples of immune complex disease in mice persistently infected with lymphocytic choriomeningitis virus (LCMV). The immune complex vasculitis seen in arteries of mice persistently infected with LCMV (**B**) was subsequently observed as hepatitis B virus antigen (**G**)/hepatitis B virus antibody (**H**) immune complex deposits in an artery of a patient with hepatitis B virus infection. The deposition of immune complexes in the glomeruli of mice persistently infected with LCMV. **C:** immunofluorescent staining. **D:** Electron microscopy of dense deposits. **F:** The deposition of immune complexes in the glomeruli of mice persistently infected with LCMV is mirrored by Epstein–Barr virus (EBV) antigen-anti-EBV antibody immune complex deposits in a human glomerulus; similar immune complex deposition is also seen in many other human infections, including those with CMV and HIV. **E:** Immune complexes being degraded by a macrophage (*arrow*) in the choroid plexus of a mouse persistently infected with LCMV (For details, see Oldstone MBA. Virus-induced immune complex formation and disease: definition, regulation, and importance. In: Notkins AL, Oldstone MBA, eds. *Concepts in viral pathogenesis.* New York: Springer-Verlag, 1984:204–2097; and Whitton JL, Oldstone MBA. Immune response to viruses. In: Fields BN, et al., eds. *Fields' virology*. Philadelphia: Lippincott–Raven Publishers, 1996:345–374.)

Opsonization of virions by antibody with or without complement may enhance virus infectivity rather than result in virus destruction by phagocytes; this situation is seen, for example, with dengue virus. When persons who are immune to one dengue serotype become infected with a different serotype of the virus, the antibodies they have are cross-reactive and nonneutralizing, and they enhance infection of mononuclear cells by the second virus via an Fc-dependent mechanism (66,67). Another example of the presence of an inappropriate, nonneutralizing antibody response with a detrimental effect is the antibody response to formaldehyde-inactivated paramyxovirus vaccines. These vaccines elicit nonneutralizing antibodies directed against the viral hemagglutinin protein, but they fail to elicit antibodies against the fusion protein, which would control the spread of virus. This process may explain the severe atypical disease that resulted when children vaccinated with inactivated RSV or measles virus vaccines subsequently became infected (119).

Yet another mechanism by which antibodies induced by a virus infection may mediate immunopathology is by reacting against normal host components; this process is called *autoimmunity*. Autoantibody responses can be induced by several mechanisms:

1. As a result of viruses interfering with normal immune regulation, by affecting T-cell control of B-cell responses or causing polyclonal activation of B cells: Some of the antibodies they secrete react with host tissues (reviewed in 2). For example, Epstein–Barr virus (EBV) infects and causes polyclonal activation of B cells (182), and autoantibody production is associated with this infection.
2. By molecular mimicry, which occurs when a viral antigenic determinant is shared with a host antigen, and so a cross-reactive immune response is generated (reviewed in 140) (Fig. 78.5): Fujinami et al. (56) identified an epitope on the capsid protein VP1 of Theiler's murine encephalomyelitis virus (TMEV), antibodies against which cross-react with galactocerebroside, a major component of myelin, and may contribute to the pathogenesis of the demyelinating disease this virus causes in mice. In humans, antibodies directed against a region of the E1b protein of adenovirus 12, which has sequence similarity to A-gliadin, a wheat protein, have been suggested to play a role in the pathogenesis of celiac disease (91).

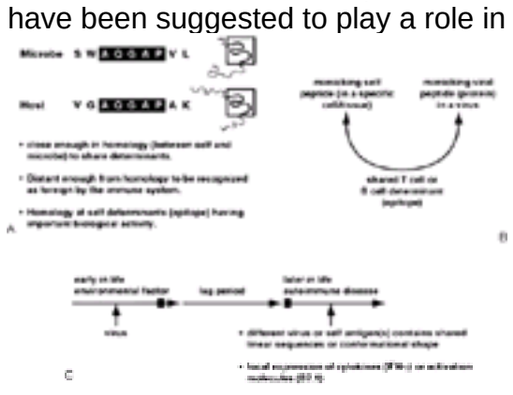

Figure 78.5. Cartoon illustrating the concept and cardinal points of molecular mimicry. **A,B:** How linear or conformational similarity between portions of a microbial protein and a host protein can lead to the induction of an autoimmune response via molecular mimicry. An immune response directed against the microbe (that is recognized as foreign) can cross-react with the self-antigen to cause an autoimmune disease. The anti-self-response initiated in this way may continue long after the triggering microbe has been cleared from the host. **C:** How a virus infection encountered early in life may predispose the host to the subsequent development of autoimmune disease via molecular mimicry. This model is based on observations made in a murine experimental models.

3. If antiidiotype antibodies against the idiotype of antiviral antibodies directed against virion surface components cross-react with proteins on host cells to which the virion can bind; for example, antiidiotypic antibodies of antibodies directed against reovirus type 3 hemagglutinin also recognize a 67-kd protein on lymphoid and neural cells, which may be the b-adrenergic receptor (40,41,48).
4. Virus infection also may lead to host components being rendered immunogenic and exposed to the immune system; this situation could occur if the virus incorporated host antigens into its envelope, if infection led to expression of new or modified host epitopes on the cell surface, or if host antigens that the immune system does not normally encounter were released and presented as a result of virus or immunopathologic damage (epitope spreading).

T-CELL–MEDIATED RESPONSES

T-cell–mediated immunity plays a critical role in virus infections. As discussed, whereas patients with defective antibody production suffer from bacterial infections (6), defective T-cell function frequently is associated with virus infection. Patients with T-cell deficiencies often develop generalized progressive vaccinia if vaccinated and are prone to infections with CMV, HSV, VZV, and measles virus. Measles virus infection of infants with defective thymic function leads to a progressive infection complicated by giant cell pneumonia, without a rash. Although disorders primarily affecting T cells also may result in defective antibody production, it is probable that the susceptibility to virus infections seen in patients who have congenital defects in cellular immunity or who are immunosuppressed results primarily from their inability to generate cytolytic and inflammatory T-cell responses to eradicate intracellular virus.

Both class I MHC–restricted (largely $CD8^+$) and class II MHC–restricted (generally $CD4^+$) T-cell responses are elicited after a virus infection. “Professional” antigen presenting cells, such as dendritic cells and macrophages, process virion proteins and viral proteins derived from infected cells for presentation in association with class II for the induction of $CD4^+$ T-cell responses and in addition possess mechanisms for capturing and presenting exogenous (usually cell-associated) antigens in association with class I for the induction of $CD8^+$ T-cell responses (68). Such “cross-priming” is essential for the induction of $CD8^+$ T-cell responses following virus infection (175). In most cells, however, peptides presented in association with class I are derived exclusively from proteins synthesized within the cell. As viruses replicate intracellularly, peptide fragments of the viral proteins become associated and presented with class I MHC molecules, as do those of normal cellular proteins, and this targets activated $CD8^+$ T cells to interact with virus-infected cells. Identification of the epitopes seen by $CD8^+$ T cells on a number of viruses confirmed that nonstructural internal virion proteins are frequently recognized in addition to virion surface proteins. Such recognition was first demonstrated with influenza virus, where the viral nucleoprotein was shown to be the principal target for T-cell recognition. There are several advantages to the recognition of nonstructural, internal viral proteins by antiviral T cells: (a) It increases the number of potential viral epitopes. (b) These proteins tend to be more conserved between viruses than virion surface proteins. For a number of viruses, T-cell responses have been shown to cross-react between strains or even subtypes (9,101), which may provide some protection against related viruses. (c) If T cells are targeted against the first nonstructural viral proteins produced in an infected cell, as has been shown to occur with herpesviruses, including HCMV, HSV, and VZV, this gives the potential for destruction of the infected cell before progeny virion production is complete, thereby

blocking virus spread.

The recent development of new techniques for quantitating antigen-specific T cells has allowed reassessment of the magnitude and kinetics of virus-specific $CD4^+$ and in particular $CD8^+$ T-cell responses in murine and human virus infections (120). Antigen-specific T cells now can be identified by staining with fluorescent-labeled tetrameric complexes of MHC molecules folded around a specific peptide or, alternatively, on the basis of their ability to produce cytokines in response to antigen stimulation in assays where cytokine-producing cells are quantitated on a single cell basis (either by ELISPOT or by intracellular staining of cells from which cytokine secretion has been blocked using brefeldin A). Using these techniques, it was shown that antigen-specific $CD8^+$ T cells can expand to far higher numbers than previously appreciated during the acute phase of a virus infection. For example, more than 50% of the $CD8^+$ T cells in the spleen of mice were virus specific at the peak of the acute response to LCMV (129,130), and in some patients undergoing acute EBV infection, more than 40% of $CD8^+$ T cells in the peripheral blood were virus specific (32). During chronic virus infections, virus-specific $CD8^+$ T cells may be maintained at high frequencies over long periods. For example, as many as 2% of peripheral blood $CD8^+$ T cells in humans chronically infected with HIV-1 or 10% of $CD8^+$ T cells in macaques chronically infected with SIV may be virus specific (8,103).

There are two principal mechanisms by which T cells contribute to control of virus infections: (a) by playing an inductive/regulatory role in the antiviral immune response and (2) by mediating antiviral effector functions. $CD4^+$ T cells more commonly perform the former role and $CD8^+$ T cells the latter, although each T-cell subset can participate in both functions.

Examples of inductive and regulatory roles principally carried out by virus-specific $CD4^+$ T cells include the recruitment and activation of T cells and cells of the innate immune system (e.g., NK cells and macrophages) at sites of infection and, in some viral infections, provision of help for the induction and maintenance of $CD8^+$ T-cell responses and provision of help to B cells for production of antiviral antibodies. Work with terminally differentiated mouse $CD4^+$ T-cell clones defined two subsets of $CD4^+$ T helper cells (Th1 and Th2) on the basis of their cytokine production patterns; cells that are not fully differentiated may exhibit an intermediate cytokine production profile. Th1 and Th2 $CD4^+$ subsets also exist in humans. Both subsets produce IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF), and TNF, which regulate the functions of hematopoietic stem cells and macrophages. In addition, Th1 cells produce cytokines such as IL-2, IFN-g, and lymphotoxin (LT), which activate T cells, especially CTLs, NK cells, and macrophages, and have a number of direct antiviral actions on tissues. Th1 cytokine production results in an accumulation of activated mononuclear cells at the site of infection, classically termed a *delayed-type hypersensitivity* (DTH) reaction, in which a number of cell-mediated responses are coordinated to combat the virus infection. By contrast, Th2 cells do not make IL-2, IFN-g, or LT but, instead, produce IL-4, IL-5, and IL-6. These cytokines again have effects on a number of cell types, the most important of which are their effects on B cells: They promote B-cell growth, differentiation, and class switching. Both Th1 and Th2 cells are activated during most immune responses, although one or the other subset may predominate. During virus infections, the Th1 response, which is more directed against intracellular pathogens, frequently predominates.

T cells mediate direct antiviral effector functions by two principal mechanisms. First, they may act as CTLs and lyse virus-infected target cells. For most virus infections studied, CTL lysis has proved to be mediated by $CD8^+$, class I MHC–restricted T cells, although $CD4^+$ CTLs also were identified in a few infections (e.g., in some instances, the human CTL response against measles virus is $CD4^+$-mediated, and human $CD4^+$ CTLs specific for HIV-1 have been documented). Two principal contact-dependent mechanisms are used by lymphocytes (both CTLs and NK cells) to kill infected cells (187). The first involves ligation and trimerization of Fas (CD95) receptors on target cells by Fas ligand (FasL, CD95L) on effector cells. This triggers the activation of caspases in the target cell, which leads to apoptotic cells death. The second mechanism involves the directional exocytosis of specialized granules in the effector cell cytoplasm; these contain a pore-forming protein perforin—that can produce membrane lesions similar to those induced by the complement C9 complex and a series of serine proteases termed *granzymes*. The granzymes, like the caspases, cleave specific target proteins in the cytoplasm and nucleus of the target cell, leading to apoptotic cell death. The second mechanism by which T cells mediate antiviral effector functions is through the production of antiviral cytokines such as IFN-g and TNF-a, which may trigger the clearance of virus from infected cells by noncytopathic mechanisms.

Numerous experiments have been carried out in animal model systems to investigate the roles of particular T-cell subsets/effector mechanisms (e.g., $CD4^+$ versus $CD8^+$ T cells and lytic versus nonlytic viral clearance mechanisms) in both protection and pathogenesis in different virus infections. These experiments have centered on two approaches: (a) *depletion*, in which a component of the immune system is selectively removed or its function is blocked by using specific antibodies or, more recently, by knockout technology; and (b) *reconstitution*, in which T-cell populations (e.g., cytokines) are adoptively transferred and the effects examined. Much less definitive evidence is available about the roles of T-cell responses during human virus infections because here only a correlative approach (i.e., measurement of the activity of different T-cell subsets at different stages of the infection and correlation of their activity levels with protection or pathogenesis) is possible. Summarizing the findings from many studies with different virus infections, it has been shown that the key effector mechanism differs depending on the infection studied, and that each type of effector mechanism has the potential to mediate pathogenesis as well as protection following virus infection. Pathogenic sequelae may occur long after the infection. Because of space constraints, we confine ourselves here to illustrating these points by just a few examples.

Lymphocytic choriomeningitis virus is a natural pathogen of mice, the outcome of infection with which depends, among other factors, on the dose and route of infection and the age of the animal at the time of infection (24). Adult mice inoculated intraperitoneally with moderate doses of virus clear the infection within 7 to 14 days. The animals mount antibody, $CD4^+$, and $CD8^+$-mediated responses to the virus, but it is the latter that is the key protective response. In acute LCMV infection, adoptive transfer of $CD8^+$, MHC class I–restricted virus-specific CTL clones into infected animals results in virus clearance (31); likewise $CD4^+$ cell–depleted animals or mice that lack CD4 or MHC class II are able to control an acute LCMV infection (36,125,158), whereas mice depleted of $CD8^+$ T cells by treatment with a CD8-specific monoclonal antibody fail to clear the virus and become persistently infected (125). Control of virus replication also is impaired in CD8 or b_2-microglobulin knockout mice, although virus clearance eventually is mediated in some of these animals. This is attributed to the action of MHC class II–restricted $CD4^+$ CTL produced in these mutant mice, in which $CD8^+$ T cells are lacking (57,127). The mechanism by which $CD8^+$ T cells mediate control of acute LCMV infection has been addressed using mice deficient in perforin, Fas, FasL, and IFN-g. Acute LCMV infection can be controlled in all except perforin-deficient mice (89,90,184), indicating that perforin-dependent lysis of virus-infected cells is the key effector mechanism by which control of an acute infection with this noncytopathic virus is mediated. This contrasts with results obtained when infections with more cytopathic viruses are studied; for example, clearance of vaccinia virus from mice can occur in the absence of perforin but is highly dependent on IFN-g production (90). It has been hypothesized that lytic clearance mechanisms make a less important contribution to the control of acute infections with cytopathic viruses because in any case destruction of infected cells ultimately is mediated by the virus.

In mice, LCMV can cause persistent as well as acute infections; the requirements for clearance of a persistent LCMV infection differ from those of an acute infection with this virus. First, although virus-specific $CD8^+$ T cells alone can control an acute LCMV infection, for long-term control of persisting virus to be achieved, $CD4^+$ T-cell help is required to sustain the $CD8^+$ T-cell response (15,117,153,184). Recently, the precise number of $CD8^+$ T cells and $CD4^+$ T cells required to clear a persistent LCMV infection have been quantitated (17). The minimum number of cells that need to be adoptively transferred to purge virus and cure a persistent infection is 3×10^6 LCMV-specific $CD8^+$ T cells and 0.7×10^6 LCMV-specific $CD4^+$ T-helper cells. Second, the key effector mechanism by which control of persistent LCMV infection is mediated differs from that of acute infection. Although IFN-g is not required for the clearance of an acute LCMV infection, it plays an essential role in the elimination of persistent LCMV infection (184). Cytokine-mediated “curative” mechanisms also been shown to constitute the means by which other persistent virus infections are cleared, for example, hepatitis B virus replication in a transgenic mouse model (64) and in chimpanzees (65).

Although clearly of key protective importance, $CD8^+$ lymphocytes also can mediate lethal immunopathologic damage in LCMV-infected mice. This situation is seen when adult mice are infected intracranially with the virus or when $CD8^+$ virus-specific CTL clones are transferred into mice persistently infected with LCMV: The animals develop a lethal choriomeningitis. The cause of death here has not been identified with certainty. Lytic damage to the central nervous system (CNS) is not apparent, and it is thought that lymphokine-mediated damage or edema related to the presence of a large cellular infiltrate in the meninges may be involved.

Although class I MHC–restricted $CD8^+$ T-cell responses are of key importance in LCMV-infected animals, in mice infected with another natural mouse pathogen, TMEV, class II MHC–restricted $CD4^+$-mediated responses are also critical determinants of protection and immunopathology. TMEV-infected mice mount $CD4^+$-dependent antibody, $CD4^+$-mediated, class II MHC–restricted DTH, and $CD8^+$-mediated, class I MHC–restricted CTL responses to the virus (37,38,113,166]. Mice depleted of $CD4^+$ T cells before infection with TMEV fail to mount an antiviral antibody response and may die of an acute encephalitic disease within 3 to 4 weeks after infection (195). Th2 cells providing help for antibody production thus have a protective role early after infection. $CD8^+$ T cells also play a role in control of virus replication (26); some mouse strains clear TMEV infection in the acute phase by a process that requires $CD8^+$ T cells and perforin (131,167). Antibody and $CD8^+$ T-cell responses are thought to make different contributions to the control of virus replication in different areas of the brain. Class I–mediated immune responses provide more protection from virus-induced damage in areas with abundant white matter, whereas regions with an abundance of neurons are protected primarily by antibody (49). Mice of strains that fail to clear the virus develop a persistent CNS infection that is associated with chronic demyelinating disease; this is used as a model for multiple sclerosis in humans. It was shown that $CD4^+$ T cells play an immunopathologic role here because mice depleted of $CD4^+$ T cells or treated with anti-class II antibody just before the start of the chronic disease phase developed less severe disease signs than did nondepleted animals (53,195). During the later stages of TMEV infection, the virus-specific DTH response peaks; Th1 cells producing DTH thus have an immunopathologic role in this phase of the infection.

Finally, it should be noted that, as with antibody production, immunopathologic T-cell responses induced after virus infection may be directed against host determinants. The mechanisms by which autoimmune T-cell responses arise are similar to those discussed for autoantibody production. These include interference with immune regulation; molecular mimicry [e.g., a region of the hepatitis B virus polymerase shares six amino acids in common with a region of myelin basic protein that is encephalitogenic in rabbits) (55), and cross-reaction of an HSV-1 gene product with a corneal antigen has been implicated in autoimmune herpes stromal

keratitis in a murine model (209)] (Fig. 78.6); neoantigen generation [e.g., CTLs recognizing cellular neoantigens have been shown to contribute to cardiac injury in mice infected with a myocarditic variant of coxsackievirus B_3) (77)]; and epitope spreading, which has been described in experimental studies of TMEV-induced demyelination and in diabetes (122,190).

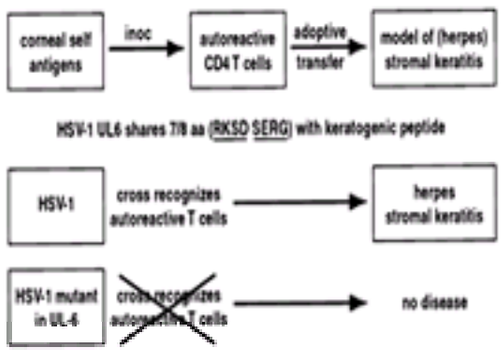

Figure 78.6. Example of induction of an autoimmune disease via molecular mimicry. Cross-reaction between the $CD4^+$ T-cell response to an epitope in the UL6 protein of herpes simplex virus type 1 (HSV-1) and a corneal antigen is implicated in the development of autoimmune herpes stromal keratitis in a murine model. Mutant HSV-1 viruses that lack this epitope do not induce autoimmune disease. (For details, see Zhao Z-S, et al. Molecular mimicry by herpes simplex virus type 1: autoimmune disease after viral infection. *Science* 1997;79:1344; for other examples of molecular mimicry in animal models and human disease, see Oldstone MBA. Molecular mimicry and immune mediated diseases. *FASEB J* 1998;12:1255).

VIRAL STRATEGIES FOR AVOIDING IMMUNE CONTROL

The immune response does not always achieve efficient clearance of virus infections. The rapid rate at which viruses are able to evolve enables them to develop mechanisms to delay or avoid clearance. A surprisingly large number of viruses are able to establish persistent infections in their hosts. The strategies by which viruses achieve these infections can be subdivided into (a) those that affect the induction or maintenance of the antiviral immune response; (b) those that affect the recognition of virions or infected cells by the immune system; and (c) those that interfere with the efficacy of antiviral effector mechanisms. Many viruses use a combination of different immune evasion strategies to maximize their chances of persisting in their hosts.

Viral Interference with the Induction or Maintenance of Antiviral Immune Responses

Viruses may induce a generalized suppression of the host immune response, or they may interfere more selectively with the induction or maintenance of virus-specific T- or B-cell responses. One mechanism by which viruses may produce a generalized immune suppression is through the production of soluble immunosuppressive factors, and the best documented example is the feline leukemia virus envelope protein p15E (116,180). Likewise, the EBV protein BCRF-1 is a homolog of the immunosuppressive cytokine IL-10 that can block the synthesis of IL-2 and IFN-g (75). Alternatively, viruses may replicate within cells of the immune system and impair their function or induce their destruction either by means of direct viral damage or by rendering them targets for immunopathologic damage by the antiviral immune response. For example, certain isolates of LCMV replicate within dendritic cells in lymphoid tissues and may infect a high proportion of these cells at early times postinfection. Although viral replication per se is noncytopathic, it renders the cells targets for destruction by the host virus-specific $CD8^+$ CTL response, which rapidly depletes these cells, leaving the host in a state of generalized immune suppression (23,135). The drawback to a virus persisting in its host by means of induction of a state of generalized immune suppression is that, if the host's immune functions are too severely compromised, it will be susceptible to many other infections that ultimately may result in its death, as occurs in the end stages of human HIV-1 infection when T-cell function is so severely impaired that death occurs from AIDS (104). Many viruses thus have evolved strategies for interfering more selectively with the virus-specific immune response.

If a virus infects its host sufficiently early in life, the virus-specific T cells will be clonally deleted in the thymus as “self”-tolerance is established. The host thus will be unable to mount a T-cell response to eliminate the virus but will be otherwise fully immune competent. This mechanism is the one LCMV most commonly uses to establish persistence during natural infection of its murine host. The virus is passed from mother to offspring *in utero*, and the offspring, having clonally deleted the LCMV-specific $CD8^+$ T cells they require to clear the infection, remain persistent viral carriers for life (151). In humans, neonatal tolerance is thought to play a role in the establishment of chronic HBV infections in children born to HBV-infected mothers (35) and may be among the factors that contribute to poor control of HIV-1 in children who acquire this infection *in utero* from HIV-positive mothers.

Viruses also have evolved strategies that enable them to interfere with the virus-specific T-cell response in adult animals. Virus-specific T cells may be deleted or rendered unresponsive. Both possibilities have been documented in LCMV persistent infection of adult mice (126,208). How antigen-specific T cells are deleted or rendered unresponsive in particular virus infections is not fully understood; it is likely that several different mechanisms may be involved. It is known that when T cells are stimulated in the presence of high concentrations of specific antigen, they may undergo apoptotic cell death. One hypothesis suggests that this occurs because the T cells acquire peptide–MHC complexes from antigen presenting cells and become sensitive to peptide-specific lysis by neighboring T cells (76). Deletion of virus-specific T cells may be induced by this mechanism if high levels of viral antigen are presented by antigen presenting cells. Alternatively, there may be deficits in T-cell stimulation. During viral infection, antigen may be presented to T cells by “nonprofessional” antigen presenting cells that fail to deliver appropriate activating signals, leading to T-cell death or nonfunction. Lack of necessary $CD4^+$ T-cell help for the induction or maintenance of $CD8^+$ T-cell responses also may lead to failure of virus-specific T-cell responses. Results from experimental models clearly demonstrate that to maintain effective $CD8^+$ T cells *in vivo* for more than a few weeks, helper $CD4^+$ cells are required (15,17,117,184). Although it is not clear how $CD4^+$ T cells function to maintain CD8 activity, one factor known to be important is IFN-g (34,52,184). Thomsen et al. (183) noted that removal of B-lymphocytes also interfered with maintaining clearance of virus and allowed infection to persist. It is not clear, however, whether the deficiency observed in the absence of B cells was due to a role for antibody, the interaction of B cells with $CD4^+$ T cells or a failure in antigen presentation by B cells. Indeed, recent data of Homann et al. (73) indicated that there was also a $CD4^+$ T-cell defect in the μMT knockout mice used by these investigators and that adoptive transfer of neutralizing antibodies played no role in limiting the viral infection.

Examples of human virus infections where interference with the induction or maintenance of virus-specific T-cell responses by the mechanisms discussed already may be involved in virus persistence are HBV and HIV-1. In the estimated 10% of persons who develop a persistent HBV infection after infection as adults, the HBV-specific CTL responses that can be detected are extremely weak compared with those seen in patients who clear the virus during the acute phase of the infection, suggesting that many virus-specific T cells may have been deleted or rendered nonfunctional (35). In HIV-1 infection, deficits in the virus-specific $CD4^+$ T-cell response are observed in all but the few patients who control virus replication efficiently over time (165) and have been suggested to result in failure to maintain effective virus-specific $CD8^+$ T-cell responses during this infection (92).

Interference with Recognition of Virions or Infected Cells by the Immune System

Viruses have evolved many strategies for avoiding recognition by the immune response. Two mechanisms that enable viruses to avoid recognition by both humoral and cell-mediated immunity are the establishment of latent infection and replication in immune-privileged sites. Latency is a strategy that is exploited particularly by herpesviruses and retroviruses. In latently infected cells, expression of viral proteins is highly restricted; for example, HSV establishes a latent infection in neurons during which there is thought to be no expression of viral proteins. Under such circumstances, the infected cells are essentially invisible to the immune system and thus are not targeted by immune effector mechanisms. Immune privileged sites are sites that are not readily accessed by the immune system. A surprisingly large number of viruses can persist in the CNS, where the blood–brain barrier restricts the presence of components of the immune system. Other favored sites for virus persistence include the epithelial cells of secretory or excretory glands, where T-cell access is restricted by a basement membrane and microvascular epithelium (e.g.,. the human polyoma viruses BK and JC); also, HCMV persists in the kidney, and both HCMV and EBV persist in the salivary gland.

To avoid recognition by neutralizing antibodies, viruses may undergo antigenic variation. With influenza viruses, which do not persist in individuals, this contributes to the persistence of the virus at the population level (150,194). Lentiviruses, including equine infectious anemia virus (EIAV), visna, caprine arthritis, enteritis virus (CAEV), and HIV, undergo antigenic variation within one host. With the possible exception of EIAV, however, in which sequential episodes of clinical disease associated with viremia correlate with the emergence of variants, this mechanism is probably not essential for persistence (39). Other viruses induce nonneutralizing antibodies; for example, lactate dehydrogenase virus (LDV) has evolved immunogenic epitopes, antibodies against which are nonneutralizing but block the action of neutralizing antibodies. Viruses that can spread by cell–cell contacts avoid exposure of the virion to neutralizing antibody altogether.

Virus-infected cells may avoid lysis by antibody-dependent mechanisms by reduced expression of viral glycoproteins on the cell surface. This action has been

observed to occur with paramyxovirus-, retrovirus-, rhabdovirus-, and arenavirus-infected cells (reviewed in 138). Neurons latently infected with HSV do not express any viral proteins (164), which avoids both B- and T-cell recognition. As mentioned, antibody itself contributes to the removal of viral antigens from the surface of measles virus-infected cells by antibody-induced modulation of viral antigens (87,88).

T-lymphocytes identify cells as being infected by recognizing viral peptides displayed on MHC molecules on the cell surface; T-cell recognition of virus-infected cells can be avoided if viruses replicate within MHC-deficient cells, such as neurons (82,83,159), or if viruses disrupt the antigen presentation pathway in infected cells (reviewed in 139) (Fig. 78.7).

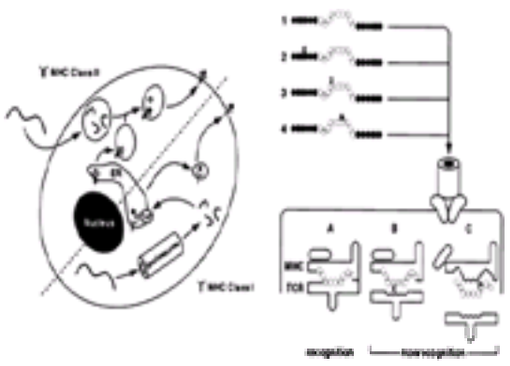

Figure 78.7. Illustratation of how amino acid changes in a viral sequence can alter the processing, presentation, or recognition of viral epitopes by virus-specific $CD8^+$ T cells. **A:** Diagram illustrating the major pathways by which antigens are processed and presented in association with major histocompatibility complex (MHC) class I and class II (for details, see Whitham S, et al. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. *Cell* 1994;78:1101). Viruses have evolved strategies to block many different steps in the class I presentation pathway (for details, see text and Whitman S, et al. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. *Cell* 1994;78:1101; and Oldstone MBA. How viruses escape from cytotoxic T lymphocytes: molecular parameters and players. *Virology* 234:179, 1997;234:179). **B:** Schematic illustrating the effects that a single amino acid mutation (X, <uftr<) either within a 9-amino-acid class I-restricted epitope (<soc>) or in the viral sequence flanking this epitope (<soc>) may have on epitope processing, presentation and recognition by $CD8^+$ T cells. Row 1 shows a viral polypeptide that is degraded by the proteasome to generate a peptide that is translocated by TAP molecules to the ER, where it binds strongly to a MHC class I molecule. This peptide–MHC complex then binds to a T-cell receptor (TCR) (motif A) and triggers T-cell activation. In row 2, a mutation in the sequence flanking the epitope prevents recognition and processing by the proteasome. In rows 3 and 4, mutations occur within the epitope itself, either at a residue that is involved in contact with the TCR (X) or at an anchor residue (<uftr>) that is critical for peptide binding to MHC. In the former case (motif B), although binding of the viral CTL epitope to the MHC and transport of the complex to the cell surface are not impeded, TCR binding or T-cell triggering does not occur. In the latter case (motif C), the viral epitope does not bind to the MHC class I heavy chain b_2-microglobulin does not lock into place, and a stable MHC-peptide complex is not formed.

Two fundamentally different approaches can be used by viruses to interfere with antigen presentation. The first is used primarily by DNA viruses associated with persistent infections, although there are also some examples of RNA viruses using this approach; they encode gene products that interfere with any of multiple steps along the antigen presentation pathway (reviewed in 139,154). If greater numbers of $CD4^+$ than $CD8^+$ CTLs are generated during a virus infection, this may signal a defect in class I antigen presentation. $CD4^+$ CTLs were found several years ago in human HSV infection (205), although $CD8^+$ CTLs also have been detected (155). By contrast, in most other viral infections studied, including HSV infection of mice (132), $CD8^+$ CTLs are generated. The reason for the generation of $CD4^+$ anti-HSV CTL in humans was unclear until others showed that the HSV ICP-47 protein efficiently blocked antigen presentation in human cells but did so poorly in murine cells (4,54,71,207). The HSV protein ICP-47 inhibits binding of its peptides to TAP, a molecule necessary for translocating peptides from the cytosol into the endoplasmic reticulum (ER).

This strategy is just one of many for interfering with the different steps in the MHC class I presentation pathway that have been described. A number of viruses can downregulate the transcription of class I genes (74) or other components of the class I presentation pathway (e.g., TAP and the LMPs). The cytosolic proteolysis required for epitope generation may be inhibited; for example, the HCMV phosphoprotein pp65 inhibits the generation of HCMV T-cell epitopes (61), and the EBV EBNA-1 protein contains a Gly-Ala repeat that frustrates proteosomal proteolysis (110). As exemplified by the HSV ICP-47 protein described previously, viral proteins also can interfere with the transport of epitope peptides from the cytosol to the ER by the TAP complex. Whereas the ICP-47 protein blocks the peptide binding site on the TAP complex from the cytoplasmic side, the US6 protein of HCMV acts on the ER–luminal side (5,69). Yet another strategy involves preventing the movement of peptide–MHC complexes from the ER to the cell surface. The adenovirus E3/19K glycoprotein was the first viral component shown to inhibit MHC class I antigen presentation (11,29,148,169). This viral protein forms complexes with MHC class I molecules and traps them in the ER; the six amino acid residues terminating the 15-membered cytoplasmic tail of its gp19 component are necessary and sufficient for retention in the ER (80,133). When these last six amino acids (DEKKMP) are transplanted onto the cytoplasmic tail of other membrane proteins that normally travel out of the ER, their ability to leave the ER is aborted. The HCMV US3 protein also binds to MHC class I molecules and sequesters them in the ER (3,86), whereas the MCMV m152 gene product, gp40, causes intracellular retention in the cis-Golgi by a mechanism that is independent of sequences in its cytoplasmic tail (210). Slater and Campbell (177) noted that the MCMV-induced defect in MHC class I synthesis and transport varied in a cell-specific manner, perhaps dependent on the state of differentiation or transcriptional activity of the infected cell. The US2 and US11 proteins of HCMV achieve the same end by a different means: They transfer the MHC heavy chain from the ER into the cytosol, where it is degraded (84,85,199). Finally, if peptide–MHC complexes reach the cell surface, antigen presentation still may be compromised. The MCMV p34 protein is present in a complex with those MHC class I molecules that reach the plasma membrane and may prevent their interaction with the T-cell receptor (TCR) (97); the HIV-1 nef protein downmodulates surface expression of class I molecules (170). Although inhibition of MHC class I antigen presentation by the adenovirus E3 complex was confirmed both *in vitro* and *in vivo* (50,193), none of the HSV, HCMV, MCMV, or HIV proteins have yet been shown to interfere with MHC class I antigen presentation *in vivo*; therefore, their biological significance remains to be established.

The second approach is used by RNA viruses, including any viruses that have an RNA step in their replication cycle. Because of their potentially high frequency of mutation, they can acquire amino acid changes in and around T-cell epitopes that interfere with epitope processing, binding to MHC class I molecules, or interaction with the TCR (reviewed in 25). Mutation in the viral sequence flanking an immunodominant epitope or within an epitope can influence its processing and block association with MHC molecules in the ER (147). For example, a single amino acid change in the p15E protein of murine leukemia viruses can alter the proteolytic cleavage site required for the generation of a $CD8^+$ T-cell epitope. The KSPWFTTL peptide encoded by AKV-MCF is processed correctly, whereas the Friend/Moloney/Rauscher (FMR) peptide RSPWFTTL is not (147). Similarly, Sevilla et al. (172) showed that a single amino acid change in the COO^- flanking region of an LCMV $CD8^+$ T-cell epitope in the Traub strain prevented the processing of this epitope and generation of epitope-specific effector $CD8^+$ CTLs. Interestingly, an *in vivo* consequence of this was the inability to generate sufficient $CD8^+$ CTLs cross-reactive to a self-viral antigen expressed in b cells of the islets to cause diabetes (172).

Viruses can escape recognition by virus-specific CTLs by acquiring mutations that prevent the binding of epitopes to MHC molecules or recognition by the TCR (45). The bound peptide sequence is linear, resulting from proteolytic fragmentation of viral protein synthesized within the cell. Several rules have been established about the structure of these peptides and their binding to MHC as well as the peptide–MHC complex binding the TCR. Studies of endogenously processed viral peptides indicate that they vary in length from 8 to 11 amino acids and display MHC allele-specific motifs. Mutational and crystallographic studies of MHC molecules complexed with viral peptide show that the molecule's flexible conformations allow these 8 to 11 amino acid peptides to bind within the MHC groove once their anchoring residues are fixed (22,60). Analysis of residues flanking the anchoring residue(s) indicated the critical importance of minor pockets of MHC-binding clefts in peptide selectivity, leading to the concept that these structural factors are likely responsible for the preferential selection of specific peptides so often observed in interactions with MHC molecules (78; reviewed in 198). Viral mutation at residues in an epitope that are important for MHC binding can ablate peptide binding to MHC altogether or reduce the affinity of peptide–MHC interaction so that the peptide-MHC complex has an extremely short half-life and is unlikely to trigger T-cell activation. Alternatively, mutations at residues involved in MHC binding can cause peptides to bind to MHC in a distorted conformation so that the TCR contact surface is altered. TCR recognition of peptide–MHC complexes also can be affected by changes in the TCR contact residues of an epitope. Altered peptide-MHC complexes may fail to interact with a particular TCR altogether, or they may be recognized but the T cell may receive a reduced or even a different type of signal when it recognizes this complex. Thus, the responding T cell may be only partly activated, for example, to proliferate but not to perform effector functions (99), or even anergy (178). Presentation of certain mutant peptides to T cells therefore can inhibit the response to the index not just by competing with it for binding to MHC but also by negatively signaling the responding T-cell population: This phenomenon is known as *T-cell antagonism*. Mutations that confer antagonistic properties on epitope sequences have been found in the persisting virus population in chronic HBV and HIV-1 infections (18,98).

Viral variants able to escape $CD8^+$ T-cell recognition have been found in a number of persistent virus infections, including LCMV and MHV-JHM in mice, SIV and HCV in nonhuman primates, and HIV-1 and HBV in humans. It not clear, however, how important is the contribution they make to viral persistence in all of these infections (review in 25). Studies with LCMV have shown that under CTL pressure *in vitro*, it is possible to select for viral variants bearing CTL escape-conferring mutations in all

three of the most immunodominant epitopes recognized in $H2^b$ mice (1,111,112). So long as at least one of these three immunodominant epitopes was not mutated, the virus was cleared efficiently and effectively with the usual kinetics *in vivo* (112). When all three immunodominant epitopes were mutated in the same virus, the host still could mount lower-affinity CTL responses against subdominant epitopes in the virus and control infection, although in this case more time was required to achieve virus clearance (112,124). Thus, under CTL pressure, escape variants can arise, but the host has other options and can bring epitopes lower down the hierarchy into play when immunodominant epitopes mutate. The inference drawn is that, unless a particular antiviral response is restricted to one epitope (for example, in LCMV TCR transgenic mice, where most of the host T cells were directed against a single viral epitope) (152), mutations in dominant CTL epitopes are unlikely to make a major contribution to viral persistence *in vivo*.

This hypothesis receives further reinforcement from recent observations of selection for CTL escape variants in HIV-1 infection (27). Within 15 days after the onset of symptoms of a primary HIV infection, a patient mounted a strong $CD8^+$ T-cell response that was highly focused on a single immunodominant epitope in gp160, at amino acids 30 through 38. Viral variants bearing mutations that conferred escape from recognition by CTLs directed against this epitope were rapidly selected for in the *in vivo* viral quasispecies and by 136 days after onset of symptoms had completely replaced the starting viral population. The kinetics of CTL escape was comparable to that of the emergence of drug-resistant viral variants in patient receiving therapy with a single antiretroviral drug and clearly indicated for the first time the biological significance of $CD8^+$ T-cell control of virus replication during HIV-1 infection *in vivo* (27). In this HIV-infected individual, subdominant CTL responses against other epitopes in gp160 and epitopes in the gag, pol, and nef proteins were subsequently detected (25,27). Because viral variants able to escape recognition by the originally dominant CTL response were selected, the viral load dropped from 200,000 to 300,000 to approximately 10,000 RNA molecules per milliliter. The viral mutations that occurred in this patient were thus not sufficient to block completely the control of virus replication; however, the fact that the viral variants were selected indicates that they must have conferred an *in vivo* replicative advantage on the virus carrying them, which may have contributed to the rapid disease course this patient subsequently underwent. Likewise, Goulder et al. (62) described selection for CTL escape viral variants in two HIV-infected patients during the late stages of disease and hypothesized that the replicative advantage conferred by the escape mutations may have contributed to disease progression.

It is thus unlikely that, during natural infection, selection of CTL escape viral variants generally constitutes the sole mechanism by which viral persistence is achieved, except when a uniquely restricted $CD8^+$ T-cell response is mounted (152). The MHC diversity and breadth of the TCR repertoire in most patients will allow for recognition of viruses, even when one or more of the most dominant epitopes have mutated (112,124).

Viral Strategies for Interfering with the Effector Mechanisms of Clearance

The important role of effector cytokines such as TNF-a and the IFNs produced both during the innate immune response and by virus-specific T cells in controlling many virus infections was discussed already. Therefore, it is not surprising that a number of viruses developed mechanisms for interfering with the function of cytokines. Adenoviruses encode at least four genes that interfere with the effects of TNF. Different members of the poxvirus family encode soluble chemokine and cytokine receptors that bind to and block the activity of factors, including TNF, the IFNs, and IL-1. The *in vivo* importance of a number of these was confirmed in animal models using viruses in which these genes have been deleted. Examples of mechanisms viruses use to avoid control by IFNs were discussed earlier.

In summary, during infection, a multifactorial and complex chess game is played between the host and the virus. A winning immune response overcomes infection, and a winning virus can effectively escape the host's immune surveillance. Defining the parameters and manipulating the players involved promise to be important strategies in the treatment of persistent viral infections.

CONCLUSION

Host defenses against virus infection consist of a broad spectrum of interreacting mechanisms targeted against both free virions and virus-infected cells. These mechanisms are still not yet fully understood, and their relative importance in vivo is even less clear, especially in virus infections in humans. Although these defense mechanisms sometimes mediate efficient virus clearance, viruses have developed strategies to escape immune clearance and therefore can establish persistent infections. Viral etiologies are being implicated in an increasing number of human diseases (e.g., multiple sclerosis, arthritis, diabetes, and chronic fatigue syndrome), making the study of virus–host interactions in animal models and in humans of key importance to increase understanding of all the possible consequences of virus infection.

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79 LYME DISEASE

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HISTORY

Lyme disease or Lyme borreliosis, which was first identified 25 years ago (1), occurs in temperate regions of Europe, North America, and Asia (2,3). It is now the most common vector-borne disease in the United States (4). Erythema migrans, the initial skin lesion (5), acrodermatitis chronica atrophicans (6), and Bannwarth syndrome (7), which are now known to be a part of the spectrum of Lyme borreliosis, were each recognized as separate entities in Europe during the early or mid twentieth century. The full spectrum of Lyme borreliosis, however, was not recognized until after the description of this entity in the United States in 1976 (8) and identification of the etiologic agent, *Borrelia burgdorferi* sensu lato, in 1982 (9). *B. burgdorferi* sensu lato has three pathogenic species: *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (10,11). Only *B. burgdorferi* sensu stricto strains have been found in the United States. Although all three species have been recovered in Europe, most illness there is due to infection with *B. afzelii* and *B. garinii*. Only the latter two species are found in Asia.

Infection is transmitted to humans by a variety of *Ixodes ricinus* complex ticks (12). In the United States, emergence of this infection in the late twentieth century coincided with reforestation of farmland and consequent increased deer populations, necessary for the natural tick life cycle (13). Expanding human residential communities and decreased hunting have probably also played a role (12). In Europe, Lyme borreliosis is already widely established in the remaining forested areas, and there is little room for spread. Within a span of 22 years, the protean manifestations of the infection were described, the etiologic agent was identified, diagnostic tests and treatments were established, the complete genome of the spirochete was sequenced (14,15), and a vaccine was developed for disease prevention (16).

IMMUNOLOGIC HIGHLIGHTS

B. burgdorferi elicits a complex immune response to multiple spirochetal proteins, including a number of membrane lipoproteins, some of which are upregulated or downregulated during the course of infection. *B. burgdorferi* lipoproteins are potent stimulants of an innate inflammatory immune response. Arthritis resistance is under control of the innate immune system, whereas control of spirochete burden is primarily the function of antigen-specific T and B cells. Failure to mount successful innate or antigen-specific responses leads to chronic infection. A subset of patients with late, active infection may develop chronic, antibiotic treatment-resistant Lyme arthritis, apparently because of molecular mimicry between the dominant T-cell epitope of outer-surface protein A (OspA) bound by DRB1*0401 and related major histocompatibility complex (MHC) molecules and a self-peptide of the adhesion molecule hLFA-1a, which is upregulated in the synovial lesion (17).

EPIDEMIOLOGY

Pathogen Genetics

The genome of *B. burgdorferi* is small (approximately 1.5 megabases), and it consists of a linear chromosome of 950 kilobases as well as 9 linear and 12 circular plasmids (14,15). The genome contains no homologs of the secretion systems, which have been described in other bacteria and which often contain toxin genes or other virulence factors. Instead, the only known virulence factors of *B. burgdorferi* are surface proteins that contribute to spirochete attachment to mammalian cells.

Although all three pathogenic species may cause infection of the skin, nervous system, heart, or joints, the frequency, duration, and severity of the involvement differ. *B. burgdorferi* sensu stricto commonly causes disseminated infection and intermittent or chronic arthritis (18,19). In contrast, *B. afzelii* less often disseminates hematogenously (20) but may cause chronic infection of the skin, acrodermatitis chronica atrophicans (21), which is rarely seen with infection with the other species (10,21,22). Finally, *B. garinii* sometimes causes a severe, chronic neurologic disorder, borrelial encephalomyelitis (21,23), which seems to occur rarely, if at all, with infection with the other species. For the most part, the mechanisms that may account for these differences are not known; however, the sequence of the immunodominant T-cell epitope of OspA bound by the DRB1*0401 molecule is found only in *B. burgdorferi* sensu stricto strains, not in *B. garinii* or *B. afzelii* strains. This may explain why, to date, chronic treatment-resistant Lyme arthritis has been reported only in the United States.

Host Genetics

Even in the United States, where Lyme disease is caused exclusively by *B. burgdorferi* sensu stricto strains, only about 60% of untreated persons develop Lyme arthritis, ranging from brief attacks lasting weeks, to chronic, persistent joint inflammation lasting several years (19). Although multiple host genes probably influence the severity and duration of Lyme arthritis, the only characterized example in humans is the association of chronic, antibiotic treatment-resistant Lyme arthritis with certain class II MHC alleles, including DRB1*0401, 0404, 0101, and 0102 alleles (24). These alleles are also associated with the severity of rheumatoid arthritis (25,26).

The murine model of Lyme arthritis seems to duplicate the acute, infectious phase of joint inflammation but not the later chronic phase seen in genetically susceptible humans. Ankle swelling in (C57BL/6xC3H) mice identified quantitative trait loci on chromosomes 4 and 5, whereas histopathologic score identified unique quantitative trait loci on chromosomes 5 and 11 (27). Candidate genes in these regions include several regulators of the innate inflammatory immune response to bacteria, such as inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-inducible protein-1, and the gene controlling the response to bacterial lipopolysaccharide (LPS) (*Lps*), among others (27).

Environmental

In the northeastern United States, *B. burgdorferi* sensu stricto exists in a horizontal cycle between *Ixodes scapularis* ticks and the field mouse, *Peromyscus leucopus* (2). This mouse is the preferred host for the immature larval and nymphal stages of the tick. The larval ticks acquire *B. burgdorferi* infection from infected mice in the late summer or early autumn. Following overwintering and molting, nymphal ticks transmit the infection back to mice in the early summer; this completes the spirochete life cycle. The nymphal ticks molt into adults and feed and breed on deer in the spring and fall. Thus, deer are important for the life cycle of the tick but not the spirochete (28). Birds may play a role in spreading ticks across geographic regions (29). Humans become infected primarily in May through July as the newly hatched nymphal ticks search for an appropriate host. The prevalence of infected nymphal ticks is directly correlated with the risk of human infection (30).

CLINICAL PRESENTATION

Signs and Symptoms; Physical Findings

After an incubation period of 3 to 32 days, Lyme disease commonly begins with erythema migrans, a slowly expanding, large annular lesion, often with a bright red

outer border and partial central clearing (stage 1: localized infection) (Fig. 79.1) (8,18,31). Within days to weeks, in patients with *B. burgdorferi* sensu stricto infection, the spirochete often spreads hematogenously to many different sites (stage 2: disseminated infection). Such patients may develop secondary annular skin lesions, severe headache, mild neck stiffness, fever, chills, migratory musculoskeletal pain, or profound malaise and fatigue. Even in untreated patients, the early symptoms usually improve within several weeks.

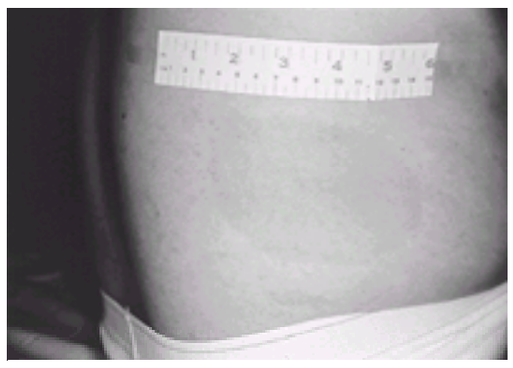

Figure 79.1. A characteristic erythema migrans skin lesion is shown. A 10-day-old lesion has gradually expanded to 10 cm and is beginning to have a brighter red border. (Reprinted from Steere AC, Bartenhagen NH, Craft JE, et al. The early clinical manifestations of Lyme disease. *Ann Intern Med* 1983;99:76, with permission.) (See Color Figure 79.1.)

After several weeks to months, about 15% of untreated patients develop frank neurologic abnormalities, including meningitis, subtle encephalitic signs, cranial neuritis (including bilateral facial palsy), motor or sensory radiculoneuropathy, mononeuritis multiplex, cerebellar ataxia, or myelitis, alone or in various combinations (32,33). The usual pattern consists of fluctuating symptoms of meningitis accompanied by facial palsy or peripheral radiculoneuropathy, lasting weeks to months. In addition, about 8% of untreated patients develop cardiac involvement. The most common abnormality is atrioventricular block (first-degree, Wenckebach, or complete heart block), fluctuating in degree and lasting weeks (34,35). Some patients have more diffuse cardiac involvement, including electrocardiographic changes of acute myopericarditis, left ventricular dysfunction on radionuclide scans, or, rarely, cardiomegaly or pancarditis.

Months after the onset of infection, about 60% of untreated patients develop frank arthritis (stage 3: persistent infection) (19). The typical pattern is of intermittent attacks of oligoarticular arthritis in large joints, especially knees, lasting weeks to months in a given joint. About 10% of patients develop chronic arthritis in knees, defined as 1 year or longer of continuous joint inflammation. Chronic neurologic involvement develops in about 5% of untreated patients months to years after the onset of infection (36). The most common form of chronic central nervous system involvement is a subtle encephalopathy that affects memory, mood, or sleep (37). These patients frequently have evidence of memory impairment on neuropsychological tests and abnormal cerebrospinal fluid (CSF) analyses (36,38). Lyme encephalopathy is often accompanied by an axonal polyneuropathy manifested as either distal paresthesias or spinal radicular pain (39,40).

Laboratory Tests

The diagnosis of Lyme disease is based primarily on the recognition of a characteristic clinical picture, exposure in an endemic area, and an elevated antibody response to *B. burgdorferi* (41). Culture of the spirochete from patient specimens permits definitive diagnosis, but with few exceptions, the procedure yields positive results only from biopsies of erythema migrans skin lesions (42). Therefore, serologic testing is the most practical laboratory test for support of the diagnosis.

For serodiagnosis, the U.S. Centers for Disease Control and Prevention (CDC) recommends a two-step approach in which samples are tested first by enzyme-linked immunosorbent assay (ELISA), and those with equivocal or positive results are tested by western blotting (41). About 20% to 30% of patients with erythema migrans have a positive response in acute samples, and about 70% to 80% have a positive response 2 to 4 weeks later, during convalescence, even after antibiotic treatment (43). After treatment, antibody responses typically remain positive for years, particularly if the disease progressed to late stages prior to treatment. In persons who have illness for longer than 1 month, a positive immunoglobulin M (IgM) response alone should not be used to support the diagnosis. After that time, the great majority of patients with active infection have a positive IgG antibody response, and a single test is usually sufficient. Patients in Europe or Asia with *B. garinii* or *B. afzelii* infection usually have less expansion of the antibody response than is observed with *B. burgdorferi* sensu stricto infection in the United States (44). Therefore, the CDC criteria cannot be used effectively in Europe or Asia.

The major limitation of serologic tests is that they do not clearly distinguish between active or inactive infection. Because culture is usually negative in Lyme disease, it had been hoped that polymerase chain reaction (PCR) testing might serve as a substitute for culture in this infection; however, this test has been readily positive only in joint fluid samples from patients with Lyme arthritis prior to antibiotic treatment. In one study, 70 of 73 joint fluid samples (96%) had positive PCR results using primer-probe sets that detect OspA gene segments (45). PCR testing is much less sensitive in detecting spirochetal DNA in CSF samples in patients with neuroborreliosis (46,47), and there seems to be little, if any, role for PCR in the detection of *B. burgdorferi* DNA from blood or urine samples.

Pathology

After injection into the skin, *B. burgdorferi* is particularly tropic for tissues of the skin, nervous system, heart, and joints, but the specific reasons for these tropisms are not yet known. Histologically, all affected tissues show an infiltration of lymphocytes and plasma cells with some degree of vascular damage, including mild vasculitis or hypervascular occlusion, suggesting that the spirochete may have been in or around blood vessels (48). The synovial lesion, which is similar to that seen in other forms of chronic inflammatory arthritis, shows synovial cell hyperplasia, vascular proliferation, and a heavy infiltration of mononuclear cells (49,50).

In patients with meningitis, CSF typically has a lymphocytic pleocytosis of about 100 cells/mm^3, often with an elevated protein but a normal glucose level (33). Electrophysiologic studies of affected extremities suggest primarily axonal nerve involvement (39,40). Histologically, the lesions show axonal nerve injury with perivascular infiltration of lymphocytes and plasmocytes around epineural blood vessels (51). In several patients with carditis, spirochetes have been demonstrated in the myocardium *in vivo* by endomyocardial biopsy (34).

Acrodermatitis chronica atrophicans, which sometimes follows years after erythema migrans, begins with red violaceous lesions that become sclerotic or atrophic (6). *B. burgdorferi* has been cultured from such lesions as long as 10 years after their onset (52). Histologically, the rete ridges of the epidermis are typically lost, and a mononuclear cell infiltrate and telangiectasia are manifest throughout the underlying dermis (53). Sclerotic lesions may resemble localized scleroderma (54).

Treatment and Prognosis

The various manifestations of Lyme disease usually can be treated with oral antibiotic therapy, except for objective neurologic abnormalities, which seem to require intravenous therapy (8,55). For patients with infection localized to skin or in those with early, disseminated infection, 20- to 30-day courses of oral doxycycline or amoxicillin are usually sufficient. Lyme arthritis often can be treated successfully with either oral or intravenous antibiotic therapy. In most cases, 30- to 60-day courses of oral doxycycline or amoxicillin or 2- to 4-week courses of intravenous ceftriaxone are adequate (56,57). Despite treatment with either oral or intravenous therapy, a subset of patients may have persistent joint inflammation for months or even several years after antibiotic therapy (56). Both nonsteroidal antiinflammatory drugs (NSAIDs) and disease-modifying antirheumatic drugs (DMARDs) have been used with some success if patients have persistent arthritis after 2 months of oral therapy or 1 month of intravenous therapy and if the PCR results for *B. burgdorferi* DNA in joint fluid are negative.

For objective neurologic abnormalities, with the possible exception of facial palsy alone, parenteral antibiotic therapy seems to be necessary. Intravenous ceftriaxone, for 2 to 4 weeks, is most commonly used for this purpose (33,36,57). In patients with high-degree atrioventricular block or a PR interval of greater than 0.3 seconds, intravenous ceftriaxone or intravenous penicillin for at least part of the course and cardiac monitoring is recommended.

The outcome of treatment with these antibiotic regimens is generally excellent, particularly in those with early Lyme disease (58). Objective evidence of relapse is rare. The most common reason for apparent lack of response to antibiotic therapy in Lyme disease is misdiagnosis (59). Patients may have subjective symptoms after Lyme disease, including fatigue, arthralgia, myalgia, headache, or cognitive difficulties, which has been referred to as *post-Lyme disease* or *chronic Lyme disease syndrome* (60,61 and 62). It is similar to chronic fatigue syndrome or fibromyalgia. In these patients, further courses of antibiotic therapy do not lead to sustained improvement.

IMMUNOPATHOLOGY AND IMMUNOBIOLOGY

Humoral Response

To maintain its complex enzootic cycle, *B. burgdorferi* must adapt to two markedly different environments: the tick and the mammalian host. This may be responsible in part for modulation of antigen expression by the spirochete (63,64,65,66,67 and 68). OspA and OspB are expressed primarily by the spirochete within the midgut of the tick (63). When the blood meal is taken, OspC is upregulated (68) and OspA is downregulated (63) as the organism traverses to the tick salivary gland and to the mammalian host. Spirochetes from ticks fed on OspA vaccinated mice died *in situ* prior to transmission into the host (69). The magnitude of the immune response to OspA elicited by the vaccine correlates with protection (16).

In the human host, antibody reactivity develops slowly to an expanding number of borrelial proteins over weeks to months (43,70,71). In a retrospective analysis of serial serum samples from patients followed up throughout the course of the infection (72), the earliest responses were usually to OspC (P23), a fibronectin-binding protein (P45, also called P35), and the flagellar protein (P41). Reactivity with OspE, OspF, P39, and P93 often developed weeks later, and months to years later, patients with arthritis frequently developed responses to OspA and OspB.

Antibodies to the flagellar protein of *B. burgdorferi* (P41) bind to a component of normal human axons identified as chaperonin-HSP60 (73). It is not known, however, whether autoreactivity causes tissue damage or is a secondary epiphenomenon. In the subgroup of patients with antibiotic treatment-resistant Lyme arthritis, the higher the IgG antibody response to OspA, the more severe and prolonged the arthritis (72). This response may be a marker for a critical T-cell response rather than an autoantibody.

Cellular Response

B. burgdorferi contacts immune cells shortly after infection at the site of entry and subsequently in the local lymph nodes, through cell surface receptors, including lipoproteins. Spirochetal lipoproteins OspA and OspB are mitogenic for B cells, macrophages, and g-d T cells, and they induce cytokine production by endothelial cells (74,75 and 76). OspA and other spirochetal lipopeptides of *B. burgdorferi* trigger LPS receptor (CD14) signaling and downstream activation of Toll-like receptor-2 (77,78,79,80 and 81). This pathway then leads to nuclear translocation of NF-kB, which upregulates cytokine production (82,83 and 84). *B. burgdorferi* binds to mammalian cells via integrins, among other pathways (85,86), which is known to upregulate NF-kB and induce interleukin-12 (IL-12), an inflammatory cytokine (87,88). Attachment to dendritic cells leads to phagocytosis, spirochete killing, and antigen presentation (89).

Activation of antigen-specific T cells follows antigen presentation. Proliferative responses of peripheral blood lymphocytes to various spirochetal antigens are significantly greater in patients with Lyme arthritis than in control subjects (90,91,92,93,94 and 95). In addition, cytotoxic $CD8^+$ T cells from both peripheral blood and synovial fluid have been elicited by OspA, OspB, and flagellin (P41) (96). These inflammatory cells may participate in spirochete control. gd T cells may be activated by *B. burgdorferi* (76,90). They also express Fas-ligand and kill activated synovial a-b T cells *in vitro*, suggesting a role in downregulating the immune response (97).

Inflammatory cytokines, particularly interferon-g (IFN-g), predominate in T cells in synovial fluid in patients with Lyme arthritis (98). T-cell clones derived from such patients have a T-helper cell type 1 (Th1) phenotype, characterized by IFN-g production, as opposed to an antiinflammatory Th2 phenotype (99). When *in vitro* recall responses were compared by a sensitive fluorescence-activated cell sorter (FACS) assay, the Th1 to Th2 ratio in synovial fluid correlated directly with the severity of arthritis, such that the higher the ratio, the greater the joint swelling (100). Peripheral blood mononuclear cells from subjects with Lyme disease secrete more IFN-g and less IL-4 than cells from control subjects (101). Proinflammatory cytokine mRNA for IL-1b, IFN-g, and TNF-a is predominant in synovium of patients with chronic Lyme arthritis (102). Finally, TNF-a is produced following *B. burgdorferi*-specific stimulation of synovial fluid mononuclear cells (98,103).

A comparison of cytokine profiles in patients with erythema migrans, a self-limited skin lesion, versus those with acrodermatitis chronica atrophicans, a chronic progressive skin condition, suggested that proinflammatory cytokines, particularly IFN-g, are important in the initial control of the infection (104). Among 27 patients who had erythema migrans alone with no associated signs or symptoms, the major cytokines expressed in T cells and macrophages were the proinflammatory cytokine IFN-g and the antiinflammatory cytokine IL-10. In the 15 patients who had associated signs and symptoms, including headache, elevated temperature, arthralgias, myalgias, and fatigue, a larger number of macrophages and greater expression of macrophage-derived, proinflammatory cytokines, TNF-a, IL-1b, and IL-6, also were found. In comparison, infiltrates of T cells and macrophages in the skin lesions of acrodermatitis patients had little or no IFN-g. Instead, they usually expressed only the proinflammatory cytokine TNF-a and the antiinflammatory cytokine IL-4.

Autoimmune Mechanisms

Joint inflammation in patients with chronic, antibiotic treatment–resistant Lyme arthritis continues after apparent eradication of the spirochete from the joint with antibiotic therapy. In an initial study, T-cell lines from patients with treatment-resistant arthritis preferentially recognized OspA (105). In a larger series, T cells from 32 treatment-responsive or treatment-resistant patients were tested for their reactivity with *B. burgdorferi* proteins in bulk culture (95). The only significant difference was the greater reactivity of treatment-resistant patients with N- and C-terminal fragments of OspA. When OspA epitope mapping was done, the responses of patients with treatment-resistant disease were significantly greater to several OspA peptides, particularly OspA peptide 154-173.

Patients with treatment-resistant arthritis were more likely than the group of treatment-responsive patients to express DR-b1-0401 or several other related alleles (24,95). Independent peptide substitution analysis of DR-b1-0401 allowed development of an algorithm to predict its binding affinity for any sequence of amino acids (106). When the OspA sequence was analyzed according to the algorithm, the best-fit peptide was predicted to be OspA165-173 (17), which was consistent with the observations in patients. Binding was confirmed with affinity studies, and the immunodominance of this peptide was shown by immunization of DR4 transgenic mice with OspA and peptide recall *in vitro* (17). When this sequence was compared with the database of sequenced genetic information (Genetics Computer Group, University of Wisconsin, Madison, Wisconsin), a single human homolog, LFA-1a332-340, was identified that had predicted binding by the 0401 molecule. Using an Elisaspot assay for IFN-g production, synovial fluid T cells from 10 of 11 patients with treatment-resistant Lyme arthritis had reactivity with the OspA or hLFA-1 peptides or, in most instances, with both, whereas patients with rheumatoid arthritis or other chronic inflammatory arthritides recognized neither peptide.

These observations led to the following hypothesis. In genetically susceptible patients, OspA165-173-specific T cells are expanded in the joint. In addition, LFA-1 expression is upregulated on activated T cells as a consequence of T-cell receptor and cytokine signals. After some of these activated T cells undergo apoptosis and are cleared by macrophages, presentation of the cross-reactive hLFA-1 peptide may continue to stimulate OspA165-173-specific T cells, enabling the immune response to continue after the eradication of spirochetes with antibiotic therapy (107). Inflammation, enhanced by inflammatory cytokines, or linked immune responses such as priming of $CD8^+$ cytotoxic T cells, may prolong the episodes of arthritis and lead to destructive joint changes.

This hypothetical autoimmune response occurs only in infected joints, although it is directed against a ubiquitously expressed self-antigen. There is precedent for this apparent paradox in a novel murine model of autoimmune arthritis, where an immune response directed against a housekeeping gene, glucose 6-phosphate isomerase, causes an arthritogenic autoimmune disease (108,109 and 110). Currently, a murine model for chronic, antibiotic treatment–resistant Lyme arthritis does not exist. The chronicity of disease observed in susceptible mice results from persistent infection and cannot be transferred with immune cells alone. DR4 transgenic mice on the B10.M/Sn background do not develop chronic, antibiotic treatment–resistant disease (111). However, the potentially cross-reactive epitope of human or murine LFA-1 is different.

ANIMAL MODELS

Mouse

The plethora of available mouse strains, including genetically modified animals, and the depth of knowledge of the murine immune system have made studies of the mouse particularly valuable in understanding Lyme disease. The mouse does not mimic all aspects of human Lyme disease, as mice do not develop erythema migrans, neuroborreliosis, or autoimmune Lyme arthritis (112). Needle inoculation, the predominant method of infection used in mice, requires a higher infectious dose and transmits cultured organisms with different antigenic expression than tick transmission. Finally, *B. burgdorferi* sensu stricto is more virulent in laboratory animals and humans than *B. garinii* or *B. afzelii* (113); this organism is the most extensively studied in laboratory rodents.

Most mouse strains can be infected with *B. burgdorferi* sensu stricto, strain N40 (Table 79.1) (112). At one extreme, young C3H/He mice develop severe destructive arthritis at all challenge doses, including challenge with fewer than 100 spirochetes (112,114). At the mild end of the spectrum, C57BL/6 mice do not develop arthritis, even at high challenge doses (114). Mice that lack T and B cells because of severe combined immune deficiency (scid) or genetically engineered recombinase deficiency (rag) fail to control spirochete replication (115,116 and 117). Efficient spirochete control is not a factor in C56BL/6 resistance, as even C57BL/6-rag mice do not develop joint inflammation (Table 79.2) (117). Most mouse strains resemble arthritis-resistant C57BL/6 mice (114). At low-challenge doses of *B. burgdorferi* strain

N40, they resist infection, whereas at higher doses, infection occurs without obvious sequelae.

Strain	Arthritis	Spirochete burden	References
Wild type			
C57BL/6 (B6)	None	Low–High[a]	114–117, 126–127
C3H/He	Severe	High	112, 117, 116–117, 119, 124, 131–132, 137, 139–144
(B6xC3H)F1	Moderate	Moderate to High[a]	27, 138
BALB/c	Dose-dependent	Dose-dependent	117, 129–130, 133, 137, 139–140
(C3HxBALB)F1	Severe	High	129–130
Naturally occurring mutants			
B6-*beige*	Severe	High	118
BALB-scid	Severe	High	117
C3H-scid	Severe	High	116–117, 135

[a] Depending on tissue tested. Scid, severe combined immunodeficiency disease.

TABLE 79.1. Infection and Arthritis Susceptibility in Selected Strains of Mice

Mutation	Strain	Finding	Arthritis	Spirochete Burden	Reference
Knockout					
Rag	B6	No B or T cells	None	High	117
IL-4	B6	Impaired Th2 response	More severe[a]	ND	127
IL-10	B6	Enhanced specific IgG2b	More severe	Low	126
Antibody specificity					
CD4	C3H	Depletion of CD4+ T cells	More severe	High	131
	BALB		More severe	High	
CD80	C3H	Impaired T- and B-cell mitogen response	Decreased	High	132
CD86	C3H	Impaired T- and B-cell mitogen response	More severe	High	132
CD80/CD86	C3H	Impaired T- and B-cell mitogen response	More severe	High	132
	BALB	Enhanced IFN-γ and impaired IL-4	Decreased	ND	133
IL-11	C3H	Not described	More severe	High	142
IL-12	C3H	Impaired IFN-γ	Decreased	High	144
	C3H-scid	Not described	More severe	High	125

IFN, interferon; IgG, immunoglobulin G; IL, Interleukin; ND, not done; Scid, severe combined immunodeficiency disease.
[a] No quantitative data given.

TABLE 79.2. Infection and Arthritis Susceptibility in Selected Strains of Genetic Knockout or Antibody-Treated Mice

MURINE INNATE RESPONSES

Mapping of chromosomal regions controlling arthritis severity in C57BL/6 mice implicated innate immune regulators (27). In C57BL/6 mice with the *beige* mutation, which causes gross defects in cell-mediated immunity, severe arthritis follows *B. burgdorferi* infection (118). High natural killer (NK) cell effector functions correlate with severe destructive arthritis, but depletion of NK cells does not alter the disease course in susceptible or resistant mice, suggesting that NK activation results from inflammation but is not pathogenic (119). In mice, *B. burg dorferi* lipoproteins are mitogenic for B cells (120) and monocytes/macrophages (121). Macrophages engulf *B. burgdorferi* and degrade the spirochetes in intracellular compartments (122,123). Although NO was thought to be the main effector molecule produced by macrophages, iNOS-deficiency does not change the course of infection or arthritis severity in susceptible or resistant mice (124).

Proinflammatory cytokines, such as IL-12, enhance macrophage function. In C3H-scid mice, anti-IL-12 treatment limits innate immunity and further exacerbates arthritis (Table 79.2) (125). IL-10, a cytokine produced by both T cells and macrophages, is an antiinflammatory macrophage suppressor. IL-10-deficient C57BL/6 mice have 90% fewer spirochetes than infected C57BL/6 control mice, probably because of sustained macrophage activation (Table 79.2) (126). Consistent with a role for antiinflammatory responses in controlling arthritis, IL-10-deficient mice develop more severe arthritis. IL-6-deficient C57BL/6 mice are also more susceptible to Lyme arthritis (127), suggesting a role for this cytokine in enhancing macrophage function. Innate immunity is the primary determinant of arthritis resistance or susceptibility.

MURINE ANTIGEN-SPECIFIC RESPONSES

BALB/c mice also can be infected with *B. burgdorferi*, and they develop arthritis in a dose-dependent manner (Table 79.1) (128). At low infectious doses, BALB/c mice resist infection; at moderate doses, they become infected and develop mild arthritis; and at the highest doses, they develop more severe arthritis. In early genetic studies of (C3HxBALB/c)F1 mice, susceptibility was dominant and associated with large numbers of spirochetes in tissues (129,130). BALB/c-scid mice develop both a high spirochete burden and severe arthritis and carditis, exceeded only by C3H-scid mice (115,116 and 117). Therefore, BALB/c mice have a similar arthritis-susceptible innate immune response to *B. burgdorferi* as C3H mice, but under normal conditions, antigen-specific T and B cells control spirochete expansion in BALB/c but not C3H mice.

In both C3H and BALB/c mice, depletion of $CD4^+$ T cells substantially inhibits the early immune response, increasing spirochete burdens and arthritis severity (Table 79.2) (131). When signaling through the T-cell costimulatory receptor CD28 is blocked with antibodies directed against CD80 and CD86 (also known as B7-1 and B7-2, respectively), or anti-CD86 alone, arthritis worsens significantly in C3H mice (Table 79.2) (132). Blocking only the CD80/CD28 interaction, however, reduces arthritis severity. CD80 has been shown to inhibit T-cell responses in a negative feedback mechanism, and blocking this signal may prolong the immune response and enhance spirochetal control, suggesting a dominant role for T-dependent responses in C3H mice. In BALB/c mice, in contrast, blockade of both CD28 costimulatory signals decreases arthritis, whereas blockade of either pathway alone does not change disease outcome (133). This suggests that humoral responses that occur in the absence of CD28 costimulatory signals, but not in the absence of T cells themselves, are most important in controlling disease and arthritis in BALB/c mice. *In vivo* expressed *B. burgdorferi* proteins induce antibodies to a 37-kD arthritis-resolving protein that are sufficient to resolve arthritis when passively transferred to infected C3H-scid mice (134,135). Anti-OspA responses are not required for arthritis resolution (135,136).

MURINE CYTOKINES

Inflammatory cytokine production contributes to spirochetal control. Highly susceptible C3H mice have lower IFN-g production early in the infection, whereas the more resistant BALB/c mice produce higher levels of IFN-g initially and then switch to IL-4 production, which presumably attenuates the arthritis (137). BALB/c and C3H mice have equal numbers of *B. burgdorferi*-specific $CD8^+$ T cells (138). Needle inoculation of high doses of spirochetes induces pathogenic Th1 responses in C3H mice (139,140), but follow-up work using tick transmission demonstrates instead a failure of C3H mice to mount an appropriate Th1 response early in infection (141). Removing proinflammatory IFN-g does not attenuate arthritis in C3H mice (142). In fact, administration of recombinant human IL-11 to C3H mice induces upregulation of IL-12 and iNOS expression and decreases arthritis severity (143). IL-12 depletion with monoclonal antibodies in C3H mice reduces immune control and enhances spirochete expansion *in vivo* (Table 79.2) (144). Nonetheless, this treatment reduces arthritis severity, demonstrating the complexity of cytokine networks and the potential pleiotropic effects of individual cytokines. Anti-IL-11 treatment increases arthritis severity in C3H mice (Table 79.2) (143). The IL-11 receptor and the IL-6 receptors share a subunit (CD130) that is expressed by both immune and somatic cells. Therefore, the same mechanism may underlie the similar findings with anti-IL-11 in C3H mice and the IL-6-deficient C57BL/6 mice. Taken together, the cytokine data suggest an important protective role for Th1 responses, in synergy with the innate immune system; however, this response is ideally balanced with an antiinflammatory response to regulate organ pathogenesis.

CARDITIS

Carditis is a frequent manifestation of *B. burgdorferi* infection in mice. C3H mice that develop severe destructive arthritis also have more severe carditis than C57BL/6 mice (145). $CD4^+$ T cells are not required for pathogenesis, and in fact macrophage infiltrates predominate in the cardiac lesions (146). Macrophage-derived IL-1 and TNF-a recruit Th1 cells to the lesion (147). Although IL-4 deficient BALB/c mice initially develop more severe carditis, resolution occurs with similar kinetics as in control BALB/c mice (148). Carditis is also an inflammatory lesion, but spirochete control seems to be the dominant factor in its resolution rather than conversion to an antiinflammatory Th2 response with antibody production.

VACCINE

Passive transfer of sera from OspA-immunized, immune competent mice to C3H-scid mice or vaccination of immune competent mice with recombinant OspA protects

on subsequent challenge with *B. burgdorferi* (149). Subsequent OspA vaccination does not worsen the disease course in previously infected mice (150). In mice, vaccination protects against tick transmission, but not needle inoculation, of multiple isolates and substrains of *B. burgdorferi* sensu stricto (151,152). In contrast, passive transfer of immune sera from infected, cured mice, which contains many specificities but few anti-OspA antibodies, protects recipients from needle inoculation (153) but not from tick-borne challenge (154). Immunity following natural infection is not cross-protective between different *B. burgdorferi* sensu lato species (113). Anti-OspA antibodies, and to a lesser extent anti-OspE and anti-OspF antibodies, are uniquely able to kill spirochetes *in situ* in the tick midgut (155).

Other recombinant borrelial proteins have been successfully used to immunize mice (156,157); however, the spirochetal lipoproteins are particularly suitable as vaccine targets because of their mitogenic qualities and ability to harness the innate immune system. The lipid moiety of OspA enhances specific T- and B-cell responses (158). Immunity to borrelial lipoproteins can develop in the absence of CD40 ligand, and even in the absence of MHC class II and $CD4^+$ T cells, suggesting that they can act as T-independent antigens (159,160). These results in mice contrast with those in humans, in which specific $CD4^+$ T-cell responses to OspA correlate with antibiotic treatment-resistant Lyme arthritis.

Hamster

Syrian hamsters vaccinated with whole killed *B. burgdorferi* are protected on subsequent infectious challenge (161). Uniquely in this model system, hamsters vaccinated with OspA develop severe destructive arthritis if challenged with cultured *B. burgdorferi* expressing OspA before they have fully developed protective immunity (162,163). Both T cells and macrophages from vaccinated hamsters with severe destructive arthritis can transfer disease in this system (164,165 and 166). Depletion of $CD4^+$ T cells from vaccinated hamsters prevents arthritis induction on infectious challenge (167). This suggests that OspA-specific T cells, amplified by immunization, may home to sproichete-infected joints, where they lead to joint inflammation.

Neuroborreliosis

Neuroborreliosis is more difficult to study in animal models because this manifestation does not occur in the mouse. Infection with *B. burgdorferi* in the rat model results in increased permeability of the blood–brain barrier within 24 hours but no clinical manifestations (168). The rhesus monkey develops neuritis, measured 3 months after infection, characterized by B-cell and macrophage infiltrates (169). Especially in tick-infected monkeys, inflammation resolves and axons regenerate even without antibiotic therapy (169).

SUMMARY

B. burgdorferi infection initiates an inflammatory immune response that has both protective and potentially pathogenic elements. Macrophages are activated by *B. burgdorferi* lipoproteins and possibly by the interaction of host integrins with other *B. burgdorferi* surface receptors to upregulate NF-kB and produce inflammatory cytokines. In the mouse model, innate immune responses are the primary determinant of arthritis susceptibility or resistance, whereas antigen-specific immunity is important in controlling spirochete expansion. Both innate and T-cell-mediated immune responses are primarily proinflammatory, ideally with an antiinflammatory component to limit organ damage. Uniquely in some human patients infected with *B. burgdorferi* sensu stricto, genetic susceptibility and ongoing active infection can lead to proinflammatory autoimmune responses to human LFA-1a, which contains a molecular mimic of the immunodominant T-cell epitope of OspA. In these patients, the autoimmune response is thought to prolong episodes of arthritis, even after eradication of the spirochete from the joint with appropriate antibiotic therapy.

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80 PARASITIC DISEASES

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Parasitic diseases are historically defined as infectious illnesses caused by unicellular protozoa or multicellular helminths as distinct from viral, bacterial, or fungal etiologic agents. They are the special health care problem of developing countries, where the extreme prevalence of malaria, filariasis, schistosomiasis, leishmaniasis, and trypanosomiasis imposes major medical and economic burdens. These diseases afflict hundreds of millions of people worldwide and are responsible for a million or more deaths every year. The importance of parasitic illness has received recent additional emphasis in both developed and developing nations by the emergence of toxoplasma and cryptosporidium as opportunistic pathogens in patients with acquired immunodeficiency syndrome (AIDS).

Control of parasitic illness remains an unfulfilled goal. Attempts at chemotherapy have been foiled by the length of treatment needed in some instances and by the emergence of drug-resistant parasites, exemplified by chloroquine-resistant malaria (1). Vector control has been similarly ineffective because of insecticide resistance and social, logistic, and economic obstacles. The hope that protective immunization against the major parasitic illnesses would circumvent the need for mass chemotherapy and vector control is now tempered by a realization that parasites have achieved a unique evolutionary arrangement with mammalian immune systems that defeats simple vaccine strategies that were previously successful against viral and bacterial targets. The immunology of parasitic disease is therefore relevant both to the development of new vaccine approaches and to the study of normal immunologic functions that may be uniquely understood by the elucidation of parasite subversive mechanisms.

SPECTRUM OF PARASITE–HOST INTERACTIONS

The inclusion of both protozoan and helminth infections in the category of parasitic disease is partially historical and embraces a heterogenous group of organisms with extremely diverse biologies. Protozoa are usually a few micrometers in size, whereas worms are typically centimeters to meters in length. Tissue-dwelling protozoa are often intracellular parasites at some stage of infection, whereas helminths, which are larger than most tissue cells, are almost always extracellular pathogens; the significant exception is encystation of *Trichinella spiralis* within mammalian muscle cells. Protozoa usually replicate during infection of a single host; helminths do not reproduce without the assistance of intermediate hosts or passage through soil or water.

Nevertheless, other aspects of the epidemiology, biology, pathogenesis, and immunology of these illnesses provide some thematic unity to justify this otherwise arbitrary grouping of diverse genera. First, both protozoan and helminth pathogens have characteristically complex life cycles with two or more developmental stages present during infection (2). For example, *Leishmania* promastigotes are introduced into mammalian hosts by biting sandflies; there they invade tissue macrophages and convert to the obligate intracellular amastigote form that replicates asexually and causes disease. In contrast, the plasmodial agent of malaria initiates a more complex biology in mammalian hosts when introduced by mosquito bite as free-living sporozoites that invade liver cells. Following this hepatocyte stage, the organism reemerges as a blood-stage parasite with alternating extracellular merozoite and intraerythrocytic trophozoite forms. Subsequently, some blood-stage parasites evolve into nonreplicating, extracellular gametocytic (sexual) forms.

Helminth life cycles can be similarly complex; in the course of a single infection, the host may be repeatedly exposed to larval, adult, or egg-stage antigens. Free-swimming cercariae of the trematode *Schistosoma mansoni* penetrate the skin of humans immersed in infested water and evolve into tissue-stage schistosomula that migrate to the liver and mesenteric veins for further differentiation into sexually dimorphic adult worms. Eggs are laid that migrate through tissues into the lumen of bowel or bladder for environmental release. Filarial infection similarly involves repeated exposure to arthropod-borne infective larva and chronic parasitization by long-lived adult worms that continuously release microfilariae that circulate in the bloodstream or migrate through subcutaneous tissues.

Because each stage of parasite development may be antigenically distinct, protozoan or helminth infections are often characterized by a series of discrete immune responses that evolve at different times during the course of disease. Protective immunity directed against a single stage may be circumvented by parasite differentiation, assisting in the survival of the pathogen and posing a significant challenge to vaccine development. Each stage of parasite development also may entail a change in tissue tropism, introducing a compartmental feature to immune responses. For example, numerous distinct cutaneous, pulmonary, and intestinal inflammatory or hypereosinophilic syndromes are associated with different stages of *Ascaris* and *Strongyloides* species as they migrate through the skin and lung before reaching adulthood in the gastrointestinal tract (3). This temporal evolution of antigenic complexity and tissue tropism is unique to parasite immunology and further distinguishes this field from that of viral or bacterial immunology.

Because complex parasitic life cycles can be maintained only by sequential passage through a sometimes inflexible choice of intermediate and definitive hosts, parasites have adapted to optimize transmission by prolonging infection. This is critical if passage through a series of intermediate hosts depends on infrequent events, such as ingestion of excreted eggs and larvae or uptake by the occasional biting insect. Perhaps because of this evolutionary pressure, chronicity and latency are hallmarks of both protozoan and helminth infections. For example, adult *Schistosoma* and *Filaria* species can survive in host tissues for as long as 30 years, producing eggs and larval stages all the time. *Plasmodia malariae* remains dormant in the liver for decades. *Leishmania* can recrudesce years after infection to cause post-kala azar dermal leishmaniasis or mucocutaneous disease; the former process may provide an important reservoir for vector-mediated transmission of disease to other humans (4). Chronicity may also reflect pressure for “balanced pathogenicity,” because extreme virulence will disrupt parasite transmission when the host dies before egg-laying or larval release can occur. In response to the evolutionary pressure for chronic infection, a broad range of evasive and suppressive strategies evolved among the parasites to maintain long-term viability in their hosts.

Adaptations for chronicity can be so successful among parasites that naturally acquired protective immunity may not be observed in areas endemic for that disease. Specifically, schistosomiasis results in an incomplete form of protection where reinfection is limited, but adult worms are tolerated for years (concomitant immunity) (5). Antimalarial immunity in highly endemic areas of the world is slow to develop and is usually nonsterilizing. Instead, clinical resistance typically manifests as a well-tolerated, limited parasitemia. Similarly, the presence of a subpopulation of hosts that are genetically susceptibile to dysfunctional immunity and prolonged infection with specific parasites may be essential for sustaining endemicity of the disease. For example, although *Leishmania donovani* often results in subclinical disease that heals with long-lasting immunity, half or more of seroconverting children in Brazil develop progressive visceral leishmaniasis and require drug therapy for cure (6). An active role of the parasite in maintaining this susceptible state is evident by the fact that curing visceral leishmaniasis by chemotherapy generates long-lasting immunity. Similar heterogeneities in host immune responsiveness may help maintain the parasite in animal reservoirs. Filarial infection similarly manifests as a range of clinical manifestations determined by divergent host immune responses. In this case, prolonged microfilaremia reflects apparent immunologic hyporesponsiveness to the parasite, whereas other hosts mount vigorous inflammatory responses that limit transmission but cause pathology (7).

MECHANISMS OF EVASION AND IMMUNOLOGIC SUPPRESSION BY PARASITIC ORGANISMS

Mechanisms of Evasion

Among the mechanisms used by parasites to avoid immune rejection are those of evasion—the use of sequestration, camouflage, and antigenic variation–and suppression or blockade of immune effector pathways.

SEQUESTRATION

Pathogens become sequestered when they enter into intracellular or extracellular compartments that are not accessible to all components of the immune response. *Echinococcus* species, *Taenia solium*, and *Trichinella spiralis* sequester themselves in mammalian tissue primarily by encystation. Whereas it was initially believed that encapsulated organisms were immunologically inert, studies have since demonstrated that macromolecules, including parasite antigens and host antibodies, can traverse cyst membranes (8). This, together with the observation that parasite death, whether spontaneous or in response to chemotherapy, provokes intense local inflammation even in the brain (9), suggests that the cyst membrane may regulate rather than prevent the transport of parasite and host molecules as a means of attenuating the host immune response.

Similarly, adult *Onchocerca volvulus* are surrounded by a relatively avascular, acellular fibrotic capsule that serves as a barrier to the host response and yet permits transport of eggs into the adjacent tissue (10). Tropism for immunologically relatively protected environments (e.g., the eye in *Onchocerca volvulus* infection and *Toxoplasma gondii* and the intestinal lumen for intestinal nematodes and protozoa) also serves to sequester the parasite physically from the host effector systems. Intracellular invasion is a common strategy used by protozoa that removes them from potentially lethal phagocytic cells and humoral factors such as complement and antibody. In the case of malaria, intraerythrocytic *Plasmodia* species may avoid immune or nonspecific destruction during splenic transit by expressing a variety of endothelial adhesion molecules on the surface of the infected red cell, thereby sequestering parasitized cells in the peripheral circulation (11).

MOLECULAR MIMICRY AND CAMOUFLAGE

Molecular mimicry and camouflage are two mechanisms that parasites use as defense strategies against the host response, the result of which is to appear “not foreign” (*self*) to the immune system. Schistosomes adsorb onto their tegument a variety of host serum and cellular proteins, including blood group factors (12), the Fc portion of immunoglobulin (13,14), and major histocompatibility complex (MHC) molecules (15). A related form of camouflage occurs when the parasite synthesizes proteins that share epitopes with host tissues; such molecular mimicry may exploit host immune tolerance against self-determinants. The ribosomal P0 proteins of both humans and *Trypanosoma cruzi* share cross-reactive epitopes for antibodies (16). Similarly, the *T. cruzi* surface antigen FI-160 exhibits an antibody binding site found on human neural tissue (17). There have also been described as antigens of *O. volvulus* that have cross-reactivity with retinal proteins (18,19).

Molecular mimicry may have other consequences. Although the host may be unable to generate protective T-cell and B-cell responses to these epitopes because of developmentally induced nonresponsiveness, autoimmunity may occur if immune tolerance is overridden. The generation of anti-FI-160 antibodies in human patients infected with *T. cruzi* has been suggested as a contributory autoimmune factor in the neural plexus destruction that occurs in chronic Chagas disease (17). Similarly, the finding of antibodies in patients with onchocerciasis that bind to retinal tissue has been suggested as a possible cause for the chorioretinitis seen in this disease (18,19).

ANTIGENIC VARIATION

Trypanosoma brucei, the cause of African trypanosomiasis, survives host immune destruction through periodic switching of the major surface protein (20). Unlike infections associated with other closely related protozoa, such as *T. cruzi* and *Leishmania* spp., *T. brucei* has no intracellular stage during human infection and is therefore vulnerable to cytolytic humoral immune responses. The variant surface glycoprotein (VSG) molecules comprise the major component of the cell surface and are encoded by several hundred VSG genes; each is antigenically distinct, and only one is expressed at a time. Periodic and sequential switches in VSG expression allow isolated clones of cells to survive despite cytocidal antibody responses to preexisting VSG epitopes. Each gene switch results in another cycle of trypanosomal growth and immune-mediated decimation, giving rise to the characteristic fluctuating parasitemia of this disease.

Antigenic heterogeneity due to strain diversity within a single species of parasite can also diminish the effectiveness of immune responses. The failure of humans to efficiently develop resistance against malaria may be related to the extreme antigenic polymorphism present among local strains of *Plasmodia* in endemic areas (21). A highly diverse family of immunogenic adhesion proteins, the var complex, also can contribute to the antigenic complexity of falciparum malaria (22). In addition to strain-related diversity, true antigenic variation within single clonal lineages also occurs because of a high mutation rate in the relevant genes, further complicating the acquisition of protective immunity (21). It has been estimated that immunologically recognizable variation in the erythrocytic phase of malaria occurs at a rate of about 2% per generation. Similar strain heterogeneity occurs in *Giardia lamblia* (23).

SHEDDING OF SURFACE ANTIGEN AND BOUND ANTIBODIES

Parasites that shed antigen on recognition by host antibody also can enhance their survival. This phenomenon has been described with *E. histolytica* (24), *T. cruzi* (25), and *S. mansoni* (26,27) parasites. Tissue- and intestinal-dwelling nematodes also periodically release portions of their outer glycocalyx coat (28), although the role this plays in avoiding immune recognition is not established. Discarded or secreted antigens also conceivably can serve as decoys to misdirect host responses.

Mechanisms of Suppression and Blockade of the Host Effector System

PARASITE-DERIVED FACTORS

Intracellular and extracellular parasites have clearly developed methods of inactivating humoral immune responses. Parasites possess surface-associated or secreted proteases that degrade host antibody molecules. Factors from cyst fluid from *Taeniae* infections have been shown to be able to activate complement (29); similarly, such factors have also been found in hydatid cyst fluid (30) and in soluble extracts of adult *S. mansoni* worms (31).

Unlike helminths that have a thick cuticle, protozoa are more susceptible to lysis following formation of the C5b-C9 membrane attack complex of complement. Several mechanisms have been defined that defeat cytocidal complement formation mediated by both alternative and classical pathways (32). The extracellular trypomastigote form of *T. cruzi* disrupts the function of C3 convertase through the expression of a protein analogous to decay accelerating factor (DAF). *T. gondii* has evolved a surface structure that is a poor substrate for activation of the alternate pathway; however, binding of specific antibody triggers fatal complement formation via the classical pathway. Activation of complement on the surface of *Leishmania* species is rendered noncytocidal because of the ability of the lipophosphoglycan coat to prevent insertion of the C5b-C9 complex (33). Similar mechanisms may operate in *Schistosoma* species (34).

Parasite-derived factors also have been isolated that interfere with T-cell function. For example, the secretion of immunodominant molecules, such as the hapten phosphorylcholine, may act as an inhibitor of T-cell reactivity (35,36,37 and 38) and secondarily as a molecule that diverts the attention of the host immune system (28). Molecules with similar effects on T-cell function have been described in schistosomes as well (31). Other molecules with similar inhibitory functions include the *Achanthocheilonema viteae* excretory-secretory (ES) antigen Av17, a cysteine protease inhibitor that accounts for much of the suppressive activity of filarial ES products on T-cell proliferation in murine studies (39), and the oligosaccharide lacto-*N*-fucopentose III, which downregulates the type 1 response in murine schistosomiasis (40).

Although their role in immunoregulation is unproven, cytokine-like molecules of parasite origin, including homologs of transforming growth factor (TGF-b (41), macrophage migration inhibitory factor (MIF) (42), and interferon-g (IFN-g) (43) also were identified in several helminth species. In the case of the IFN-g homolog of *Trichuris muris*, receptor binding and biologic activity also have been demonstrated *in vitro* (43).

Acute infection with *T. cruzi* is associated with suppressed T-lymphocyte proliferation and lymphokine production, apparently in response to soluble parasite products (44). Of interest, parasites such as *Brugia malayi* (one of the causative agents of lymphatic filariasis) and *Taenia* both utilize endogenous and exogenous arachidonic acid to produce and release prostanoids [prostaglandin E_2 (PGE_2) and prostacyclin] molecules capable of inhibiting T-cell proliferation (45,46 and 47). In another instance, parasite-derived cytocidal activities of *Entamoeba histolytica* contribute to the virulence of this protozoan parasite during invasive infection by lysing host effector cells, including neutrophils and T-lymphocytes (48).

Parasites can modulate the ability of the host to present soluble antigen to the immune system. Schistosome infection appears to render antigen-presenting cells less competent (49). The release of parasite-derived protease inhibitors, such as filarial cystatin (50) or aspartic protease inhibitor from *Ascaris lumbricoides* (51), may block efficient antigen processing dependent on proteolytic lysosomal enzymes. Accessory cell functions also may be disrupted when these cells are themselves the target of parasitization. *Leishmania* species are obligate intramacrophage parasites that can block presentation of parasite antigen by infected cells and that disrupt signal transduction pathways essential for IFN-g-mediated upregulation of MHC II and monokine synthesis (52). Leishmania lipophosphoglycan (LPG) can interrupt signal transduction pathways necessary to interleukin-12 (IL-12) production and inducible nitric oxide synthase (iNOS) expression (53). Infection also induces the release of T-cell-suppressive cytokines from infected macrophages (54).

Other parasite products have antioxidant properties that limit the destructive potential of activated granulocytes or macrophages in the local extracellular milieu. Helminths susceptible to oxidant-mediated killing express both secreted and membrane-associated enzymes, such as superoxide dismutase (55), glutathione-S-transferase (56,57), and glutathione peroxidase (58), molecules thought to play a significant biologic role in assisting parasite survival in inflamed tissues. Protozoa that invade macrophages have evolved similar strategies. Again, the lipophosphoglycan surface molecule of *Leishmania* organisms has antioxidant properties

that may assist in intramacrophage survival (33). *T. gondii* generates glutathione peroxidase and catalase that permit survival in parasitized macrophages (59).

HOST-RELATED FACTORS

The inability to clear parasitic infection often is related to inefficient or counterprotective host responses elicited by helminths or protozoa. These pathogens may elicit cytokines, such as TGF-b, IL-4, IL-13, or IL-10, that antagonize microbicidal cellular immune responses. In some cases, parasitic infection may cause genetically persons to mount cellular immune responses that effectively misdirect host energies toward nonprotective responses. Thus, susceptible hosts may constitute a large enough population to sustain the local prevalence and transmission of disease. Although the suppression of protective immunity is host related, parasitic products actively shape these responses. For example, nonhealing leishmaniasis (including visceral disease) (60), the microfilaremic form of lymphatic filariasis (61,62,63 and 64), and generalized onchocerciasis (65,66,67,68 and 69) and schistosomiasis (70,71 and 72) all are associated with a defective immune response that can be restored when the parasitic burden is diminished by drug therapy (72,73,74 and 75). Infection with *T. cruzi* triggers generalized T-cell immunosuppression mediated by increased synthesis of IL-10 and TGF-b (76,77). Similarly, schistosome egg antigens elicit increased production of the cytokine IL-10 in infected tissue that consequently downregulates Th1-type $CD4^+$ T-lymphocyte responses necessary for the elimination of eggs or adult worms (78).

In addition to suppressing cellular responses, host-related factors may assist in parasite survival by providing a biologic signal necessary for parasite development. Specifically, *S. mansoni* ovulation is delayed in hosts that are unable to mount cell-mediated immune responses; however, reconstitution with proinflammatory molecules, possibly TNF-a, promotes earlier fecundity in some studies (79), although other factors may be necessary (80).

Parasite-induced perturbations of the specific antibody isotype generated during infection may additionally diminish the effects of a given immune response. Helminth infections, particularly lymphatic filariasis and schistosomiasis, result in the production of high levels of immunoglobulin E (IgE) and IgG4. The expression of antibodies of distinct isotypes directed against the same antigens may be beneficial to the host when IgG4 blocks IgE-mediated allergic responses (81); however, the increased magnitude of IgG4 and IgE responses commonly seen in schistosomiasis (82) and filariasis also may diminish the protective response mediated by other antibody isotypes directed against the parasite. Similarly, antibodies of the IgM and IgG2 isotypes also have been able to block antibody-dependent killing of schistosomula (83). Finally, the presence of these blocking antibodies has been associated with susceptibility to reinfection with schistosomiasis following chemotherapy (84).

VECTOR-RELATED FACTORS

Insect bites that convey infectious parasites may contribute to infectivity through biochemical manipulation of the local host environment. Sand fly saliva contains proteins with potent vasodilatory and macrophage-deactivating effects that may increase the availability of macrophage targets permissive for infection with *Leishmania* organisms and that enhance the infectivity of *Leishmania braziliensis* in normally resistant experimental hosts (85,86).

PATHOLOGY ASSOCIATED WITH IMMUNE RESPONSES IN PARASITIC INFECTION

Although the pathologic findings associated with each of the parasitic infections are different and most commonly relate to the presence of the parasites in the host tissue, there are pathologic reactions that stem directly from the host immune response. Although it is not the purpose of this chapter to review the pathologic and immunopathologic aspects of the parasitic diseases, the nature of some of the most common immunopathogenic mechanisms are described.

Immune Complexes

Immune complexes, potent mediators of localized inflammatory processes, form in many parasitic infections, most likely because of the chronic low-dose antigen release seen in these infections. The best-characterized example is the glomerulonephritis associated with *Plasmodium malariae* infection, in which parasite antigen has been demonstrated in the kidneys of patients with this manifestation of infection (1). Circulating immune complexes have clearly been identified in both experimental (87) and human filarial (39) and schistosomal (88) infections. These complexes have been shown to induce lymphatic inflammation and vasculitis in at least one filarial infection as a result of their deposition (89). Furthermore, observations of immune-complex glomerulonephritis have been made in patients with hepatosplenic schistosomiasis (90), loiasis (91), and onchocerciasis (92). Notably, the clinical syndrome of acute schistosomiasis also has been attributed to the presence of immune complexes (88).

Antoantibodies

Autoantibodies have been implicated as causing disease both in protozoal and in helminth infections, presumably reflecting a polyclonal B-cell activation that often accompanies these infections. Whereas this immunopathologic mechanism has been proposed to contribute to the central nervous system (CNS) and cardiac lesions of African trypanosomiasis, the cardiac and neural lesions seen in Chagas disease, and the anemia and nephropathy seen in malaria, the evidence for its existence in helminth infection is less well founded, despite being implicated in filariasis (93,94 and 95), schistosomiasis (96), and hookworm infection (97). Nevertheless, autoantibodies against nuclear material were found in a vast majority of patients with chronic schistosomiasis and much more frequently than in patients with diseases such as systemic lupus erythematosus (SLE) (98). In addition, calreticulin (antibodies to which are found in SLE) (99) have partial (64%) structural identity with the *Onchocercus volvulus* antigen RAL-1, a 42-kd antigen expressed in infective-stage larvae and adults (100). Furthermore, patients with onchocerciasis have antibodies that cross-react with calreticulin, suggesting that autoimmunity may play a role in the pathologic responses in onchocerciasis.

Autoimmune reactions have been considered to underlie some of the pathology seen in filarial infections, most notably in the chorioretinitis seen in onchocerciasis. Evidence was initially provided that demonstrated the presence of antibodies specific for human retinal antigens in *O. volvulus*-infected patients with chorioretinitis (101). Antibodies to several antigens of the inner retina also have been described, but the relationship between chorioretinitis and the presence of these antibodies could not be established (18,102). More recently, an antigen has been found in *O. volvulus* (22 kd) that induces antibody that cross-reacts with a 44-kd antigen component of bovine retinal pigment epithelial cells (19).

T-Cell-Dependent Pathology

Granulomatous reactions can be seen in many protozoal and helminth infections. Parasitic granulomata have been best studied in *S. mansoni* infections, where T-cell regulation of size and development has been well documented (103). Whereas such granulomas presumably act normally to isolate and help eradicate infectious or irritative challenges, these foci of intense immunologic activity often damage normal tissue. This damage may be essential for completing the life cycle of schistosomiasis by allowing eggs to escape through tissue barriers into the intestinal or urinary tract and their subsequent dispersal into the environment; however, granulomatous responses also contribute to the pathology of this disease. For example, scarring of the hepatic portal tracts resulting from fibrosis associated with granuloma formation around schistosome eggs can lead to cirrhosis and portal hypertension. Lymphatic filariasis is associated with similar fibrotic reactions where adult parasites reside in the lymphatic channels and lymph nodes (104); this “scarring” is believed to be partially responsible for the lymphedema, elephantiasis, hydrocele formation, and chyluria found in this condition. The mutilating facial lesions of mucocutaneous leishmaniasis occur in the setting of exuberant delayed-type hypersensitivity and aggressive granulomata formation emerging years after the primary cutaneous infection (usually caused by *L. braziliensis* species) has healed (105). This process is thought to represent attempted immune destruction of small numbers of parasites that persist within the nasal and oral tissues.

The blood stage of malaria is associated with the neuropathologic crisis of cerebral malaria that is partially dependent on T-cell-regulated cytokine responses. Cytoadherence of parasitized erythrocytes within the capillaries of the CNS causes seizures, coma, and death when they obstruct blood flow (106). Because many of the vascular receptors for parasite ligands are upregulated by circulating TNF and other cytokines, the presence of these factors in the blood may predispose to this crisis. TNF may be produced in a nonspecific fashion; schizont rupture directly induces the synthesis of monokines by nonimmune blood mononuclear cells, and serum levels of TNF are associated with the severity of cerebral disease (107). As a consequence, proinflammatory T-lymphocytic responses against malaria that normally contribute to killing of parasite (see later) also can contribute to host mortality. Similarly, $CD4^+$ T cells that produce inflammatory cytokines contribute to the pathology of *Toxoplasma* encephalitis, although they are also important for containment of the dormant parasite (108).

Immediate Hypersensitivity Responses

Many of the immediate hypersensitivity responses are modulated as helminth infections become chronic; during the early and acute phase of infections with invasive helminth parasites, such as ascaris, hookworm, schistosomes, or filariae, patients may manifest symptoms suggestive of allergic reactivity, such as wheezing or urticaria (109). Furthermore, in the clinical syndromes associated with *Loa loa* infection (with its angioedematous Calabar swellings) (110), with tropical pulmonary eosinophilia (3,111), and with larvae currens in strongyloidiasis (112), IgE-mediated reactions are believed to reflect the underlying mechanism of these signs and symptoms.

Eosinophil-associated pathology, unlike IgE-mediated hypersensitivity, is found even more frequently in response to helminth infection. Evidence has been accumulating in parasitic diseases associated with profound or extreme hypereosinophilia, as in endomyocardial fibrosis associated with loiasis in expatriates (110,113)

or in the tropical (filarial) pulmonary eosinophilia syndrome (3), that the major cause of the tissue destruction appears to be the eosinophil and its toxic molecules. In contrast, eosinophilic responses during protozoan infection are rarely observed.

HOST RESPONSES AGAINST PARASITES

Helminth Infections

Although the number of pathogenic helminth infections for humans is large and the biology of each is diverse, helminth infections frequently are accompanied by immune responses with all the features of immediate-type hypersensitivity reactions. These reactions are characterized by IgE antibody production, tissue and peripheral blood eosinophilia, and the participation of inflammatory mediator-rich basophils and mast cells. Whereas in the atopic state these immediate hypersensitivity responses have clearly been implicated in the pathogenesis of allergic diseases, in parasitic helminth infection—although these types of responses can certainly induce pathologic reactions–they also have been implicated in functioning to provide a degree of immunologic protection from the helminth parasites.

A major advance in understanding the regulation of these types of responses was in the identification of functionally distinct populations of $CD4^+$ T cells designated T-helper cells type 1 (Th1) and type 2 (Th2). Th1 cells produce IL-2 and IFN-g in response to antigen or mitogen, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10 (114). Th1 and Th2 cells develop from uncommmitted precursor $CD4^+$ cells with less distinctive cytokine profiles. Naive $CD45RA^+$ precursor cells develop into Th0 cells with multipotent or intermediate secretory profiles and further differentiate into Th1 or Th2 cells following repeated antigenic stimulation. A generally stable relationship between Th1 and Th2 $CD4^+$ populations occurs in part through the production of cross-regulatory cytokines (e.g., IL-10 and IFN-g) that suppress function and growth of the opposite cellular subset. This achieves significance when helminth infections bias Th1/Th2 development toward a Th2 type of cytokine production that may be causally linked to the immediate hypersensitivity responses associated with these infections.

IGE RESPONSES

Although IgE antibody responses are regulated both quantitatively and qualitatively in most persons, infection with helminths perturbs these regulatory mechanisms and consistently allows high levels of IgE to be produced *in vivo* (109). Most of the IgE produced is not antigen specific, perhaps representing nonspecific potentiation or deregulation of a normally well-controlled immune response (115,116 and 117). This potentiating effect seems to be selective for the IgE (and perhaps the IgG4) isotype (118). This was demonstrated most definitively in studies in which animals previously sensitized to a nonparasite antigen (e.g, ovalbumin) subsequently were infected with a helminth parasite; they produced IgE antibodies directed not only against the parasite but also against the nonparasite antigen (116).

Elevated total serum IgE levels in parasitized patients have been documented in many studies (119,120). Characteristically, individuals with invasive helminth infections have serum IgE levels approaching 100 times normal values (109). The factors responsible for this degree of elevation are not well defined, although it most likely reflects a clonal expansion of IgE-producing B cells related to an imbalance of T cells producing either IL-4 or IFN-g (7). There seem to be, however, not only quantitative differences but also qualitative ones. In contrast to atopic individuals whose IgE tends to be directed toward only a few antigens, patients with invasive helminth infection produce IgE antibodies with specificity against an incredibly broad range of parasite antigens (121). It has been proposed that such potentiated polyclonal responses lead to the production of irrelevant IgE that saturates the mast cells' high-affinity FceR, rendering it refractory to triggering by parasite antigen.

EOSINOPHIL RESPONSES

Blood and tissue eosinophilia are also characteristic responses in helminth infection and probably are mediated by increased production of IL-5, possibly in synergy with IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF). Eosinophil-associated pathology can be found in helminth infections and may be caused by eosinophil cationic proteins that are toxic to various normal tissues and cells both *in vitro* and *in vivo* (122,123). Release of proinflammatory eicosanoids, most notably LTC4 and PAF-acether (124), has been implicated in the localized pathology found in tissues with invading helminth infections. Evidence has been accumulating in helminth infections associated with hypereosinophilia, such as onchocerciasis, endomyocardial fibrosis associated with loiasis in expatriates (110,125), or in the tropical (filarial) pulmonary eosinophilia syndrome (111), that the major source for the tissue destruction appears to be the eosinophil and its toxic molecules. Nevertheless, these same molecules can clearly mediate larval killing of many helminths. Chemokines, including MIP-1a, eotaxin-1, eotaxin-2, and regulated on activation normal T cell expressed and secreted (RANTES) (among others) all have been implicated in providing the signals necessary to recruit eosinophils specifically to the sites where helminths or helminth antigens are present (reviewed in 126).

MAST CELL AND BASOPHIL RESPONSES

Mast cells also can contribute to inflammatory reactions directed against invasive helminth parasites. These cells express high-affinity Fce receptors that are sensitized with specific antiparasite IgE and that can be triggered by parasite antigen. Thus, the presence of the mast cell may represent an evolutionary adaptation to infection with parasitic helminths in that they confer a biological advantage to the human host (127,128,129 and 130). Indeed, it has been postulated that mediators specifically released by sensitized mast cells contribute to (a) the recruitment and activation of effector eosinophils; (b) increased local concentrations of antibody and complement; and (c) mucus hypersecretion and increased peristalsis in the gastrointestinal tract that could interfere with the attachment process of certain parasites that would be necessary for chronic infection (reviewed in 128). Despite such apparent benefit, these same effector molecules may also mediate gastrointestinal pathology associated with intestinal parasites.

Basophils are a major source of the T-cell polarizing cytokines IL-4 and IL-13 and produce it in response to either FcÎR crosslinking or to antigen itself (131,132,133 and 134). This suggests that circulating helminth antigens or those helminth parasites that are bloodborne, by inducing basophil-derived IL-4/IL-13, can provide a constant source of those cytokines influencing T-cell subset development and B-cell isotype switching. This may explain how IgE and type 2 T-cell elevations are maintained chronically in helminth infections.

HYPERGAMMAGLOBULINEMIA AND POLYCLONAL ACTIVATION

Hypergammaglobulinemia with elevated levels of parasite-specific antibodies of most (if not all) isotypes has been recognized in many helminth infections, particularly in filarial infections (104). Indeed, in a study of patients with tropical (filarial) pulmonary eosinophilia (111) and one on individuals acquiring loiasis (110), hypergammaglobulinemia was a common finding. Although presumably reflecting a polyclonal expansion of B cells, the mechanism for this polyclonal activation is not well defined. Work examining the interaction between parasite antigen and B-cell responses *in vitro* indicated that polyclonal immunoglobulin responses occur as a result of this interaction almost without exception (135); further, this polyclonal activation occurs among a broad range of helminth parasite infections. In addition, several investigators have isolated antigens from various helminths that appear to have mitogenic activity. These mitogenic factors, however, seem to act indirectly on B cells in that their primary effect is directed at the T-cell arm, which then mediates helper function (136).

SUSCEPTIBILITY TO HELMINTH INFECTION

Because of their tissue-invasive properties, direct chemical and physical damage to the host tissue can initiate inflammatory responses that are not dependent on specific immune interactions. The components mediating this inflammatory response include complement components, a variety of soluble mediators, granulocytes, and T-lymphocytes; the end result is an alteration in the local environment acting to limit the movement and differentiation of the parasite. These inflammatory reactions can clearly encapsulate the worms; but, in many cases, the parasites escape from these inflammatory reactions.

Rapid progress has been made, largely by the use of inbred strains of mice, in defining the genetic basis of susceptibility to protozoal infection, but relatively less work has been done in infections with helminth parasites. Nevertheless, interest in this particular area has been growing because epidemiologic studies in areas where these helminth parasitic diseases are endemic have revealed differential susceptibilities to infection both within the entire population and within families studied (137). Thus, genetic predisposition to innate resistance to parasitic helminth infection must be assumed.

Although the cause of differential susceptibility to clinical expression of helminth infections has been addressed in only a few human studies, the data have implicated, in part, the MHC (138,139). Several large-scale studies attempted to make more compelling the argument of an underlying genetic susceptibility for disease outcome in both filarial and schistosome infection (140,141,142,143,144,145 and 146). For schistosomiasis at least, a genetic locus for susceptibility and resistance has been physically mapped (145).

PROTECTIVE IMMUNITY AGAINST HELMINTHS

Although acquired immunity is difficult to demonstrate definitively in many helminth infections, three important lines of evidence for the presence of acquired, immunologically mediated resistance exist. First, on epidemiologic grounds, there is evidence in schistosomiasis (147,148 and 149) and in each of the filarial infections (150,151) that resistance occurs and that it is acquired with increasing age. Whereas some age-related changes in parasite-specific antibody levels or isotypes have

been reported (82,152,153), the mechanism underlying this age-related acquisition of immunity is not well understood. Second, concomitant immunity, a concept suggesting that despite chronic (and patent) infections, hosts are protected from reinfection by newly invading larvae, also has supporting evidence. Originally described in experimental infections with schistosomes (5,154), other nematodes (155), and *Taenia* organisms (156) in animal models, there is reasonably compelling evidence for its existence in human helminth infection. Finally, genetic predisposition to mounting a specific and effective immune response must be considered and certainly has been demonstrated in several human helminth infections, including intestinal helminths (157,158 and 159) and schistosomes (84,142).

Cytokines and Protective Immune Responses

The characteristic features of helminth infection (eosinophilia, elevated serum IgE, and mastocytosis) are controlled primarily by the type 2 cytokines IL-4, IL-13, and IL-5. In animal models of intestinal helminth infection, $CD4^+$ (but not $CD8^+$) cells are necessary for worm expulsion (160,161), and this expulsion is associated with a dominant Th2-type cytokine response (162,163, reviewed in 164). Abrogation of the type 2 response by administration of soluble IL-4 receptor, anti-IL-4 antibodies, recombinant IFN-g, or IL-12 leads to delayed worm expulsion and a delayed IgE response to infection in these models (165,166,167 and 168). The effect of IL-12 on protective immunity is dependent on IFN-g production and is markedly diminished if treatment is initiated 6 to 8 days after primary infection or, in secondary infection, once a type 2 cytokine response has already been established (167). These data suggest that it is the skewing of the immune response away from a type 2 response rather than a direct effect of IFN-g that is responsible for the decrease in protection in this model.

Although $CD4^+$ cells also play an important role in immunity to tissue-invasive helminth infection, the precise roles of $CD4^+$ helper subsets are not as clear in immunity to these helminths (e.g., filaria, schistosomes, *Strongyloides* species) and may depend on the particular cytokine, life-cycle stage, parasite species, and host studied. Increased adult worm (but not egg) antigen-induced production of IL-5 and IL-4 by $CD4^+$ cells has been correlated with resistance to reinfection in human schistosomiasis (169,170). Multiply vaccinated mice also develop a Th2-type response that can be passively transferred by immune serum (171). In contrast, singly vaccinated immune mice and mice with prepatent infection develop an immune response characterized by Th1-type responses directed primarily at the infective stage (schistosomula), and interference with this response (i.e., by administration of antibodies to IFN-g) has been shown to abolish protective immunity to challenge infection (172,173). Studies examining antigen-induced cytokine production by lymphocytes isolated from strains of mice resistant or susceptible to *Trichinella* infection also demonstrated skewing toward Th1 cytokine production in resistant mice (174).

In murine models of protective immunity to filarial infection, spleen cells from mice immunized with radiation-attenuated *Brugia* L3 larvae proliferate and secrete high levels of IL-4 and IL-5 (but little IFN-g) in response to parasite antigens (175). Both immunity and parasite-induced Th2 cytokine secretion are reduced in mice treated with anti-CD4 antibody (176). A similar correlation between parasite burden and Th2 cytokine production by $CD4^+$ cells was demonstrated in mice infected with *Litomosoides sigmodontis*, a filarial parasite of rodents (177). Long-term residents of filaria-endemic areas without clinical or laboratory evidence of filarial infection (putatively immune) also exhibit heightened lymphocyte proliferation to parasite antigens (69), although the correlation between immune status and parasite-specific Th2 cytokine production remains somewhat controversial (178,179).

Despite these complexities, it appears that Th2-type responses play a significant role in immunity to both intestinal and tissue-invasive helminths, but the data are more compelling for the intestinal helminths.

Antibodies and Protective Responses

Antibodies appear to play a minor role in mediating protection to helminth infection. Indeed, in both B-cell-deficient animals or those with FcR deletions, infections proceed without differences from their immunologically competent wild-type control animals (126,180). Nevertheless, the IgE isotype bears particular mention because IgE has been implicated in mediating some protection in both human and animal studies. In schistosomiasis, studies of *S. haematobium* in Gambia showed that persons with high serum levels of parasite-specific IgE after antischistosomal chemotherapy were less likely to become reinfected or have lighter reinfections compared with those with lower IgE levels (82). Additionally, it was observed that there was an associated increase in serum levels of parasitic-specific IgE with increasing age, which was closely correlated with development of acquired resistance. Similar studies of *S. mansoni* in Brazil pointed to the same conclusion of an association between elevated levels of anti-schistosomal IgE and acquired immunity (181). In support of these findings in humans are animal studies suggesting a protective role for IgE in rats (182) infected with filarial parasites (182), *T. spiralis* (183), or *S. mansoni* (184), filarial-infected cats (185), or baboons infected with *S. mansoni* (186). In contrast, however, are findings in human trichuriasis in which gastrointestinal pathology was associated with the presence of degranulated surface IgE^+ mast cells capable of releasing histamine in the presence of parasite antigen (187). In addition, mice incapable of making IgE (by anti-IL-4 monoclonal antibody therapy) can clearly be vaccinated to *S. mansoni*, suggesting that IgE alone is not mediating protection to schistosome infection (188).

Eosinophils and Protective Immunity

The role of the eosinophil in mediating protection to helminth infection is also under scrutiny. In studies of resistance to reinfection with *S. mansoni* and *S. hematobium*, eosinophilia has been correlated with resistance to reinfection (189,190). There has been conflicting evidence (188,191) on the protective efficacy of eosinophils in animals depleted of eosinophils and subsequently challenged with *S. mansoni* or the enteral parasite *Heligmosomoides polygyrus* (192); in *T. spiralis* (183) and *S. venezuelensis* (193), however, eosinophils were capable of mediating resistance to migrating larvae. Overall, the discrepancy seen in the study of the role of eosinophils in protective immunity in these different animal models of parasite infections may result either from a redundancy of effector immune responses or from the basic differences in animal models and mouse strains.

Epidemiologic studies in humans and animal models demonstrated correlations between eosinophilia and resistance to helminth infection (189,194). Eosinophils (or purified eosinophil granule proteins) are able to kill many different species of tissue invasive helminth larvae *in vitro* in the presence of antibodies and complement (195,196,197,198 and 199). The mechanism of eosinophil-mediated larval killing is incompletely understood but may involve eosinophil peroxidase-mediated formation of active oxygen species, as has been reported in eosinophils from granulomas isolated from the livers of mice infected with *S. mansoni* (200), complement, and antibodies (201).

Results of *in vivo* studies are conflicting with respect to the role of eosinophils in resistance to helminth infection. Mice depleted of peripheral eosinophils using antieosinophil antibodies show increased susceptibility to infection with a variety of helminths (202,203), whereas mice treated with anti-IL-5 antibodies have no decrease in immunity to these same helminths despite a lack of blood or tissue eosinophilia (204,205 and 206). Furthermore, IL-5 transgenic mice, which have increased levels of eosinophilia, are more (rather than less) susceptible to schistosomiasis and show delayed clearance of primary *Trichinella* infection compared with normal mice (207,208).

The most conclusive *in vivo* evidence for the role of eosinophils in helminth killing comes from experiments with diffusion chambers containing infective helminth larvae implanted in the subcutaneous tissue of mice. Not only were eosinophils the only cells that accumulated in the chamber concomitant with larval killing (209), but such killing could be prevented by inhibition of eosinophil migration into the chamber or treatment of the mice with anti-IL-5 antibody (209,210 and 211).

Mast Cells and Protective Immunity

Although the accumulation of mast cells in parasitized tissues has been used as an argument in favor of a role for mast cells in protective immunity, it is not at all certain that these cells serve a protective function in parasite infection. Nevertheless, animal models of immunity to challenge infection provided some experimental evidence for a salutary role of the mast cell. These types of secondary challenge studies provided evidence that mast cells are responsible for the prevention of reinfection (212) in gastrointestinal parasites and for the cytotoxicity against early schistosomula in the epidermis of previously vaccinated primates (213). Further, depletion of mast cells has been shown to abrogate the cytotoxic responses against schistosomula (214). *In vitro*, mast cells, in concert with peroxidase and iodide, also have been shown to be capable of mediating cytotoxicity to schistosomula (215).

Expulsion of intestinal parasites is temporally associated with an increase in the number of mast cells in the intestine and secretion of mast cell proteases and leukotrienes into the tissues and serum (216,217,218 and 219). This mastocytosis is dependent on both T-cell-derived cytokines (IL-3, IL-4, IL-9, and IL-10) (220,221) and T-cell-independent factors [stem cell factor (SCF)] (222). The mechanism by which mast cells are activated by helminth parasites remains unclear, but the high-affinity IgE receptor does not appear to be essential to the process (220,221).

Despite the strong association between mastocytosis and intestinal helminth infection, mast cell-deficient mice (W/Wv mice genetically deficient in the SCF ligand, *c-kit*, or mice treated with antibodies to *c-kit* or SCF itself) exhibit variable responses depending on the infecting species. Administration of monoclonal antibodies to SCF completely abrogates the intestinal mastocytosis (and eosinophilia) seen in murine *Trichinella* infection and results in delayed expulsion of adult parasites (223). In contrast, *Nippostrongylus* adult worms are expelled at a similar rate by wild-type mice, mast cell-deficient W/Wv mice (224) and mice treated *in vivo* with antibodies to IL-3 and IL-4, which have an 85% reduction in the number of mast cells in the gut compared with wild-type mice (225).

The mechanisms by which mast cells might cause worm expulsion remain uncertain. Changes in intestinal muscle contractility and fluid dynamics in response to mast cell release of leukotrienes and prostaglandins have been implicated in some studies (see review by Finkelman et al., 1997 [164]), although expulsion of worms does

occur, albeit in a delayed fashion, in the absence of these alterations in gastointestinal physiology in mast cell-deficient and SCID mice treated with a long-acting preparation of IL-4 (164,226).

Protozoan Infection

Important protozoan causes of human disease include invasive organisms with distinct intracellular and extracellular forms as well as others that cause infections restricted to the gastrointestinal lumen and epithelium. The resulting character of host immunity is greatly influenced by these factors. Generally, extracellular parasites are susceptible to killing mediated by neutralizing or opsonizing antibody, whereas intracellular parasites are controlled by T-lymphocyte responses that activate microbicidal mechanisms in phagocytic and nonphagocytic cells. However, this is an oversimplification of real events. Most intracellular protozoa have brief, obligatory extracellular phases, and some intestinal parasites, such as *Entamoeba histolytica*, are also invasive at times and can cause visceral disease. Furthermore, host responses can be innately heterogeneous, working to the benefit of the parasite if some proportion of the infected population is genetically incapable of mounting protective immune responses. Notably, activation of Th2 $CD4^+$ T cells may promote B-cell and antibody responses to the detriment of protective Th1 $CD4^+$ T-cell responses that are required for control of intracellular parasites (227).

CELL BIOLOGY OF INTRACELLULAR PARASITIZATION

Organisms that invade host cells are provided with some sanctuary against complement-mediated lysis and ingestion by polymorphonuclear phagocytes. An intracellular location may fulfill metabolic and developmental requirements, such as access to hemoglobin for the nutritional use of blood-stage malaria. Whatever the adaptive advantages for intracellular parasitization, it is a common theme of protozoan infection and poses a challenge to host defense strategies distinct from that of extracellular infection. Specifically, T-cell-mediated immunity becomes increasingly important in the control of these organisms, as is clinically apparent by the increased incidence and severity of *Toxoplasma*, *Trypanosoma cruzi*, *Leishmania*, and *Babesia* in immunocompromised AIDS and transplant patients (228,229).

Intracellular parasitism requires that the invasive protozoa attach to specific target cells and cross the plasma membrane. Many parasites accomplish both these goals by binding to cellular receptors that mediate phagocytosis when engaged. *Leishmania* organisms can attach to multiple macrophage receptors via their surface gp63 and lipophosphoglycan molecules; partial activation of complement on the surface of the parasite contributes additional ligands for macrophage complement receptors (230). In other protozoan infections, cellular entry is actively mediated by the parasite without the use of normal host cell phagocytic mechanisms. For instance, *Toxoplasma* organisms enter by an unusual form of induced phagocytosis that actively excludes host membrane proteins from the parasitophorous vacuole that forms, although the identity of the cellular receptor is unclear (231). Uptake may be enhanced by coating with serum-derived fibronectin and engagement of the appropriate integrins on host cells. Both *Toxoplasma* and *Plasmodia* species possess a secretory organelle, called the *rhoptry*, that plays a key role in cellular adherence and invasion. Although mechanistic details are unclear, it is probable that amphipathic proteins and lytic enzymes released by rhoptries help to destabilize the host cell plasma membrane and favorably condition the subsequent parasitophorous vacuole (232,233).

It is surprising that tissue macrophages are the preferred target of several protozoa, including *Leishmania*, *Trypanosoma*, and *Toxoplasma*, considering the ability of these phagocytes to generate microbicidal oxygen and nitrogen products during phagocytosis of opsonized particles. Protozoa avoid these potent nonspecific responses by infecting resident tissue macrophages with diminished oxidative and nitric oxide synthetic functions and by selectively engaging receptors that mediate uptake without triggering these antimicrobial defenses (32,230). *Leishmania* species induce their uptake by engagement of CR1 and CR3 complement receptors that do not activate the oxidative burst. The entry of *Toxoplasma* and *T. cruzi* into macrophages also circumvents activation of cytocidal responses, although the exact nature of the ligands engaged is not known; however, there are limits to the extent that macrophages can be safely invaded. Prior opsonization of protozoa by specific IgG can lead to uptake by Fc receptors that efficiently trigger a microbicidal oxidative burst (32). At least one species of *Leishmania*, however, has adapted this method of cellular entry to facilitate infection by amastigotes that become coated with IgG during intracellular transfer (234); only macrophages with relatively deactivated microbial responses are permissive for intracellular parasitization. Activation of macrophages by exposure to IFN-g or TNF generated as a result of antigen-specific T-lymphocytic responses induces microbicidal functions that can control infection. Although nonmacrophage cell types such as fibroblasts and hepatocytes do not have oxidative burst capability, they can be activated by cytokines to generate nitric oxide capable of killing intracellular *Plasmodia* and *Toxoplasma* organisms (235,236). As a consequence, cell-mediated immunity is usually the critical component of the host response that leads to control of infection and subsequent immunity against reinfection.

Once internalized, parasites must be able to avoid or tolerate the acidic, proteolytic lysosomal environment if phagocytosis is used as means of entry (32). *Toxoplasma* organisms accomplish this by modification of the phagocytic vacuole to prevent acidification and lysosomal fusion (232). *T. cruzi* and *Theileria parva* pass through the phagosomal membrane and replicate within the cytoplasm of the host cell. *T. cruzi* accomplishes this exit by secreting an acid-active hemolysin (Tc-TOX) that disrupts the phagosome membrane; another secreted enzyme with neuraminidase activity may facilitate release by desialating the vacuole interior surface (237). This hemolysin is antigenically similar to human pore-forming complement protein, C9 (238). In contrast, *Leishmania* species are metabolically adapted for survival and replication within the acidic phagolysosome (239). Further protection may be provided by a dense lipophosphoglycan surface coat that can scavenge reactive oxidative products and that suppresses activation of potentially microbicidal responses (33). Products of the C3 complement protein on the surface of *Leishmania* organisms also seem to enhance survival of the parasite within the phagolysosome, although the mechanism involved is not understood (240).

Whatever the mechanism of intracellular survival, the compartmentalization of parasite antigen in the lysosome or cytoplasm may influence how antigen is processed and whether MHC I- or MHC II-restricted activation of $CD4^+$ or $CD8^+$ T-lymphocytic responses dominates cellular immunity (241). Not surprisingly, a greater role for $CD8^+$ T cells in protective immunity against cytoplasmic *T. cruzi* and *T. gondii* is evident in comparison to the obligate phagolysosomal parasite, *Leishmania*.

NONSPECIFIC HOST DEFENSES AGAINST PROTOZOAN INFECTION

Infectious threats, whether intracellular or extracellular, are countered by an orchestrated cascade of nonspecific (*innate*) and antigen-specific (*adaptive*) host defenses. In some cases, however, resistance is mediated by genetic traits of the host that uniquely prevent intracellular entry or survival of parasites. This has been well characterized in areas endemic for malaria, where the frequent appearance of abnormal hemoglobin genes, such as sickle cell trait, results in erythrocytes less able to support parasite replication (242). The loss of the Duffy antigen from the erythrocytes of Africans similarly prevents *P. vivax* infection by removal of the ligand required for red cell invasion (242). As another example, Cape buffalo are protected against infection with *T. brucei*, which causes fatal disease in all other bovine species. In contrast to other bovines, Cape buffalo are capable of markedly downregulating serum levels of catalase during systemic inflammation, an usual response that permits xanthine oxidase-derived oxidative products to accumulate in the bloodstream and kill trypanosomes (243).

Other nonspecific host defenses are part of the innate humoral immune response, which includes the alternative pathway of complement, pattern-recognition molecules, neutrophil responses, and proinflammatory cytokines (244,245,246 and 247). These are rapidly activated in the critical period of infection prior to recruitment of antigen-specific immunity. Serum carbohydrate binding proteins (collectins) and complement are central to the innate humoral immune response; however, as described earlier, pathogenic protozoa may have the ability to evade, inhibit, or subvert the activation or function of the alternative complement pathway. As a result, these parasites are poorly opsonized and are relatively resistant to phagocytosis by polymorphonuclear cells. Microbes that invade host cells further limit their exposure to extracellular complement and professional phagocytes.

In contrast, the innate cellular immune response is centrally directed by macrophages and dendritic cells during infection. Both cell types utilize a wide range of receptor molecules—such as the mannose receptor, advanced glycosylation end-product receptor, toll receptors, and integrins—that bind highly glycosylated fungal and protozoan surfaces and that subsequently mediate cellular responses. These responses include phagocytosis or endocytosis of offending particles identified as microbial by pattern-recognition receptors (246,248). Phagocytosis by macrophages is sufficient to kill most microbes, a process assisted by production of microbicidal-reactive oxygen intermediates and nitric oxide induced during phagocytosis or surface receptor ligation. Because dendritic cells and macrophages also serve as efficient antigen-presenting cells, the scavenging and ingestion of microbial glycoproteins serve an important immune surveillance function for priming antigen-specific immunity in addition to mediating an early nonimmune defense.

The same pattern-recognition molecules that activate phagocytosis also critically stimulate secretion of proinflammatory cytokines, including IL-12, TNF, IL-6, IL-1, and chemokines. These factors may incite fever, which can help limit parasite expansion during infection with *T. cruzi* and malaria (249), direct leukocyte immigration into areas of infection, or elicit production of IFN-g. Although innate cellular cytokine responses are best described in bacterial infection (247), protozoan products with intrinsic inflammatory properties (adjuvant-like effects) can also directly elicit cytokines, although the involvement of Toll receptors in the process is not yet defined. For instance, the erythrocytic phase of malaria is associated with elevated serum TNF levels triggered in response to plasmodial molecules and the by-products of massive hemolysis (107,250). TNF synthesis under these conditions probably benefits the host by activating phagocytic cells in the spleen to ingest and lyse infected blood cells; however, TNF produced in excess is detrimental to the host. At the extreme, it may help precipitate the immunopathologic crisis of cerebral malaria discussed earlier. *T. gondii* also contains glycolipids that strongly activate IL-12 production and systemic inflammation by host dendritic cells (251).

Although usually thought of as a product of mature T-cell immunity, IFN-g can be acutely and nonspecifically secreted by T lymphocytes and NK cells in response to microbial challenges (252). This cytokine response is dependent on the prior production of IL-12 and IL-18 by accessory cells and is antagonized by TGF-b or IL-10 (253). In combination with TNF, IFN-g activates nitric oxide in a wide range of infected cells, with additional stimulatory effects on the oxidative burst and phagocytic functions of phagocytic cells. In the setting of toxoplasmosis, which induces strong innate IL-12 synthesis, IFN-g responses can be rapid, T-cell independent, and

capable of sharply limiting early parasite load (254,255).

ANTIGEN-SPECIFIC IMMUNITY AGAINST PROTOZOAN INFECTION

Although innate immune responses may ameliorate the early stages of parasitic infection, cure of intracellular infection typically is associated with the onset of antigen-specific $CD4^+$ and $CD8^+$ T-lymphocytic responses (reviewed in 227). In the case of parasites that are predominately intracellular, such as *Leishmania* or *T. gondii*, cellular responses may be the only effective type of immunity available to the host, with antibodies providing little protective effect against sequestered protozoa (60). Where both extracellular and intracellular forms of parasite coexist during infection, such as during malaria, humoral and cellular responses may have comparable importance in the control of infection.

As described for antihelminthic responses, functionally distinct subsets of $CD4^+$ and $CD8^+$ T cells variously mediate cure, susceptibility, or tissue pathology in the cellular immune response to protozoa. Whether $CD4^+$ or $CD8^+$ cells predominate in response to intracellular parasitism may depend on the location of the pathogen in infected cells, with lysosomal sites more conducive to MHC II expression of parasite antigen and cytosolic sites more likely to provide antigenic peptides for loading of MHC I complexes (241). Infection of cell types such as fibroblasts and hepatocytes that express little or no MHC II may also account for the dominance of $CD8^+$ T-cell responses against *T. cruzi* and liver-stage *Plasmodia* organisms.

The Th1 and Th2 $CD4^+$ T cell subsets have important roles in the control or progression of intracellular infection (227). As described earlier, Th1 and Th2 cells are defined by mutually exclusive functional properties, notably cytokine release. They derive from precursor cells with less distinctive cytokine phenotypes. The antagonistic roles of Th1 and Th2 cells in the control of intracellular infection has been appreciated using a mouse model of leishmaniasis (60). Strains of inbred mice experimentally infected with *L. major* reproduce a spectrum of disease similar to that seen in humans infected with *L. donovani*, providing a convenient model for the examination of protective and exacerbating host responses. Resistant C57BL/6 and C3H mice heal local infection, develop delayed-type hypersensitivity to leishmania antigens, and exhibit long-lasting immunity against reinfection. BALB/c mice develop progressive infection and die of visceral dissemination that resembles human visceral leishmaniasis. Cure or progression of infection is mediated by Th1-type or Th2-type responses, respectively. The roles of IFN-g and TNF in healing were established by the exacerbating effect of anti-IFN-g and TNF monoclonal antibodies when administered during infection of resistant mice (249,256). Similarly, recombinant human IFN-g has been used clinically to treat drug-resistant visceral leishmaniasis (257). In contrast, the IL-4 product of Th2-type cells proved necessary to progressive infection: Anti-IL-4 cured normally susceptible mice (258). The IL-4-like cytokine IL-13 provides redundant inhibitory effects on leishmania immunity due to signaling through a shared IL-4/IL-13 receptor complex (259).

Cytokine effects on the outcome of infection are twofold. First, Th1 and Th2 cell products have opposing effects on macrophage activation requisite to the efficient killing of leishmania in infected tissues. IFN-g and TNFb activate nitric oxide and reactive oxygen intermediate synthesis, whereas the Th2 product IL-4 blocks these microbicidal functions (260,261). Second, IFN-g and IL-4 are required in the first week of infection to permit subsequent differentiation and amplification of Th1 and Th2 cells, respectively (262,263). The immunologically important cellular source of these cytokines in infected tissue appears to be $CD4^+$ T cells (264). Although NK cells generate IFN-g early in infection, they are not required for the development of protective adaptive immunity (265). A similar involvement of IFN-g and IL-4 in the spectral expression of disease in humans infected with *Leishmania donovani* has been suggested, although murine models of *L. donovani* infection suggest that increased TGF-b activity is necessary and sufficient for progressive infection (266). To summarize, distinct forms of progressive leishmaniasis rely on activation of different suppressive cytokines that antagonize IFN-g-dependent immunity as a common feature.

In contrast to the dominant immunoregulatory role of $CD4^+$ T cells in leishmaniasis, other protozoan infections elicit mixed $CD4^+$ and $CD8^+$ T-cell responses active in cure and prevention of disease. This has been well established for *T. cruzi*, the cause of Chagas disease, which afflicts millions of persons living in Central and South America (228). Extracellular trypomastigote forms enter macrophages, muscle cells, and fibroblasts, where they reproduce as intracellular amastigotes. Acute parasitemia in murine and human hosts resolves in association with the appearance of antigen-specific antibody and T-cell responses. Extracellular trypomastigotes actively inhibit C3 convertase assembly, but specific antibodies may allow parasiticidal opsonophagocytosis and antibody-dependent cellular cytotoxicity. The appearance of amastigotes in the blood during some infections may allow persistence of the parasite despite effective antitrypomastigote defenses because amastigotes are resistant to killing by the C5b-C9 complement attack complex (32). A role for MHC I-restricted parasite killing was demonstrated by the appearance of fatal parasitemia in b_2-microglobulin-deficient mice that do not possess mature $CD8^+$ T cells (267). $CD8^+$ T cells also were involved in the pathogenesis of acute myocarditis; however, both $CD4^+$ and $CD8^+$ T-cell responses are required for optimal killing of the parasite (268). The protective effects of T cells is dependent on production of IFN-b to activate macrophage parasite killing (269,270).

Despite the potential effectiveness of humoral and cellular immunity in control of acute American trypanosomiasis, infection usually persists for the lifetime of the host and can result in chronic pathology. Latency of *T. cruzi* also was demonstrated by the recrudescence of disease following immunosuppression (228). The failure to clear infection totally may be linked to nonspecific immunosuppression of both B- and T-cell function that appears after several weeks of infection. This appears to be caused by activation-induced cell death of responding lymphocytes (271) and is directly linked to the production of IL-10 and TGF-b, which downregulate the synthesis and protective action of IFN-g (76,77).

Antibodies directed against *T. cruzi* also can cross-react with human proteins, reflecting the presence in both protozoa and humans of proteins with conserved sequences, such as the ribosomal P0 protein (16) and the heat shock protein HSP70 (272). Other cross-reactivities are shared by functionally nonhomologous proteins, such as the sharing of an epitope between a *T. cruzi* 160-kd protein and a mammalian neural 48-kd protein (17); however, the role of these cross-reactive immune responses in the mediation of chronic intestinal or cardiac pathology is uncertain because parasites are detectable in these same tissues well into the chronic phase and might be the important target of immune destruction (273).

Toxoplasma gondii is an obligate intracellular parasite that causes asymptomatic infection in 40% to 90% of humans in developed countries. The importance of latent infection, especially contained within dormant cysts in the brain, has been well appreciated as a result of disastrous reactivation following immunosuppression associated with organ transplantation, leukemia chemotherapy, and AIDS. *T. gondii* has a broad range of tissue preferences, with macrophages a frequent target for invasion. As discussed earlier, survival is facilitated by entry without triggering an oxidative burst or fusion of the parasitophorous vacuole with lysosomes (232). Both $CD4^+$ and $CD8^+$ T cells are involved in protective immunity (274). $CD4^+$ T-lymphocytes contribute both to the control of acute infection and to continued dormancy of encysted *T. gondii* because human immunodeficiency virus (HIV)-related $CD4^+$ T-cell depletion in humans and anti-CD4 antibody therapy in infected mice results in recrudescent brain infection (275). $CD8^+$ T cells may be equally important in the generation of native and vaccine-mediated immunity, perhaps through the cytolysis of infected cells (276,277). Regardless of the type of T cell necessary for cure, IFN-g is a central requirement for killing *T. gondii* in macrophages, endothelial cells, and fibroblasts. Recent findings suggest that the mechanisms are not strictly dependent on nitric oxide or superoxide production (278). Other IFN-g-inducible microbicidal responses have been identified that might also contribute to toxoplasma clearance (279).

HOST RESPONSES TO EXTRACELLULAR PROTOZOAN INFECTION

All invasive protozoan parasites have an extracellular stage at some time. This stage may be transient, as in the case of intracellular parasites that briefly enter the extracellular space at intervals when spreading to adjacent target cells. Usually these short forays do not result in appreciable damage to the parasite mediated by humoral immunity. In contrast, the protozoan cause of African sleeping sickness, *T. brucei*, does not have an intracellular stage and is always in contact with serum during infection. Consequently, the generation of antibody can result in clearance of parasitemia through Fc receptor-mediated phagocytosis or by activation of complement; however, as discussed earlier, *T. brucei* uses antigenic variation to escape protective humoral surveillance. Although T-helper cells are required for the generation of cytocidal antitrypanosomal antibodies, IFN-g is also critical for cure of trypanosomiasis and indicates a dual role for $CD4^+$ T cells in recovery (280).

Malaria generates a protozoan infection with a complex series of developmental stages that can be either predominately extracellular or intracellular. Sporozoites introduced into vertebrate hosts by mosquito bite circulate briefly in the bloodstream and then invade hepatocytes. After a variable incubation period of days to years, merozoite forms emerge, invade red blood cells, and engage in multiple cycles of asexual replication in the bloodstream. Symptoms of disease usually occur at this time as a result of the repeated lysis of erythrocytes and margination of parasitized cells within capillaries. Extracellular gametocyte forms develop sporadically to perpetuate the life cycle when subsequently ingested by mosquitoes. Four species of malaria—*Plasmodia falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*, cause human disease. Of these, *P. falciparum* poses the greatest clinical threat because of its worldwide distribution, the often fatal intensity of parasitemia that develops during infection, and its acquisition of chloroquine and mefloquine resistance. At least a million deaths a year are attributed to this single species of parasite.

Acquired immunity against falciparum malaria develops in the inhabitants of highly endemic regions, but such resistance is incomplete in that it takes years to develop, serves to reduce rather than prevent parasitemia, and does not persist following emigration out of endemic areas (281). The delay in acquisition of immunity likely reflects the considerable antigenic polymorphism and variation within the local population of *P. falciparum*. Sporozoite-specific antibodies have been intensively studied because of their production by malaria-immune individuals and because immunization with irradiated sporozoites is beneficial (282,283). Antibodies are directed against tandem peptide repeats in the central portion of the circumsporozoite (CS) protein. Regions of the CS protein that flank these tandem repeats contain the ligand for recognition and invasion of hepatocytes (284). Although antibodies directed against the heptaocyte ligand prevent liver-stage infection, this region is poorly immunogenic in intact sporozoites. One suggestion has been that the intensely immunogenic nature of the tandem repeats, which probably activate B cells in a T-cell-independent fashion, may misdirect the immune response away from more protective epitopes. Although antisporozoite antibodies may activate cytocidal

complement and facilitate Fc receptor-mediated killing of parasites by splenic macrophages, clinical trials of sporozoite vaccine studies have been disappointing (285).

Sporozoites also elicit $CD8^+$ cytotoxic T cells that act to limit hepatic sequestration. Mice immunized against *P. falciparum* require $CD8^+$ T cells and IFN-g for disease resistance (reviewed in 286). IFN-g activates nitric oxide synthesis in infected hepatocytes and kills intracellular plasmodia (235). Possession of the human leukocyte antigen (HLA)B53 MHC background was associated with acquired immunity against *P. falciparum* in endemic regions and permitted immune recognition of a unique peptide derived from the liver stage-specific antigen, LSA-1. This antigen is not expressed by sporozoites and directs specific cytotoxic T-lymphocyte (CTL) responses that can limit hepatic infection (287).

Both humoral and T-cell immunity against blood-stage malaria have been documented. Antibodies directed against antigens of the extracellular merozoite stage may help to limit disease, as suggested by high titers of these immunoglobulins in malaria-resistant humans living in highly endemic areas and by the protective effect of passive immunization with sera from immune donors (288). These effects may be mediated by neutralization of parasite cytoadhesion receptors (289). Several of the parasite-encoded molecules on the surface of parasitized erythrocyte are ligands for the vascular adhesion molecules CD36, thromobospondin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1) and may assist parasite survival by preventing circulation of infected red blood cells through the spleen, where deformed or opsonized cells would be removed (21,290). Consequently, antibodies that neutralize adherence of parasitized cells to the peripheral vasculature would be expected to assist in the clearance of blood-stage infection. Multiple ligand–receptor interactions with redundant functions may have evolved in response to immune pressure, suggesting that heterogenous antibody responses are needed for protection. Much of this variability can be ascribed to the antigenically diverse family of var cytoadherence proteins (22).

T-cell immunity against blood-stage *P. falciparum* is easily demonstrated and appears to be important, but the particulars are not well defined (289). Primate and mouse models of malaria clearly demonstrate a requisite role for $CD4^+$-mediated immunity. Intraerythrocytic death of malaria, termed *crisis*, may be causally linked with release of lymphokines and monokines dependent on T-cell activation. Activation of splenic macrophages by IFN-g, a Th1 product, would be expected to help clear parasites and infected red blood cells; however, $CD4^+$ depletion as a result of AIDS has not yet caused recognizable exacerbation of falciparum malaria in endemic areas (291).

In summary, immunity against malaria represents the sum of complex, overlapping cellular and humoral responses to distinct stages of the parasite. Although specific immune protection against homologous strains of parasite can be demonstrated in defined experimental systems, cross-reactivity in the field is profoundly limited by strain heterogeneity and the ability of the parasite to vary critical antigenic targets over time. Naturally acquired immunity is consequently a state of relative disease tolerance and limited parasitemia without evidence of sterilizing immunity.

PROTOZOAN INFECTION OF THE GASTROINTESTINAL TRACT

A variety of pathogenic and nonpathogenic protozoa live within the mammalian gastrointestinal tract. *G. lamblia*, *Entamoeba histolytica*, and *Cryptosporidium parvum* are the most clinically important of these parasites. Of these, *E. histolytica* has the additional ability to invade through the mucosal surface and cause hepatic abscess. Protective immunity against luminal parasitism is restricted in choice of immune mechanisms compared with those available in tissues. There is little evidence that macrophages or polymorphonuclear cells perform a major defensive role on the gastrointestinal epithelia. Although $CD8^+$, ab^+ intraepithelial lymphocytes that can mediate cytocidal and delayed-type hypersensitivity responses are present in the human intestinal epithelium, a role for these cells in protective immunity against parasites has not been defined. T cells present in the deeper lamina propria and Peyer patches have all the characteristics of Th2 cells and support IgA production but probably are inefficient mediators of proinflammatory cellular immune responses. Indeed, the ability to secrete large quantities of dimeric IgA is the best-defined immunologic feature of the gastrointestinal mucosa that may regulate luminal parasitization and that is amenable to study (292).

The role of IgA in neutralizing intestinal protozoan infection is supported by studies of giardiasis (293). In humans, IgA deficiency and other hypogammaglobulinemias have been repeatedly associated with inability to clear intestinal giardiasis, whereas patients with cellular immunodeficiencies are not similarly predisposed. Infection with *Giardia* organisms, which are not known to cross the intestinal epithelium, nevertheless triggers production of *Giardia*-specific IgM, IgG, and IgA in the serum. This likely represents transport of antigen across the mucosal surface to Peyer patch lymphatic tissue by M cells. Oral treatment of experimentally infected mice with anti-*Giardia* IgA preparations has a protective effect. Although $CD8^+$ T-lymphocyte depletion in infected mice does not affect recovery, anti-CD4 antibodies do, but this may be due to inhibition of T-cell-dependent help required to generate IgA (294). Because IgA is not cytolytic and because IgA receptor-positive phagocytes are not a prominent feature of intestinal mucosal immunity, it seems most likely that IgA is disrupting attachment of parasite to epithelial receptors. Further research into clinical effective anti-*Giardia* vaccines may need to address possible antigenic variation in different isolates of this protozoa.

During most of its life cycle in humans, *Cryptosporidium parva* is covered with a thin layer of epithelial cell membrane where it attaches to the gut wall, although actual invasion of the cytoplasm does not occur (295). Replication requires exit into the lumen of the gut as oocysts and as free-dwelling sporozoites that attach to the gut wall. A major role for cellular immunity has been suggested by the increased severity and chronicity of this illness in patients with AIDS (296). A mouse model confirmed that $CD4^+$ T cells and IFN-g are required for protection (297). The importance of gut IgA is less certain, although anecdotal evidence suggests some benefit from specific IgA contained in colostrum.

The enteroinvasive parasites *Entamoeba histolytica* cause a spectrum of disease ranging from asymptomatic colonization to erosive colitis to hepatic abscess (48). Both attachment and lytic factors are central to the pathogenicity of *E. histolytica*. Antibodies that prevent engagement of colonic mucosal cells by the parasite's 260-kd galactose-specific lectin are thought to mediate some degree of protection against intestinal infection (298,299); however, strong amebicidal cell-mediated immunity has been correlated with recovery from colitis and amebic abscess (300). Antigen-specific T-cell responses produce IFN-g, which promotes killing of ameba by activated macrophages *in vitro* (301). *In vivo* studies have been compromised by an inability to recreate the full range of human pathology in experimental animals, although T cell–dependent and nitric oxide–dependent protection against hepatic abscess formation in rodents has been described (302,303).

VACCINE DEVELOPMENT AGAINST PARASITES

Despite the considerable progress achieved in understanding the molecular biology of parasitic infection and the nature of antiparasitic immunity, few clearly effective vaccines have been fielded for prophylaxis against the major parasite threats of the world; however, there is a more comprehensive appreciation of the components of protective or deleterious immune responses necessary for the pursuit of this goal. Specifically, the way in which immunodominant antigens are presented during immunization may critically affect the type of immune response that results. Indeed, single antigen epitopes may elicit either protective or exacerbating immunity against *Leishmania* in different hosts (304). To summarize, the development of effective parasitic vaccines will require the use of (a) carrier molecules or adjuvants (305) that will overcome the innate heterogeneity of immune responses in human populations and specifically induce protective immunity against parasitic infection; (b) cross-reactive, nonpolymorphic antigens where strain heterogeneity is a problem; and (c) multicomponent vaccines to target each of several parasite forms when disease is caused by more than one stage of a parasite life cycle. Efforts to develop an effective malaria vaccine have been particularly intense, and the following discussion focuses on these efforts as a generic model for vaccine design against other protozoan or helminth pathogens (reviewed in 180).

Because many parasites are not easily cultured for large-scale production of immunogenic antigen and because the generation of stable, attenuated parasite strains safe for human use does not seem to be imminent, recombinant proteins will continue to be a key component of parasite vaccine design; however, special delivery systems may be required to minimize the expense of production and to increase tolerance to heat and humidity prevalent in parts of the world where parasitic illnesses are most damaging. Furthermore, recombinant antigens may be highly dependent on the use of adjuvants to generate clinically significant immune responses, as demonstrated during human efficacy studies of a recombinant *P. falciparum* sporozoite vaccine (306). In this regard, the advent of recombinant viral vaccines, either attenuated poxvirus (307) or the comparatively safer fowlpox virus (308), may allow for mass vaccinations without requiring extensive purification of antigen beforehand. Self-limited viral infection promotes specific immunity because it generates antigen at the site of vaccination and because it triggers local inflammatory responses that can substitute for adjuvants in enhancing T-cell and antibody production. Furthermore, because proteins encoded by the virus are produced in cytoplasm and targeted for MHC I coexpression, recombinant viral vaccines are of special interest for those diseases where $CD8^+$ T-cell immunity is protective.

Another approach may be to use recombinant bacille Calmette-Guérin (BCG) (309). Considerable effort has suggested the feasibility of this approach, although clinical information is not yet available on human efficacy and safety. Because BCG may provide a strong adjuvant effect, particularly in support of proinflammatory $CD4^+$ and $CD8^+$ T-cell responses, the development of this vaccine system may overcome the predisposition of some recipients to develop Th2-type immune responses. The use of BCG combined with *Leishmania* antigens as immunotherapy for nonhealing cutaneous leishmaniasis supports the feasibility of this approach (310). A similar technique applied in animal models has been the use of nonpathogenic *Salmonella*-expressing parasite antigen (311). These, too, may generate antigens for stimulation of both $CD4^+$ and $CD8^+$ T-cell immunity, although humoral responses are often poor.

Regardless of the delivery system used, monocomponent vaccines are unlikely to be of use in the setting of multistage parasitic disease and in the face of antigenic heterogeneity. This has been a major obstacle for antimalarial vaccine design, and current efforts are focused on the inclusion of nonpolymorphic sporozoite, merozoite, and gametocyte-stage antigens to limit infection at several sites as well as to provide “altruistic immunity” in the form of transmission-blocking antibody responses (312). Multimeric peptide complexes using a variety of conserved sequences have been explored for their ability to stimulate antiparasitic immunity. B-cell and T-cell epitopes are chemically combined to maximize cognate interactions required for strong humoral and cellular immunity (313); however, despite early reports

of success, a *P. falciparum* synthetic vaccine (SPf66) that uses a “cocktail” of blood-stage antigenic peptides (314) was ineffective where local parasite strains were not antigenically related to the peptides used in SPf66 (315).

Ultimately, many of these objectives might be best met through use of DNA vaccines, which can incorporate multiple antigen genes under the control of strong viral promoters and which have demonstrated long-lasting protection in animal models (316). Expression of plasmid-encoded antigen within the host cell assures strong $CD8^+$ T-cell responses necessary for prevention of intracellular infection, although strong $CD4^+$ T-cell and humoral responses are also observed. The ability to combine different plasmids in a single vaccine is especially well suited for malaria prevention, where a mix of stage specificities is important (317). Furthermore, DNA vaccines provide self-adjuvant properties through the presence of CpG motifs that induce accessory cell function and immunoregulatory cytokine synthesis (318,319). Phase I studies of influenza- and malaria-specific DNA vaccines in humans have already commenced (320,321), potentially inaugurating a new class of vaccines that can be adapted for protection against the wide range of parasitic infections that bedevil humankind.

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81 THE IMMUNOCOMPROMISED HOST

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Normal immune competence relies on the coordinated activity of a variety of hematopoietic cells [granulocytes, monocyte/macrophages, specialized antigen-presenting cells, natural killer (NK) cells, T cells, and B cells] and hematopoietic cell-derived factors (complement, cytokines, and antibodies). Both innate and adaptive (Chapter 1) immune mechanisms are essential for generating a robust and effective response to microbial or viral challenge.

The clinical spectrum of disease in patients with deficiencies in single or multiple components of this complex system demonstrates the importance of each of these components in maintaining immune competence. Some immune deficiency states arise as a result of *primary* (congenital) defects (Chapter 28). These include immunodeficiencies affecting T cells, such as DiGeorge syndrome, whose etiology lies in defective thymic development, or B cells, such as combined variable immunodeficiency, in which antibody production is impaired. Other primary immunodeficiency states, such as Chediak-Higashi disease, affect predominantly neutrophils, in which neutrophil chemotaxis and granule function are defective. Although primary immune deficiencies are rare, they have provided important information about the role of specific components of the immune response as well as interactions between different elements of the immune system.

Acquired immune deficiencies are more commonly encountered, either as a result of systemic illness or secondary to therapy for other illness. The sequelae of infection by human immunodeficiency virus (HIV), in which $CD4^+$ T cells are progressively depleted, is an illustrative example of a systemic illness that results in acquired immune deficiency. Other systemic illnesses that result in immune compromise include collagen-vascular disease (Felty syndrome, systemic lupus erythematosus), hematologic malignancy [multiple myeloma, chronic lymphocytic leukemia (CLL)], aplastic anemia, and nutritional deficiency.

Modern therapies for serious systemic illnesses can cause significant immune impairment. Immunocompromise due to neutropenia after chemotherapy is a common problem in oncologic clinical practice, and increases the risk of life-threatening infection. Bone marrow transplantation (BMT) results in a prolonged period of immune deficiency as the transplanted immune system reconstitutes from hematopoietic progenitor cells. Immunosuppressive agents (e.g., cyclosporine, tacrolimus, and corticosteroids) are used extensively after bone marrow and organ transplantation as well as in the treatment of rheumatologic and oncologic disease, and significantly compromise normal immune function. Systemic illnesses often affect multiple components of the immune system, and reflect the coordinated nature of the immune response against pathogens. Nonetheless, several acquired immune-deficient states can affect predominantly one arm of the immune response, and this can serve to highlight the importance of these particular functions in host defense. Regardless of whether immune compromise is due to primary or acquired deficiency, improved understanding of which immune components are impaired can help identify the possible pathogens to which the immunocompromised host is susceptible, and direct therapy. In the following sections, components of the immune response are considered individually, and the acquired deficiency states associated with each, and possible treatments are described.

ACQUIRED COMPROMISE OF NEUTROPHIL FUNCTION

Manifestations

Clinically, acquired neutropenia as a result of therapy is among the most commonly seen complications after treatment with cytotoxic agents for oncologic and rheumatologic disease. Granulocytopenia is a serious complication because (a) it predisposes the patient to infections caused by a wide array of pathogens, and (b) it can be associated with rapid progression, often in the absence of classic signs and symptoms of infection. Thus when neutropenia is severe, infections rapidly become life threatening, and require prompt diagnosis and treatment. In patients with cancer, the correlation between number of circulating granulocytes after the administration of cytotoxic therapy and risk of infection has been well established. In 1966 Bodey et al. (1) described the course of 63 patients with acute leukemia (age range, 1 to 77 years) and demonstrated that the incidence of proven infection and the absolute number of circulating granulocytes were clearly related. Risk of infection increased progressively with both duration and magnitude of granulocytopenia <1,000 cells/μL. At 100 cells/μL, the incidence of infection increased dramatically. Nevertheless, severe infections occurred more frequently during relapse than during remission, suggesting that other factors also played a role in predisposing to infection (1). Other investigators have demonstrated that patients on the verge of recovery are at considerably lower risk of bacteremia than are those who have recently received cytotoxic therapy and are expected to be neutropenic for an extended period (2). Although these early studies were performed in patients with cancer, these findings have also been found to apply to other neutropenic patients.

Bacterial infections represent the main source of infection in patients with acquired neutropenia. The spectrum of infectious agents has evolved over the past three decades, and reflects changes in clinical practice (3). In the 1950s and 1960s, when cytotoxic treatments were first being developed, gram-positive infections were most commonly encountered. In the 1970s, gram-negative organisms emerged as the most common pathogens in neutropenic patients. Since the 1980s and 1990s, gram-positive bacteria are again commonly encountered (4). Despite these general trends, there is significant institutional variation in the prevalence of particular pathogens, and therapeutic interventions must be adapted to reflect these variations (5). With prolonged neutropenia, invasive fungal infections also occur with increased frequency (6). Viral infection and parasitic infection, in the setting of isolated granulocytopenia, are less common.

Etiology

CHEMOTHERAPY AND RADIATION THERAPY

Cancer treatment with most cytotoxic therapies and radiation therapy commonly results in transient suppression of bone marrow hematopoiesis. This results in a decrease in the number of hematopoietic cells circulating in the periphery, including granulocytes, which typically lasts for a few days up to 1 week or longer. In some cases, the degree of neutropenia is mild, with no clinical sequelae; in others, it may be quite profound, thus markedly increasing the risk of life-threatening infection. The depth and duration of treatment-related neutropenia is related to the dose and the specific cytotoxic agents used, as well to the intrinsic bone marrow reserve of the patient. Thus neutropenia generally becomes more severe, and recovery is delayed after repeated cycles of chemotherapy deplete the hematopoietic reserve of the bone marrow.

Although quantitative deficiency in neutrophils after chemotherapy is clearly an important cause of the susceptibility to infection, *in vitro* studies also have demonstrated that the drugs used to deliver cytotoxic treatment can directly impair phagocytic function. Various investigators have demonstrated that cytotoxic agents can inhibit specific granulocyte functions, including hexose monophosphate shunt activity, superoxide production, phagocytosis, chemotaxis, and microbicidal activity. Implicated agents include methotrexate, 6-mercaptopurine, vincristine, vinblastine, anthracyclines, cyclophosphamide, carmustine, and platinum compounds (7,8,9,10,11,12 and 13). Many cytotoxic regimens also include glucocorticoids, which are known to have many effects on immune effector cells. The major effect of glucocorticoids on granulocyte function is a decrease in chemotactic activity. This inhibits accumulation of granulocytes at the site of infection and decreases the localized inflammatory response (14). This may contribute to the clinical observation that many of the signs and symptoms of infections are “masked” in patients taking glucocorticoids. Steroids also have important effects on circulating monocytes, including monocytopenia and functional defects in monocyte chemotaxis, phagocytosis, and killing of bacteria and fungi (15).

BONE MARROW APLASIA

Aplastic anemia (AA) is a disease of primary bone marrow failure. The initial presentation of these patients is typically related to severe or recurrent infection, with subsequent discovery of neutropenia and marrow aplasia (16). Infection also is the leading cause of death in this patient population. A retrospective evaluation of 150 patients with AA, admitted to the Clinical Hematology Branch of the National Institutes of Health (NIH) between 1978 and 1989, revealed that 36 (62%) of 58 deaths were attributable to infection (17). Sixty-seven percent of these were bacterial infections; 23% fungal, 7% viral, and 3% were parasitic infections. AA patients vary with respect to the degree of neutropenia, and this study demonstrated that the single most important factor for developing severe and fatal infections is the degree of neutropenia and monocytopenia. Marrow aplasia also is a manifestation of paroxysmal nocturnal hemoglobinuria (PNH), and these patients similarly can have neutropenic fever (18).

Pure white-cell aplasia (PWCA) is a rare syndrome characterized by severe pyogenic infections and neutropenia. PWCA is generally caused by autoantibodies directed against myeloid progenitor cells, and approximately 70% of cases are associated with thymoma. In some cases, PWCA may occur several years after removal of thymoma. Bone marrow examination shows almost complete absence of myeloid precursors, with normal erythroid precursors and megakaryocytes. Marrow inhibitory activity is seen in both immunoglobulin G (IgG) and IgM fractions of patients' sera and disappears as the marrow recovers. PWCA has been reported in association with ibuprofen therapy, in which *in vitro* serum inhibitory activity required both drug and complement, and the clinical syndrome resolved once ibuprofen was discontinued. Cyclophosphamide, steroids, cyclosporine, and intravenous immunoglobulin (IVIG) have provided effective treatment of PWCA in some patients (19).

MALIGNANT DISEASE OF THE MARROW

In acute myeloid leukemia (AML), myeloid cells are arrested in an early stage of differentiation and cannot develop into mature neutrophils. In addition, several studies have documented significant functional defects in mature granulocytes from patients with AML (20,21). Abnormalities in granulocyte myeloperoxidase activity have been correlated with subsequent development of infection (22). Patients with acute lymphocytic leukemia (ALL) have been reported to display similar functional defects (23). At the time of diagnosis, nearly two thirds of children with ALL have fever, and the majority are neutropenic due to bone marrow replacement with malignant cells (24).

IDIOSYNCRATIC DRUG REACTIONS

Drug reactions that cause leukopenia range from mild to profound, and are characterized by the reduction or disappearance of neutrophilic granulocytes from the peripheral blood. Patients with drug-induced agranulocytosis are at high risk of life-threatening infections, and studies report a mortality rate of 6% to 30% (25). Agranulocytosis is associated with the use of various categories of drugs summarized in Table 81.1. The incidence of drug-induced agranulocytosis is estimated to be approximately 2.6 to 10 cases/million drug exposures/year, although it is difficult to know the precise incidence because these cases are often not reported (26).

Class of Medication	Examples of Specific Drugs	References
Antithyroid agents	Propylthiouracil Methimazole	(27,28)
Phenothiazines	Chlorpromazine Prochlorperazine Promazine Mepazine Thioridazine	(29–31)
Sulfonamides	Trimethoprim-sulfamethoxazole Sulfasalazine	(32–37)
Antibiotics	Cephalosporins Aztreonam Aminoglycosides	(38,39)
Antiarrhythmic agents	Procainamide Quinidine Hydralazine Captopril	(40–43)
Anticonvulsant drugs	Phenytoin Carbamazepine	(44,45)
Antiinflammatory agents	Phenylbutazone Oxyphenbutazone Indomethacin Colchicine Gold salts Penicillamine	(46–49)
H_2-receptor antagonists	Cimetidine	(50)

TABLE 81.1. Medications Associated with Idiosyncratic Agranulocytosis

Drug-induced agranulocytosis can result from at least two different mechanisms. Most instances result from drug-induced antibodies that, in the presence of the drug, react with granulocytes, their bone marrow precursors, or both (51,52 and 53). The abrupt onset of granulocytopenia, usually beginning 1 to 3 weeks after the first dose of the drug, is a characteristic feature of this mechanism. The development of drug-induced antibodies is frequently associated with severe systemic symptoms such as chills, fever, extreme malaise, skin rash, and severe sore throat. After recovery, there is a prompt recurrence of these abnormalities if the patient is reexposed to the drug. If a bone marrow biopsy is obtained early in the course, a marked depletion of mature granulocytes and granulocyte precursors but adequate numbers of erythroid precursors and megakaryocytes is seen. If performed after recovery has begun, the bone marrow biopsy reveals large numbers of promyelocytes and early myelocytes with prominent azurophilic granules that may resemble the marrow of a granulocytic leukemia. In the presence of drug, antibodies in the serum may react with mature granulocytes in agglutination or other test systems and may inhibit growth of granulocyte colonies *in vitro* (54,55 and 56).

Some drugs, such as phenothiazines, produce agranulocytosis by a different mechanism, which involves suppression of differentiation of committed progenitor cells of all three marrow lineages (41,57,58). However, because of the short intravascular residence time of granulocytes, the disorder often is seen as agranulocytosis with only mild anemia and thrombocytopenia. The typical clinical setting in which this is seen is a psychiatric patient in whom an insidious onset of leukopenia develops 2 to 15 weeks after beginning a new medication. Alternatively, agranulocytosis is discovered because of a routine blood count in an asymptomatic patient or because of a blood count prompted by evidence of infection. Recurrence slowly develops over days with reexposure to the offending drug or when a different phenothiazine is administered. Bone marrow biopsy reveals a markedly hypocellular bone marrow resembling that of aplastic anemia. There is no evidence of drug-induced serum antibodies. Recently it was suggested that the reactive metabolites of drugs implicated in agranulocytosis may interact with specific components of the extracellular matrix and interfere with the normal regulation of granulopoiesis. This results in decreased production of granulocytes because normal myeloid maturation is dependent on certain components of the stromal microenvironments [intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, CD11b/CD18, heparin sulfate proteoglycans, fibronectin, hemonectin] (59).

In addition to the drugs associated with agranulocytosis, many commonly used medications have documented effects on phagocyte function. These agents include nonsteroidal antiinflammatory drugs (NSAIDs) (60,61), opiates and narcotic analogs (62), ethanol (63), benzodiazepines (64), methylxanthines (65), b-adrenergic blocking agents (66), phenytoin (67), inhaled and intravenous anesthetics (68), gold compounds (69), nicotine (70), allopurinol, cyclosporine (71), heparin (72), calcium-channel blocking agents (73), and antibiotics (74).

VIRAL INFECTIONS

Neutropenia after viral infection is seen more commonly in children than in adults, especially after infection by measles, chickenpox, rubella, influenza, infectious hepatitis, yellow fever, sand fly fever, HIV, Colorado tick fever, or dengue fever. The mechanisms for neutropenia in these patients have not been well defined but may result from nonspecific suppression of marrow hematopoiesis by cytokines secreted in response to the viral infection. In addition, rickettsial disease, miliary tuberculosis (TB), malaria, kala-azar, and relapsing fever have been associated with neutropenia. In contrast to that after viral infection, neutropenia after bacterial infection is unusual but can occur after salmonella infection, tularemia, and brucellosis. In this setting, neutropenia often indicates exhaustion of bone marrow reserves as well as margination and localization of neutrophils to sites of bacterial infection.

RHEUMATOLOGIC DISEASES

Felty syndrome is a complication of rheumatoid arthritis, and is characterized by the following manifestations: (a) severe, long-standing arthritis; (b) splenomegaly; (c) severe neutropenia; (d) serologic abnormalities, including elevated gamma globulins, high titer of rheumatoid factor, antinuclear antibodies (ANAs), and evidence of mixed cryoglobulins (cold precipitable immune complexes); and (e) normocellular or hypercellular bone marrow with increased numbers of early myeloid forms. The neutropenia of Felty syndrome is not entirely understood. Several mechanisms have been implicated including impaired granulopoiesis, increased destruction of granulocytes secondary to immune complexes or antineutrophil antibodies, and excessive margination (75). Whereas the appearance of the bone marrow usually suggests increased granulocyte production, granulopoiesis may be depressed in some individuals. In some patients, the bone marrow has been found to contain T lymphocytes that suppress normal marrow granulocyte colony growth *in vitro* (76). In other patients, increased amounts of immunoglobulin bound to granulocytes have been demonstrated as well as increased amounts of immunoglobulin in serum that can bind to granulocytes of other individuals. In these patients, accelerated

clearance of immunoglobulin-coated neutrophils and suppression of granulopoiesis by serum immunoglobulins appear to contribute to the neutropenia (77,78 and 79).

Patients with systemic lupus erythematosus (SLE) have increased risk of infection, often due to alterations in both the number and the function of granulocytes (80). This risk increases with use of corticosteroids and other immunosuppressive agents and in the presence of impaired renal function. Approximately 47% of these patients are leukopenic, due to neutropenia (81). Neutropenia may occur because of immune destruction, marrow suppression, hypersplenism, or drugs. SLE serum has been shown to increase neutrophil aggregation, and this may contribute to the neutropenia (79,82). The evidence for bone marrow suppression by SLE sera comes from the examination of effects of SLE sera on bone marrow colonies growth *in vitro*: 18 (34%) of 42 SLE sera retarded bone marrow colony growth, whereas no effect was seen with 40 control sera (83). Conversely, 12 (40%) of 30 sera from patients with other inflammatory diseases also retarded growth. It has been demonstrated that there is a normal chemotactic response of neutrophils from patients with SLE, but the generation of chemotactic factors was decreased in ten (43%) of 23 patients (84). Serum from 22 (73%) of 30 SLE patients interfered with phagocytosis and lysosomal release in normal neutrophils (85,86).

NUTRITIONAL DEFICIENCY

Neutropenia can be seen in association with anemia of nutritional deficiency of vitamin B_{12}, folate, and copper (87). These nutritional deficiency states are characterized by ineffective myelopoiesis and megaloblastic changes in the bone marrow.

Diagnostic Evaluation of Granulocytopenia

Neutropenia can be generated by decreased production of granulocytes, a shift of granulocytes from the circulating to the marginating pool, peripheral destruction, or a combination of these factors. In general practice, granulocytopenia is usually defined as an absolute neutrophil count (ANC) <500 and is calculated as follows (88):

$$\text{ANC} = \text{WBC} \times (\%\text{bands} + \%\text{mature neutrophils}) \times 0.01 \quad (1)$$

In a patient newly discovered to have neutropenia, a careful history should be elicited to determine the approximate onset of neutropenia (5). In particular, recent exposure to cytotoxic therapy, or drugs that are associated with neutropenia or agranulocytosis, should be determined. On physical examination, the presence of splenomegaly is helpful and might suggest that increased destruction of granulocytes contributes to neutropenia. Additional examination of peripheral blood can determine if other hematopoietic lineages are affected. In many cases, it will be necessary to examine the bone marrow to determine whether there is any evidence of failure to generate myeloid precursors, or whether there is an arrest in myeloid maturation, or marrow infiltration with leukemic cells. One mechanism by which neutropenia is generated is the development of antibodies to neutrophil antigens such as NA1, NA2, ND1, ND2, and NB1, as well as antigens shared by erythrocytes and to human leukocyte antigens (HLAs) (51). These antibodies, usually of IgG and IgM type, can be detected by a variety of assays, such as leukoagglutination, opsonization, immunochemical assays, direct antibody binding, and complement activation (54,89). Detection of antineutrophil antibodies (ANAs) is helpful in establishing the diagnosis of immune neutropenia, although a negative assay does not exclude the diagnosis. Although not commonly used, detection of increased serum lysozyme or lactoferrin reflects increased destruction of neutrophils.

If fever appears in a granulocytopenic patient, successful management depends on early intervention. Once fever develops, aggressive diagnostic efforts should be undertaken to identify specific causes of febrile illness, with the goal of identifying potential sources of infection to optimize therapy. Special attention should be directed at intravascular-access devices, wounds, and the perirectal area. Standard assessments include cultures of urine, blood, sputum, stool, and cerebrospinal fluid (if mental status changes or signs of meningismus are present). A chest radiograph should be obtained to determine whether there is any evidence of pneumonia. If an intravascular-access device is present, blood cultures from each lumen of the device should be obtained. Even with a comprehensive evaluation, a definite infectious cause is found in only 30% to 50% of cases. In a prospective study evaluating >1,000 consecutive episodes of febrile neutropenia, no features were found that reliably distinguished bacteremic patients from those with unexplained fever (24).

Various *in vitro* studies have been developed to examine neutrophil function (e.g., respiratory burst, phagocytosis), and are described in detail in Chapter 20. Outside of the setting of research or diagnosis of congenital defects, these tests are not used in general clinical practice.

Treatment of Granulocytopenia

EMPIRIC ANTIBIOTICS

Because bacteremias in severely neutropenic patients are often rapidly fatal, empiric antimicrobial therapy should be promptly instituted in patients with ANC >500/µL, even if no clinical signs of infection are detected other than fever (90). Moreover, empiric antibiotics are often continued until neutropenia has resolved, even if specific pathogens are not identified. Increased awareness of the necessity of timeliness of intervention has resulted in improved outcomes. Over the past decade, more intensive chemotherapeutic protocols have been developed, with consequent increased degree of immunosuppression and prolonged use of antibiotics. These developments have resulted in the increased incidence of invasive fungal infections, as well as the emergence of antibiotic-resistant bacteria (3,91).

The choice of empiric antibiotics is designed to maximize activity against organisms that are commonly encountered or particularly virulent or both. Because gram-negative bacteria have been the most frequently isolated pathogens in neutropenic patients, and these tend to be quite virulent, broad-spectrum antibiotics that provide effective therapy against these organisms are an essential component of any empiric regimen. Other required features of empiric antibiotic regimens include the ability to achieve high serum bactericidal levels that provide increased effectiveness in the absence of neutrophils and an acceptable toxicity profile. To prevent the emergence of resistance, the use of a combination of agents has been recommended. Early experience using an aminoglycoside and expanded-spectrum penicillin such as carbenicillin demonstrated a synergistic effect against *Pseudomonas aeruginosa* and resulted in improved clinical outcome (92). Such combination regimens are still widely used, but the development of more effective cephalosporins has led to further evaluation of the need for combination regimens (90). A meta-analysis of 12 randomized controlled trials comparing ceftazidime as single-agent therapy with combination therapy for treatment of febrile neutropenia did not demonstrate any advantage of combination therapy over monotherapy (93). As newer antibiotics are continuously being developed, the role of these agents for empiric therapy of severely neutropenic patients should be evaluated in prospective randomized trials. In addition to efficacy, important factors that influence the widespread use of these new agents in this setting include toxicity profile, emergence of resistance, ease of administration, and cost.

Empiric treatment of gram-positive bacteria in severely neutropenic patients also is important. Nevertheless, vancomycin is generally not indicated as part of the initial regimen. In a randomized study of a vancomycin-containing versus a non–vancomycin-containing regimen, the incidence of secondary gram-positive infections was reduced in the vancomycin-containing group. However, no differences in morbidity were attributed to gram-positive infections between the two groups, and all gram-positive infections in the non–vancomycin-treated group were successfully treated by the addition of vancomycin after the organism was identified (94). Similarly, a large retrospective analysis showed no excess morbidity when the use of vancomycin was delayed by waiting for either a clear microbiologic or clinical indication (95,96). The reduced empiric use of vancomycin in this setting limits the emergence of vancomycin-resistant bacteria and also reduces the toxicity associated with this agent.

Patients with severe neutropenia and persistent fever also are at risk for development of invasive fungal infection (97). Two prospective randomized trials demonstrated the efficacy of empirical amphotericin B in persistently febrile neutropenic patients (98,99). In both studies, patients who received amphotericin B after a course of antibiotics had superior outcome to patients who were treated with antibacterial agents alone. Deaths in a significant number of patients who did not receive amphotericin B were due to fungal infections. Other randomized trials have demonstrated similar efficacy of intravenous amphotericin B and oral ketoconazole (100,101). However, use of ketoconazole was complicated by difficulty in tolerating oral medication and erratic absorption in this patient population (102). New liposomal formulations of amphotericin B have been developed with similar efficacy and with decreased toxicity (103).

To determine the source of bacterial infection in neutropenic patients and whether identification of colonizing bacteria might help in the initial antibiotic management of neutropenic patients, nose, throat, urine, and stool cultures were obtained serially in 652 febrile patients. In those patients that developed sepsis and a specific causative pathogen was isolated, 62% were colonized with the infecting organism (104). These findings demonstrate that serious infections in patients with severe neutropenia are most often caused by organisms that have colonized the individual before the development of neutropenia. However, surveillance cultures at a single body site were not predictive of subsequent infection, and several pathogens were often isolated in addition to the organism responsible for infection. Moreover, the costs of performing routine surveillance cultures are enormous (105,106). Thus routine surveillance cultures are not recommended in general practice.

Traditionally, patients with febrile neutropenia remain hospitalized with antibiotic therapy until fever and all signs of active infection have resolved and neutropenia has resolved. The possibility that certain favorable subgroups of patients may be effectively treated with antimicrobial regimens as outpatients (107,108 and 109) is being currently explored.

The management of fever in patients with AA is a particular clinical challenge because the appropriate duration of the empiric antibiotic regimen has not been defined. In contrast to cancer patients in whom febrile neutropenia develops after cytotoxic therapy, neutropenia in AA is not self-limited, and the neutropenia may last for months or years. Patients with ANC <100 are at highest risk, and the high incidence and grave outcome of fungal infection merit special consideration. In most patients,

antibiotic therapy is continued for only 10 to 14 days, followed by careful observation. This recommendation is based on a series of randomized studies in which patients with fever and neutropenia were assigned to either discontinue antibiotics on day 7, or day 14, or until resolution of granulocytopenia. In a large percentage of afebrile patients who stopped antibiotics on day 7, recurrent fever developed, and in an alarmingly high percentage of persistently febrile patients whose antibiotics were discontinued, hypotensive episodes developed. In contrast, patients who stopped antibiotics on day 14 often responded to reinstitution of their initial regimen if fevers recurred (110).

MYELOID COLONY-STIMULATING FACTORS

Granulocyte colony-stimulating factor (G-CSF) is a lineage-specific growth factor that increases circulating neutrophils by stimulating release from storage pools and by shortening the maturation time in bone marrow. Granulocyte-macrophage (GM)-CSF has broader hematopoietic effects due to the stimulation of a less committed progenitor cell population and other cells such as mature monocytes and dendritic cells. Functionally, neutrophil respiratory burst; adherence, phagocytosis, and bacterial killing are normal if not enhanced *in vivo* after treatment with either agent.

Before the advent of CSFs such as G-CSF and GM-CSF, the treatment of patients with neutropenia was limited to the administration of antibiotics and to the occasional use of granulocyte infusions. In three major randomized trials in adults receiving moderately myelosuppressive chemotherapy, CSFs were shown to enhance recovery of granulocytes and to reduce the incidence of febrile neutropenia by approximately 50% (111,112 and 113). The value of CSFs has not been established in less myelosuppressive regimens (114,115), and their use is not currently recommended with many chemotherapy regimens (114). Exceptions are those patients who have had a history of recurrent febrile neutropenia during earlier cycles of chemotherapy and conditions that markedly increase the risk of systemic infection (e.g., ongoing active infection or open wound). There is evidence that CSF administration can decrease the probability of febrile neutropenia in subsequent cycles of chemotherapy after documented occurrence in earlier cycles. CSFs also can be used to prevent prolonged neutropenia causing excessive dose reduction or delay in chemotherapy administration (111,116,117).

Because most myeloid leukemia cells express receptors for CSFs, there has been concern whether these agents could induce leukemic transformation when used after chemotherapy in patients with myeloid malignancies or myelodysplasia. As a result, several studies have examined the outcome of postchemotherapy CSF administration in patients with AML. In one trial conducted in older patients (55 to 70 years) with AML, patients randomized to receive GM-CSF were found to have a modest but significant reduction in duration of severe neutropenia and incidence of severe infection (118). There was a trend toward higher complete response rates and a significant prolongation of median survival in patients receiving GM-CSF. A subsequent trial conducted in similar patients was less encouraging (119). A statistically significant but clinically inconsequential reduction in the duration of severe neutropenia was seen, but there was no significant difference in the incidence of serious infection, days of hospitalization, acute mortality, or complete response rate.

In adults, the recommended dose of G-CSF is 5 µg/kg/day, and of GM-CSF is 250 µg/m^2/day, subcutaneously or intravenously as clinically indicated (114). CSFs are generally started 24 to 72 hours after completion of chemotherapy and continued until ANC >10,000/µL after neutrophil nadir. The predominant side effect of G-CSF is medullary bone pain, and appears to occur more frequently at higher doses. In randomized trials, 15% to 39% of patients receiving 5 µg/kg/day have had this symptom (111,112 and 113,120). Less frequent side effects include exacerbation of preexisting inflammatory conditions (eczema, psoriasis, vasculitis) (121), occasional rashes, rare allergic reactions, acute febrile neutrophilic dermatosis (Sweet syndrome) (122,123), transient leukemia cutis in CML patients, and rare injection-site reactions. Laboratory abnormalities occur frequently and include elevations in lactate dehydrogenase (LDH), uric acid, and serum and leukocyte alkaline phosphatase, presumably secondary to increased myeloid cell turnover. In patients with advanced cancer, a 25% to 50% decrease in platelet counts has been reported with doses greater than 10 µg/kg/day (124).

Whereas the largest experience with CSFs has been in patients receiving cancer chemotherapy and BMT, CSFs also have been successfully used for treatment of neutropenia not associated with cancer. Prolonged administration of G-CSF and GM-CSF has been used to maintain normal neutrophil counts in patients with Felty syndrome (125) and SLE (126). Of concern, in several patients with Felty syndrome, leukocytoclastic vasculitis and flares of arthritis developed after the use of CSFs. However, this was rare if G-CSF was administered at lower doses (126,127). CSFs are now commonly used in the treatment of Felty syndrome and are preferred to previous treatments such as methotrexate, lithium, testosterone, cyclosporine, and splenectomy (128). CSFs also have been used to overcome HIV-mediated leukopenia and to circumvent the myelosuppressive effects of antiviral agents (129,130).

GRANULOCYTE TRANSFUSIONS

Granulocyte transfusions were first used more than 30 years ago. At that time granulocytes collected from patients with CML were infused into patients with severe neutropenia, and infusions of large numbers of cells often effected resolution of life-threatening infections (131). Subsequent studies suggested that clinical response and survival correlated directly with the number of granulocytes infused (132). Although effective in some settings, the routine use of granulocyte transfusions was quite problematic. Granulocytes were difficult to collect from normal donors, as the number of circulating granulocytes available for collection was relatively small. Moreover, granulocyte function deteriorated rapidly during storage, and granulocyte infusions were frequently associated with severe complications. Thus, even though several encouraging randomized clinical studies advocated the use of granulocyte transfusions in the setting of life-threatening infection and neutropenia, they largely fell out of favor as a therapeutic modality (133).

Over the past decade, there has been renewed interest in granulocyte transfusions because patients are receiving increasingly intensive myelosuppressive regimens, which are associated with prolonged periods of neutropenia. In addition, administration of G-CSF can increase circulating granulocytes more than tenfold and mobilize granulocyte progenitors (134,135). Thus large numbers of normal granulocytes can now be harvested from normal donors with relatively little toxicity.

Several controlled studies have evaluated the use of therapeutic granulocyte transfusions. As a group, these studies are limited by small numbers and heterogeneous patient populations, and clinical results varied (136,137,138,139,140,141 and 142). In general, the studies appear to support the use of granulocyte transfusions for patients with persistently severe neutropenia. Studies that showed no benefit appeared to reflect inadequate granulocyte transfusion support. To be effective, the dose of granulocytes transfused should be greater than 2 to 3 × 10^{10} PMN (not less than 1 × 10^{10}). Daily granulocyte transfusions should be continued until infections have resolved or until ANC increases above 500/µL. In addition to cell dose, the effectiveness of granulocyte transfusions is influenced by the extent of recipient alloimmunization and donor histocompatibility, likelihood of endogenous granulocyte recovery, and whether the organism is sensitive to antimicrobial agents. Toxicity to transfusions can be very severe, ranging from low-grade fevers to rigors, dyspnea, pulmonary infiltrates, and hypoxia. Some degree of toxicity can be prevented by administering granulocyte transfusions slowly, at 1 to 2 × 10^{10} cells per hour. Increased toxicity with concomitant administration of amphotericin B has been reported (143). In general, granulocyte transfusions should be considered for persistently neutropenic patients with unusually severe and progressive infections that cannot be controlled with antimicrobial agents. This includes patients with progressive bacterial infections, systemic fungal infections, congential disorders of granulocyte dysfunction, fungal infections after BMT, and newborns with bacterial infections (133). The use of prophylactic granulocyte transfusions has not been supported by prospective studies and is not recommended.

ACQUIRED COMPROMISE OF T-CELL FUNCTION

Manifestations

T-cell immunity is necessary for control of viral, protozoan, bacterial, and fungal infections. Essential to the development of mature T cells is surface expression of rearranged T-cell receptor (TCR) genes in conjunction with surface expression of CD3. Individuals are able to respond to diverse pathogens and stimuli, new and old, because of the very high degree of complexity of the TCR repertoire. Once the TCR is engaged, in the presence of costimulatory signals, a series of intracellular events leads to activation of the T cell. Peripheral T cells are subdivided into the $CD4^+$ (T helper) and $CD8^+$ [cytotoxic T lymphocytes (CTLs)] subsets that recognize and respond differently to processed peptide antigen. In normal immunocompetent adults, the total number of $CD4^+$ and $CD8^+$ T peripheral lymphocytes is remarkably stable. These T-cell subsets comprise both naive and memory cells, whose numbers are maintained by separate homeostatic regulators. The thymus normally involutes during adolescence, and the production of new $CD4^+$ and $CD8^+$ T cells decreases with increasing age. Recent thymic emigrants express CD45RA, whereas T cells in the memory compartment express CD45RO (144).

The specific pathogens encountered and the spectrum of clinical findings in patients with acquired defects of T cell–mediated immunity vary depending on the underlying disease, the degree of immunosuppression, and the presence or absence of other host defense abnormalities. Typically, patients with acquired T-cell deficiency are susceptible to infections caused by intracellular organisms, which are capable of surviving and replicating within macrophages, B cells, or other cell types. This observation attests to the importance of T-cell function for macrophage activation and subsequent microbicidal activity. Many of these agents are not pathogenic in individuals with normal T-cell function. Infections found in hosts with defective T-cell immunity typically fall into the following categories: (a) bacterial (e.g., *Mycobacteria*, *Legionella*, *Nocardia*, *Salmonella*); (b) fungal (e.g., *Clostridia neoformans*, *Histoplasma capsulatum*); (c) viral [e.g., varicella zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV)]; and (d) protozoan (e.g., *Pneumocystis carinii*, *Toxoplasma gondii*, *Strongyloides*).

Etiology

CHEMOTHERAPY FOR MALIGNANT AND NONMALIGNANT DISEASES

Previous reports have examined the phenotypic immune reconstitution of patients receiving intensive chemotherapy and found that return of $CD4^+$ T-cell numbers is inversely proportional to age. Younger patients promptly recovered total $CD4^+$ T cells, including subsets of $CD45RA^+$ (naive) cells, presumably because of output of new T cells from the thymus (145). In older patients in whom the thymus is involuted, recovery of $CD4^+$ T cells was slow and numerically incomplete. Moreover, most $CD4^+$ T cells expressed the memory marker CD45RO, suggesting that they were derived from the expansion of residual peripheral T cells that survived cytotoxic chemotherapy, rather than from the production of new T cells in the thymus (146,147).

RECONSTITUTION AFTER MYELOABLATIVE THERAPY AND HEMATOPOIETIC STEM CELL TRANSPLANTATION

Myeloablative therapy followed by hematopoietic stem cell transplantation is frequently used in the treatment of patients with hematologic malignancies. Mature T and B cells present in the stem cell product (bone marrow or peripheral blood stem cells) contribute to immune function after transplant, but full and long-lasting immune reconstitution after myeloablative therapy is dependent primarily on the differentiation of new immune cells from undifferentiated hematopoietic progenitor cells. After transplantation, the T-cell compartment typically reconstitutes slowly, with the $CD4^+$ population lagging behind the $CD8^+$. This results in inversion of the normal CD4/CD8 ratio for prolonged periods after BMT (148,149 and 150). $CD8^+$ T cells reach normal numbers by 3 to 4 months after BMT, whereas $CD4^+$ numbers do not normalize until 6 to 12 months after BMT. NK cells are among the first cell populations to return. In the first month after transplant, NK cells often represent the predominant lymphoid population, especially in patients who receive marrow depleted of mature T cells. Approximately 30% of NK cells express CD8, and this likely contributes to increased CD8 numbers early after BMT (151,152). In addition to the presence of mature $CD3^+$ $CD4^+$, and $CD3^+CD8^+$ T cells, detectable populations of T cells in the peripheral circulation are normally present only during fetal life or in the adult thymus, reflecting recapitulation of lymphoid ontogeny after BMT. These include $CD3^{dim}CD4^-CD8^-$ double-negative, and $CD3^+CD4^+CD8^+$ double-positive T cells, as well as $CD1^+$ cells and increased numbers of TCRgd T cells (153,154).

Although thymic function declines with age, normal individuals are able to maintain a highly diverse TCR repertoire. Without a highly diverse T-cell repertoire, the ability of foreign antigens to escape detection increases, and the subsequent development of an effective T-cell response to external pathogens decreases. The complexity of the TCR repertoire in an individual can be measured by quantifying the use of various TCR Vb genes in peripheral T cells (155). Moreover, the diversity of the CDR3 regions generated within populations of T cells can be measured by the method of spectratyping (156,157). Recent studies from our laboratory and others have directly examined reconstitution of the T-cell repertoire after myeloablative therapy and hematopoietic stem cell transplantation by using spectratyping (158,159,160,161 and 162). In this method, polymerase chain reaction (PCR) with TCR Vb family-specific primers is used to amplify the CDR3 regions of all T cells that have rearranged TCR Vb genes. Because normal polyclonal T cells each have unique CDR3 regions, the size display of normal T cells with a diverse TCR repertoire demonstrates a series of eight to ten peaks with a gaussian distribution. As shown in Fig. 81.1A for a typical patient 3 months after allogeneic BMT, the CDR3 pattern demonstrates a large number of clonal and oligoclonal peaks as well as absent use for some of the TCR Vb families. However, by 1 year after BMT, this profile has normalized, and most of the CDR3 patterns demonstrate normal polyclonal profiles (Fig. 81.1B). In patients who have engrafted completely with donor cells and have no posttransplant complications, TCR Vb repertoire reaches normal levels of diversity by 12 to 18 months after transplant (158). This occurs more rapidly in pediatric patients, presumably because of a higher level of thymic function in these individuals (163,164). Reconstitution of normal TCR repertoire appears to be delayed in patients who have posttransplant complications such as graft-versus-host disease (GVHD) (162) and in patients who have mixed hematopoietic chimerism rather than complete donor hematopoiesis (158).

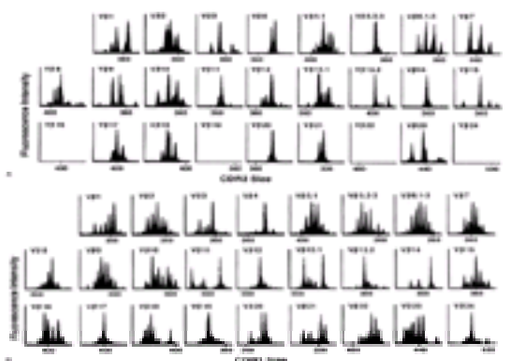

Figure 81.1. T-cell receptor Vb spectratype. CDR3 regions are amplified by reverse transcription/polymerase chain reaction (RT-PCR) from peripheral blood T cells by using 26 different Vb family primers and a single constant region primer. The PCR-amplified product is separated on a DNA sequencing gel and analyzed by using GeneScan software. **A:** CDR3 spectratype is derived from peripheral blood T cells obtained from a single patient 3 months after allogeneic stem cell transplantation. **B:** Spectratype is derived from the same patient 1 year after transplant and demonstrates marked improvement, with most patterns showing a normal polyclonal CDR3 profile.

Rearrangement of the TCRa gene during normal thymic differentiation results in the excision of a well-defined fragment containing the TCRd gene. As shown schematically in Fig. 81.2, this excised genomic DNA becomes circularized and is retained within the maturing T cell as a stable episomal fragment. This episomal fragment, which has been termed a TCR excision circle (TREC), is neither degraded nor replicated with subsequent cell division (165,166). The presence of TRECs is therefore a unique marker of a cell that has undergone TCR gene rearrangement and has been used to identify those T cells that have recently emigrated from the thymus. Quantitative PCR assays for TRECs have been developed and used to measure thymic output of new T cells (165). In normal individuals, TREC levels are high in cord blood and remain relatively high in peripheral blood T cells for the first two decades of life (167). TREC levels subsequently decline, but TRECs remain detectable in peripheral blood throughout adult life (168). This observation provides evidence for continued T-cell neogenesis in adults despite thymic involution (169). In patients who have undergone allogeneic stem cell transplantation for congenital immune deficiency, TREC levels increase rapidly after transplantation, indicating successful generation of new donor T cells (170,171). In adult patients, TREC levels remain low for at least 3 months after allogeneic BMT, and in many cases, TRECs are not detectable. As shown in Fig. 81.3, TREC levels begin to recover by 6 months after BMT and achieve normal levels by 1 year after BMT (172). This demonstrates that T-cell neogenesis from hematopoietic donor stem cells recovers in adult individuals, but the recovery period is prolonged. Adult patients usually remain susceptible to opportunistic infections for at least 1 year after BMT, and, in part, this is due to the slow reconstitution of T-cell neogenesis. This results in a markedly abnormal and deficient T-cell repertoire and inability to respond effectively to many external antigens.

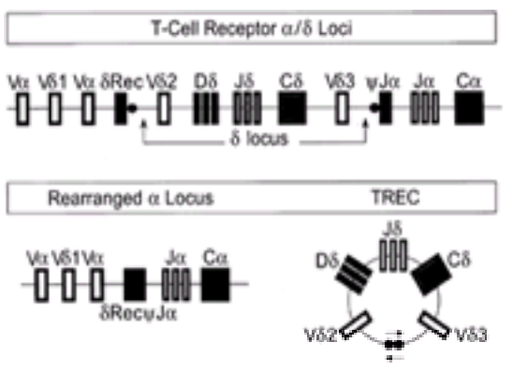

Figure 81.2. Derivation of T-cell receptor excision circles (TRECs) during rearrangement of the T-cell receptor a gene. (See Color Figure 81.2.)

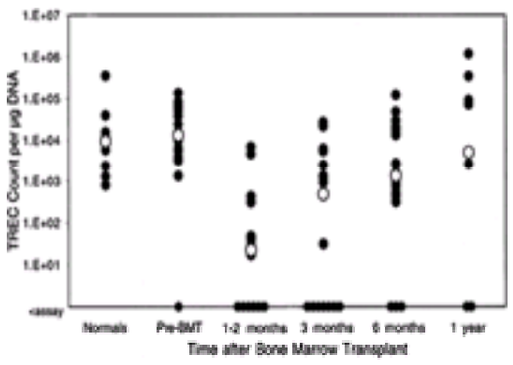

Figure 81.3. Quantitative assessment of T-cell receptor excision circles (TRECs) in peripheral blood lymphocytes during the first year after allogeneic stem cell transplantation in adult patients. The number of TREC copies/µg RNA were determined by quantitative reverse transcription/polymerase chain reaction. Patients received myeloablative conditioning followed by infusion of T cell–depleted bone marrow from human leukocyte antigen (HLA)-matched siblings. Peripheral blood samples for TREC analysis were obtained at various times after stem cell infusion and results compared with age-matched normal donors. *Solid circles* represent values for individual patient samples. *Open circles* represent median values for all samples at each time point.

After hematopoietic engraftment of donor stem cells, acute and chronic GVHD represent the most common immunologic complications of allogeneic BMT. Although a variety of host factors influence the clinical manifestations and severity of these reactions, both acute and chronic GVHD are mediated primarily by donor T cells. In the context of this potentially severe immunologic reaction, GVHD is associated with a variety of defects of T-cell function. For example, $CD4^+$ helper T-cell number and function are decreased, and the number of activated (DR expressing) $CD4^+$ and $CD8^+$ T cells is increased (173,174 and 175). In addition, patients with GVHD have a reduced capacity to produce new $CD4^+$ CD45RA T cells (176,177). Because patients with severe GVHD are always treated with immune-suppressive agents, it is difficult to determine whether the functional impairment of T cells is due primarily to GVHD or to immune suppressive therapy. Nevertheless, patients with GVHD represent a population at very high risk for opportunistic infections, and uncontrolled infections represent the most common cause of death in these individuals (178).

Because T cells are the primary mediators of GVHD, depletion of mature T cells from the donor marrow is an effective strategy for prevention of GVHD after allogeneic BMT. Several different methods have been developed for *ex vivo* depletion of T cells (179,180). These methods vary greatly in the extent to which mature $CD3^+$ cells are depleted and in the extent to which other cell populations are affected. In patients who receive T cell–depleted marrow, the incidence of GVHD is relatively low, and few patients are required to also receive immune-suppressive medications for GHVD prophylaxis. Nevertheless, reconstitution of T-cell immunity is delayed in these individuals because it is primarily dependent on the generation of new T cells from hematopoietic progenitor cells rather than from the transfer of functionally mature T cells from the donor (149,181). Mature donor T cells also play an important role in eliminating residual recipient leukemia cells after BMT. This effect has been termed “graft versus leukemia” (GVL), and patients who receive T cell–depleted donor marrow also have decreased GVL and increased risk of leukemia relapse after BMT (182,183). Recent studies have suggested that infusion of donor T cells after BMT can restore this GVL effect, but the ability of donor T-cell infusion to restore T-cell immunity against opportunistic infections has not been examined (184,185,186,187,188 and 189).

In addition to deficiencies in the number and diversity of mature T cells, previous studies have also demonstrated substantial functional abnormalities in peripheral T cells after myeloablative therapy. These functional deficiencies include impaired responsiveness to mitogens, soluble antigens, and allogeneic cells (149,190). These functional defects have been attributed to defects in various intracellular signaling pathways as well as to the impaired secretion and ability to respond to exogenous cytokines such as interleukin (IL)-2 (191,192,193 and 194). These functional cellular deficiencies have also been observed in recipients of autologous stem cells, although the extent and duration of deficiency is less pronounced than that in recipients of allogeneic stem cells (161,190,195). These functional abnormalities persist for prolonged periods but generally resolve gradually and often recover to normal levels by 12 to 18 months after transplant.

IMMUNOSUPPRESSIVE DRUGS

Because T cells are important effectors of the immune response, many therapies have been designed to suppress T-cell function in patients with immune overreactivity. Immunosuppressive drugs are commonly used in clinical practice after allogeneic bone marrow and solid organ transplantation to prevent GVHD or graft rejection, respectively. Immunosuppressive agents also are used extensively in rheumatologic and autoimmune disease, and in the treatment of various hematologic malignancies. In all of these clinical situations, the use of these drugs is associated with impaired T-cell immunity and increased susceptibility to a variety of pathogenic agents. In general, the degree of immune suppression is dependent on the duration of therapy and the specific agent that is used, as well as on the underlying disease. Moreover, although the effects of immunosuppressive agents on T-cell function are discussed in this section, these drugs often also affect other cellular systems as well, such as B cells, monocytes, and phagocytes. These other cellular effects increase the level of immune deficiency and also affect the susceptibility to opportunistic infections.

CORTICOSTEROIDS

Corticosteroids (e.g., prednisone, methylprednisolone, dexamethasone) are among the most commonly used immunosuppressive agents. At very high doses, these drugs are directly lympholytic. They also inhibit the immune system by directly blocking the activation of lymphocytes and macrophages. With corticosteroid use, early events in the induction of antigen-specific T-cell stimulation are not inhibited, but subsequent events leading to transcription and production of lymphokines are blocked (196,197 and 198). Thus corticosteroids effectively inhibit IL-2 production, with subsequent inhibition of T-cell proliferation and expansion of effector CTLs.

Corticosteroids also inhibit production of lipid mediators of inflammation by macrophages, such as leukotrienes and prostaglandins (199,200). Macrophage production of cytokines, tumor necrosis factor (TNF), IL-1 and chemokines, and nitric oxide also is inhibited (201,202 and 203). These effects on macrophages are the probable cause of the lack of fever in patients with pyogenic infections taking steroids. Other effects of corticosteroids on lymphocytes and macrophages include alteration in trafficking and availability of these cells for recruitment into sites of infection or delayed-type hypersensitivity (DTH) skin tests (204,205 and 206).

CYCLOSPORINE AND TACROLIMUS

Although dissimilar in chemical structure, both cyclosporine and FK506 (tacrolimus) block calcium-dependent events in lymphocyte signaling by forming a ternary complex with one or more specific binding proteins, and the calcium-dependent phosphatase calcineurin B (207). This results in blockade of T-cell activation, inhibition of IL-2 production and other lymphokines, and thus inhibition of T-cell proliferation and expansion of CTLs (208). There is also inhibition of T-cell interactions with B cells, and thus decreased antibody production in response to antigen stimulation (209). The prolonged use of cyclosporine and tacrolimus in solid organ and marrow transplant recipients has been associated with an increased risk of EBV lymphoproliferative disease (210,211,212 and 213).

ANTITHYMOCYTE GLOBULIN AND ANTI–T-CELL MONOCLONAL ANTIBODIES

T cell–specific antibodies such as OKT3 (anti-CD3), antithymocyte globulin (ATG), and Zenapax (daclizumab) are used extensively in solid organ transplant recipients as well as in the treatment of GVHD after allogeneic stem cell transplantation (214,215,216,217 and 218). OKT3 is a murine monoclonal antibody, whereas ATG is a polyclonal antibody prepared in sheep, horses, or rabbits immunized with human T cells or thymus. Daclizumab is a humanized IgG1 monoclonal antibody, which binds to the a chain of the IL-2 receptor (CD25) (219). Humanized monoclonal antibodies specific for other antigens expressed on T cells such as CD2 (220) and CD52 (221,222 and 223) also are undergoing evaluation as immune-suppressive agents in clinical trials. Preparations of polyclonal antibodies vary in their potency and selectivity for T cells, and their antigenic specificity is not well defined or controlled (180). Monoclonal antibodies have the advantage of having well-defined specificity, but the target antigens can be expressed on various cell types. Thus OKT3 has very specific reactivity for CD3-positive T cells, but antibodies targeting CD2 also are reactive with undifferentiated T cells in the thymus and NK cells, which express this antigen. Antibodies targeting CD52 effectively deplete T cells *in vivo* but also target many other hematopoietic cells that express this common adhesion molecule. In contrast to broadly reactive anti–T-cell antibodies, daclizumab is reactive only with activated T cells and NK cells that express high-affinity IL-2 receptors.

The immunosuppressive activities of anti–T-cell antibodies are, in large part, dependent on their ability to deplete T cells *in vivo*. These reagents generally bind to circulating T cells rapidly after intravenous administration and subsequently also bind to T cells in lymph nodes and tissues. Binding of antibody to the T-cell surface results in complement activation and deposition of complement components on the target cell membrane. To varying degrees, target cells are depleted by complement-mediated cell lysis, opsonization, and antibody-dependent cellular cytotoxicity (ADCC). Monoclonal antibodies also are capable of direct functional effects on T cells mediated by the specific target antigen. Thus OKT3 also can inhibit T-cell activation by dysregulation of the TCR/CD3 complex, which renders T cells anergic to subsequent activation by specific antigens (224). Similarly, antibodies to adhesion molecules or cytokine receptors also may exhibit functional effects through

inhibition of cell trafficking or cytokine signaling.

FLUDARABINE

Fludarabine is a newly developed nucleoside analog used in the treatment of CLL, which has been proven to provide effective therapy for this disease (225,226). Patients who receive fludarabine are known to develop low levels of CD4 T cells and are at increased risk for development of opportunistic infections (227,228). Low CD4 counts often persist for months after completion of fludarabine therapy (228). The mechanism whereby fludarabine induces prolonged immune deficiency is not entirely known, but recent studies have demonstrated that levels of cellular STAT1 protein become markedly depleted after *in vitro* exposure to fludarabine (229). T cells from patients receiving fludarabine also have low levels of STAT1. Depletion of STAT1 protein appears to be secondary to reduced expression of STAT1 mRNA, but it is not known how fludarabine induces this effect. STAT1 is an important cellular mediator of the interferon (IFN) signaling pathway, and mice deficient in STAT1 have defects in T cell–mediated immunity similar to that observed in mice deficient in IFN-g. This functional defect also is similar to that observed in patients receiving fludarabine, and this may be responsible for at least some of the impaired T-cell function in these patients. Other nucleoside analogs such as pentostatin also are associated with impaired T-cell function (230,231), but it is not known whether these other agents have similar effects on cellular STAT1.

TOTAL LYMPHOID IRRADIATION

Total lymphoid irradiation (TLI) has been used in Hodgkin disease, as well as in solid organ transplants. Its immunosuppressive effect results from its ability to reduce circulating T and B cells by 50%, with a similar reduction in lymphocyte proliferative responses *in vitro* (232,233). Circulating levels of lymphocytes are reduced during the first 6 to 12 months after treatment, and return to baseline after 2 to 3 years (233). Patients subjected to TLI are at increased risk of CMV and varicella infection (234).

OTHER CHEMOTHERAPEUTIC AGENTS

Cytotoxic agents such as azathioprine, cyclophosphamide, chlorambucil, and methotrexate inhibit the development of primary antigen-specific responses by inhibiting DNA synthesis and by blocking the proliferation of cycling cells (235).

HODGKIN DISEASE

Patients with early-stage Hodgkin disease often demonstrate impaired T-cell proliferative responses to mitogen stimulation *in vitro* (236). Patients with more extensive disease who have otherwise good performance status frequently exhibit impaired delayed hypersensitivity with negative skin tests against multiple antigens (e.g., tuberculin, coccidioidin, histoplasmin, and mumps antigen), defective signaling pathways, and abnormal ADCC (237,238,239 and 240). Patients with advanced Hodgkin disease often exhibit generalized depletion of tissue lymphocytes and typically demonstrate lymphopenia and a depressed CD4/CD8 ratio (241). Radiotherapy for Hodgkin disease also results in depletion of T-helper cells, resulting in increased incidence of herpes zoster infection during the first and second years after completion of radiotherapy (242,243). Untreated patients generally have normal antibody responses, but this can change after treatment with chemotherapy and radiation. The most common infections in this population are characterized by granuloma formation (*Mycobacterium tuberculosis*) and by persistence within macrophages and monocytes (*Listeria monocytogenes* and salmonella). There is a correlation between measured abnormalities and the predisposition to infections such as varicella-zoster or *L. monocytogenes* (244,245).

VIRAL INFECTION

HIV infection is a prototypic example of an infection that selectively targets T cells. HIV infection leads to the preferential depletion of memory $CD4^+$ $CD45RO^+$ T cells, which are more likely than naive T cells to be engaged by antigen (246,247,248 and 249). $CD8^+$ T cells undergo an initial peripheral expansion followed by gradual loss of memory $CD8^+$ cells. As viral infection progresses, the T-cell repertoires of both $CD4^+$ and $CD8^+$ T cells undergo shrinkage (250). Recent studies have shown that infection with HIV is associated with impaired generation of naive T cells in the thymus as well as memory T cells, resulting in a markedly deficient T-cell repertoire (251). This effect is reversible after the institution of effective antiviral therapy (165).

Diagnostic Evaluation of Acquired T-Cell Deficiency

The evaluation of suspected T-cell deficiency begins with the immunophenotypic analysis of peripheral blood lymphocytes by flow cytometry. Established methods to measure relative and absolute numbers of cells present in various T-cell subsets are based on phenotypic assessment using monoclonal antibodies against surface antigens specific for each subset. These include measuring the total number of $CD3^+$ cells as well as $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. Measurement of $CD4^+$CD45RO and $CD4^+$CD45RA subsets can be used to determine the number of memory versus naive T cells, respectively.

Assessment of T-cell function *in vitro* can often demonstrate deficiency even when numeric evaluation of T cells and T-cell subsets is normal or detects only minimal deviation from normal. These assays include evaluation of proliferation after TCR triggering with anti-CD3 monoclonal antibody or stimulation with mitogens [phytohemagglutinin (PHA)], soluble antigens, or allogeneic cells. T-cell secretion of IL-2, IFN-g, TNF, or other cytokines can also be measured after appropriate *in vitro* stimulation. The ability to generate a CTL response to allogeneic target cells can be measured with cytotoxicity assays after *in vitro* priming and coculture with the target cells.

More recently, several techniques have been developed to measure antigen-specific T cells accurately (252,253). Antigen-specific T cells are generally present at low frequency in peripheral blood but increase after *in vivo* exposure to antigen. Before antigen exposure *in vivo*, antigen-specific cells are found within the naive T-cell compartment. These rare cells reflect the frequency of T-cell precursors capable of responding to antigen presented by appropriate major histocompatibility complex (MHC) molecules. After antigen stimulation and selection *in vivo*, antigen-specific T cells are primarily found within the memory T-cell compartment. The ELISPOT assay quantifies the number of T cells that secrete INF-g after stimulation with a specific antigen or peptide (254,255 and 256). Individual responding T cells are identified as spots in methylcellulose plates coated with anti–IFN-g antibodies. Alternatively, responding T cells can be enumerated by flow-cytometric techniques that stain INF-g–secreting cells after *in vitro* stimulation with a defined antigen (257,258,259 and 260).

It also is possible to enumerate $CD8^+$ T cells with receptors for specific antigenic peptides (261). Because the TCR expressed by $CD8^+$ T cells only recognizes specific peptides presented by HLA class I molecules, these cells can be identified by molecular constructs consisting of soluble HLA class I molecule with specific peptide bound in the antigen-presenting cleft. These constructs are engineered as tetrameric complexes with fluorescent tags and can be used with flow-cytometric methods to quantify the number of T cells expressing peptide-specific TCR. Because TCRs recognize only HLA-bound peptide, definition of the HLA class I restricting element as well as the bound peptide is required before this method can be applied. Despite these requirements, this method is increasingly used to monitor specific $CD8^+$ T-cell responses *in vivo* (262,263,264 and 265). Although it has been technically more difficult to construct peptide/HLA class II tetramers, recent reports suggest that it will also be possible to apply similar methods to monitor specific $CD4^+$ T-cell responses (266).

Molecular techniques have been developed to measure directly the diversity of the T-cell repertoire *in vivo*. One method described previously examines T-cell repertoire by characterizing the spectrum of rearrangements of the TCR Vb genes. This technique, called CDR3 spectratyping, uses PCR to amplify the Vb gene rearrangement of the CDR3 region of the TCR (Fig. 81.1) (156,157). TCR spectratyping of T cells from normal donors demonstrates a highly diverse and polyclonal TCR repertoire with a typically gaussian distribution of CDR3 species of approximately eight sizes for each Vb region. In contrast, strong immune responses such as acute GVHD, solid organ transplant rejection, infection, and autoimmune diseases are associated with oligoclonal or monoclonal CDR3 patterns in peripheral blood as well as in the affected tissue (160,267,268,269,270,271,272,273,274,275 and 276). Patients with HIV infection have been found to normalize their T-cell repertoire after effective antiretroviral therapy (250,277). CDR3 spectratyping also has been used to characterize the T-cell response after donor lymphocyte infusion (DLI) (159,160,278). Patients with relapsed CML after allogeneic BMT were found to have markedly abnormal TCR repertoires, but T-cell repertoire complexity was restored after DLI. Moreover, the response to DLI was associated with the appearance of new clonal T-cell populations in peripheral blood. Taken together, these studies demonstrate that severe deficiencies in the T-cell repertoire can be reversible and that CDR3 analysis provides a sensitive method for characterization of the T-cell repertoire as well as for following changes in individual patients over time.

Another recently developed molecular technique is detection of TRECs (Fig. 81.2) (165,166). (See under section on thymic function.) The process of TCRa gene rearrangement, which is central to the differentiation of all T cells expressing conventional TCRab receptors, results in the generation of an episomal DNA fragment termed TREC. TRECs become stable intracellular episomes, which are neither degraded nor replicated with cellular division. TRECs are found in almost all maturing T cells within the thymus and at very high levels in cord blood T cells (167). Relatively high numbers of TRECs are found in peripheral blood T cells in the first two decades of life, and TREC levels subsequently decline with increasing age (167). Levels of TRECs within a mature T-cell population decline with ongoing cell division, and as a result, TREC levels are higher in naive T cells (168). These studies suggest that TRECs can be used as an accurate measure of thymic function and the general level of T-cell neogenesis in an individual. After effective therapy, TREC levels can increase and return to normal age-dependent levels (165,169). This

indicates that T-cell neogenesis is not a static process in adults and that the output of new T cells with diverse repertoire can improve with appropriate therapy.

Treatment of Acquired T-Cell Deficiency

PROPHYLACTIC THERAPY FOR OPPORTUNISTIC INFECTIONS

T-cell immunocompetence is required for protection against protozoan and viral infections. Prophylactic therapy is indicated in patients with documented and persistent T-cell deficiency. Patients with HIV infection and patients reconstituting after allogeneic stem cell transplantation are examples of the exclusive use of prophylactic therapy in which clear clinical benefit has been documented (279). In these patients, prophylactic therapy is directed at common causes of opportunistic infections. This most commonly includes prophylaxis for HSV and HZV with acyclovir and prophylaxis for *P. carinii* with trimethoprim/sulfamethoxazole. Alternative regimens are available for patients who are allergic to or unable to tolerate these medications. Prophylactic regimens for other viral infections such as CMV also are commonly used, especially in patients who harbor latent virus and are at high risk for development of active disease.

ADOPTIVE T-CELL IMMUNOTHERAPY

Over the past decade, the notion that T cells are critical to protection against viral infections has been validated by the demonstration that infusion of virus-specific T cells can effectively treat viral infection (280). Riddell et al. (281) administered CMV-specific $CD8^+$ T-cell clones to recipients of matched sibling stem cell grafts, and demonstrated that CMV-specific immune responses were reconstituted. In none of the treated patients did CMV disease develop. No adverse effects were observed from the adoptive transfer of these clones. Subsequently, Walter et al. (282) isolated virus-specific $CD8^+$ CTL clones from the blood of immunocompetent allogeneic stem cell donors, expanded them to large numbers *in vitro*, and transferred them into recipients early after transplant. Infused doses of 10^9 cells/m^2 were well tolerated and resulted in good restoration of CTL responses in the recipient. However, consistent with the notion that maintenance of $CD8^+$ T-cell immunity to chronic viral infections requires a concurrent $CD4^+$ T-helper response, they found that strong CTL responses persisted only in patients who also recovered $CD4^+$ CMV-specific T cells.

Other investigators have used adoptive T-cell immunotherapy to prevent and treat EBV-associated lymphoproliferative disease (EBV-LPD) (283). Bone marrow and solid organ transplant patients are at high risk for EBV-LPD, especially in the early posttransplant period when functional donor/recipient T cells are scarce. Risk factors for the development of posttransplant EBV-LPD include the use of marrow from a mismatched related or closely matched unrelated donor, T-cell depletion of donor marrow, and intensive immunosuppression (284). The incidence of EBV-LPD ranges from 5% to 25% in patients with these predisposing characteristics, and patients with EBV-LPD respond poorly to chemotherapy or IFN-a (285,286). Reduction in the degree of immunosuppression can induce regression of EBV-LPD, and this underscores the importance of immune competence for control of EBV infection.

Posttransplant EBV-LPD is a good model for evaluating the efficacy of adoptively transferred antigen-specific CTLs, because tumor cells express all the latent-cycle virus-encoded antigens, most of which are targets for virus-specific immune responses [e.g., EBV-associated nuclear antigen (EBNA) 1, 2, 3a, 3b, 3c, LMP1, 2a, and 2b] (287,288). Moreover, most normal donors are immune to EBV and carry a high frequency of EBV-specific CTL precursors (289). The use of T-cell infusions for EBV-LPD was first reported by Papadopoulos et al. (290,291), in which single infusions of donor T cells at doses of 0.35 to 1.0 × 10^6 $CD3^+$ cells/kg induced clinical remissions in five allogeneic marrow transplant recipients. The therapeutic effect was attributed to the reconstitution of EBV-specific immunity by repletion of CTL precursors present in the donor lymphocyte infusion. They observed that responses occurred within 2 weeks after infusion and that there was shrinking of lymph nodes 3 to 4 weeks after cell infusion. Using genetically marked EBV-specific CTLs, other investigators have shown that these virus-specific effectors can persist in the recipient for at least 18 months and can expand *in vivo* in response to renewed challenge with EBV-infected cells (292,293). In one series, none of 26 patients who received prophylactic CTLs from allogeneic donors developed EBV-LPD, whereas patients who did not receive donor cells had a cumulative risk of 14%. More recently, Gustafsson et al. (294) monitored quantitative levels of EBV DNA in posttransplant patients, and showed that administration of prophylactic autologous EBV-specific CTLs can prevent the development of overt disease.

Although the first line of treatment for HIV infection is combination antiretroviral therapy (see Chapter 74), several lines of evidence indicate that $CD8^+$ cells are the primary effector cells in the host defenses against HIV (295,296,297,298 and 299), and thus adoptive transfer of HIV-specific T cells can be considered. Some investigators have demonstrated the ability to expand polyclonal or HIV-specific CTL clones *in vitro*, and the safety of infusion of the expanded T cells (299,300 and 301). Nevertheless, infused $CD8^+$ T cells appear to be short-lived and result in only transient responses because of the limited survival of CTLs *in vivo*. This observed effect may be due to inadequate T-helper cell activity (302).

Donor lymphocyte infusions have been shown to be especially efficacious for treatment of some hematologic malignancies after allogeneic BMT (187,188,303). In patients with relapsed CML after allogeneic BMT, remissions can be achieved in up to 80% of patients with unfractionated or CD8-depleted donor lymphocyte infusions (184,185 and 186). T cells mediate this antileukemia effect, but it is unknown whether the donor T cells are directed against leukemia-associated antigens or against minor histocompatibility antigens (mHAg) (304). In patients in whom this has been analyzed in detail, the immune response initiated by infusion of mature donor T cells appears to be a coordinated response involving both B- and T-cell immunity to a variety of antigens (278,305,306,307,308,309 and 310). Thus both leukemia-associated antigens and mHAg may be targets of this immune response. In addition to initiating an antitumor response, an important observation in several studies has been that donor T-cell infusion also leads to consistent improvement in broad measures of T-cell function. This includes improvement in the generation of new T cells from the thymus measured by TREC assays (172) as well as improvement in the complexity of the T-cell repertoire measured by spectratyping (159,160,278). The mechanism for this reversal of T-cell immune deficiency is not known. In particular, it is not known whether this is a direct effect of donor T-cell infusion or whether the improvement in T-cell function is secondary to the elimination of leukemia cells that may have had an immune suppressive effect *in vivo*.

INTERLEUKIN-7

Although the thymus undergoes progressive involution in adults, recent studies have demonstrated that the generation of new T cells persists throughout adult life in normal individuals. Currently, this can best be measured by quantitative TREC assay, and several studies suggest that depressed levels of thymic function and T-cell neogenesis can improve with appropriate therapy. Stimulating thymic generation of new T cells may be a novel method for improving T-cell function *in vivo* and for enhancing the ability of an individual to respond to a variety of antigenic stimuli. As a result, there has been renewed interest in administration of cytokines or hormones to augment thymic function. Examples include epidermal growth factors, which can stimulate proliferation of thymic epithelium or enhance thymopoiesis before thymic involution [i.e., growth hormone (GH), insulin-like growth factor (IGF)-1]. Alternatively, cytokines such as IL-7, which play an important role in early B- and T-cell development, may also provide a mechanism for enhancing thymic function in adults (311). Several murine models of BMT have demonstrated that exogenous IL-7 can enhance thymopoiesis and accelerate immune reconstitution (312,313). In murine models, IL-7 has been shown to augment the generation of antiviral CTLs (314,315). Thus far, however, clinical trials of IL-7 have not yet been initiated.

ACQUIRED COMPROMISE OF B-CELL IMMUNITY

Manifestations

The most important functions of B cells are to generate antibodies and to act as antigen-presenting cells (APCs) that can assist in T cell–mediated immune responses. Antibody responses are essential for cure and prevention of many bacterial and viral infections, and immunodeficiencies related to B cells primarily result in inadequate production of specific antibodies. Immunoglobulins and complement each have associated opsonic, lytic, and neutralizing activities and function predominantly against bacterial infections. Thus patients with impaired B-cell function tend to have recurrent and chronic infections. In contrast to patients with T-cell deficiency, these infections are most often due to encapsulated bacteria, but other organisms such as enteroviruses and *Giardia lamblia* also can cause significant disease in these individuals.

As discussed in other chapters, T cells play an important role in the regulation of B cells and in the generation of appropriate antibody responses to many antigens. Patients with significant T-cell deficiency will therefore often also have deficiencies in humoral immunity. There are, however, several well-documented instances in which patients primarily acquire significant deficiencies of B-cell function, and T-cell function remains largely intact.

Etiology

HEMATOLOGIC MALIGNANCIES

Deficiencies in antibody production have been described in patients with CLL and plasma cell dyscrasias (multiple myeloma, Waldenström macroglobulinemia) (316,317 and 318). These deficiencies of antibody production can be clinically significant, and patients with these diseases frequently have recurrent severe bacterial infections. These infections are often due to polysaccharide-encapsulated pathogens such as *Streptococcus pneumoniae*, *Haemophilus influenzae,* or *Neisseria*

species. In patients with progressive CLL or multiple myeloma, infections are a major cause of death.

Patients with CLL have an intrinsic defect in B-cell function, which leads to unbalanced immunoglobulin chain synthesis and hypogammaglobulinemia (317). This persistent and often profound hypogammaglobulinemia was first recognized in the 1940s and 1950s (319). The likelihood of developing hypogammaglobulinemia correlates with the duration of disease and is as high as 70% by 7 years after diagnosis (320,321). This immune defect is not reversed by therapy with conventional agents, despite a concomitant hematologic response in the primary disease process (322). It is estimated that infectious complications will develop in up to 80% of patients with CLL, ranging in severity from moderate to life threatening, and infection accounts for up to 60% of deaths in these patients (317). The incidence of infection correlates with the duration and stage of the disease, as well as serum levels of immunoglobulins (particularly IgG) (323). Serious infections are most often due to encapsulated bacteria, but infections caused by staphylococci and enteric gram-negative bacilli also occur (317). The risk of infection increases with administration of corticosteroids and cytotoxic drugs. Other immune defects in this patient population include alteration in the normal ratio between T4 and T8 cells, especially with advanced disease (324).

Multiple myeloma has been associated with suppressed antibody responses by nonmalignant B cells. This defect is, at least in part, related to decreased numbers of normal B cells. In addition, immune feedback mechanisms are triggered in response to the high levels of monoclonal immunoglobulin seen in almost all patients with myeloma (318). It has been proposed that increased catabolism of immunoglobulins in these patients also contributes to decreased levels of nonmalignant immunoglobulins (318). As a result of this acquired defect in B-cell number and function, patients with multiple myeloma are at increased risk of developing serious infections. Savage et al. (325) found a biphasic pattern of infection, with infections due to *S. pneumoniae* and *H. influenzae* occurring earlier in the course of disease (first 8 months of diagnosis) or in patients responsive to therapy. In contrast, gram-negative infections occurred more frequently in patients with refractory or advanced disease.

Patients with acute leukemia have been found to have lower levels of antibody to the core glycolipid of the Enterobacteriaceae than do noncancer patients, and their antibody levels decrease after intensive cytotoxic therapy (326). Cytotoxic chemotherapy, radiotherapy, and steroids all adversely affect B-cell as well as T-cell functions, and can result in diminished opsonizing activity, inadequate agglutination and lysis of bacteria, and deficient neutralization of bacterial toxins. Cytotoxic therapy also can blunt the humoral response to vaccine administration, but adequate responses have been documented for patients in remission receiving maintenance chemotherapy. Thymomas are associated with marked aberration of the immune response, most severely affecting humoral immunity (327). These patients may have antibody-mediated autoimmune diseases, such as myasthenia gravis (328), or recurrent infections (sinopulmonary infections due to encapsulated bacteria), and may have a profound depletion of B cells and serum immunoglobulins (329,330).

ALLOGENEIC BONE MARROW TRANSPLANTATION

Myeloablative therapy followed by allogeneic BMT results in a prolonged period of B-cell as well as T-cell deficiency. $CD20^+$ B cells are often entirely absent from the peripheral blood early after stem cell transplantation, but these cells return to normal levels by 1 to 2 months after BMT (151). The proliferative response of B cells to mitogenic stimulation returns to normal by 2 months after BMT and parallels the recovery of phenotypic B cells (149). Donor-derived antibody production can be detected as early as a few months after BMT. However, BMT recipients are more typically incapable of normal antibody production to capsular polysaccharide antigens of encapsulated respiratory bacteria for prolonged periods after BMT (331,332 and 333). Analysis of variable heavy-chain (V_H) genes present in circulating B lymphocytes early after transplantation demonstrate V_H use similar to that seen in fetal B lymphocytes, consistent with recapitulation of B-lymphocyte ontogeny (334). Moreover, there appears to be a recapitulation of the transient hypogammaglobulinemia of infancy. After stem cell transplantation, IgM recovery precedes IgG and IgA recovery. IgM recovery takes 9 to 12 months; IgG recovery starts at 9 months; and IgA levels return to normal by 2 to 3 years after BMT (335). Without replacement of immune globulin, transplant recipients have reduced IgG, IgA, and IgM during the first 6 months after BMT (333).

In the absence of specific immunosuppressive treatment, acute GVHD has little effect on the tempo of lymphoid reconstitution, as measured by absolute lymphocyte count. In contrast, patients with chronic GVHD demonstrate multiple immune deficits, such as the decreased capacity to develop an antigen-specific T-cell response and to produce specific antibodies, especially to polysaccharide antigens, while having increased incidence of autoantibodies (336). These effects are exacerbated by the use of immunosuppressive agents. In patients with chronic GVHD, immunizations with recall antigens (e.g., tetanus, diphtheria toxoids) or new antigens [e.g., keyhole limpet hemocyanin (KLH)] reveal sustained defects in antibody production, including decreased primary antibody production, decreased immunoglobulin switching, and decreased secondary antibody production (336). These patients also have prolonged inability to produce antibodies to polysaccharide antigens, which are the primary antigenic determinants of the encapsulated respiratory bacteria (337). Before routine administration of prophylactic antibiotics and IVIG after stem cell transplantation, patients with chronic GVHD showed an increased incidence of infections with encapsulated respiratory bacteria (178). With the routine administration of prophylactic antibiotics and IVIG, the incidence of infectious complications has decreased in these patients (338).

Diagnostic Evaluation of Acquired B-Cell Deficiency

Quantitative assessment of immunoglobulin production can be measured by direct measurement of immunoglobulin levels. Normal immunoglobulin levels vary with age, and laboratories should provide age-adjusted normal levels. Interpretation of results may be difficult because low levels of immunoglobulin do not always correlate with patients' ability to generate specific antibody responses against new antigens. Moreover, most people with low IgG subclass levels have clinically adequate antibody responses to defined antigens.

The number of circulating B cells can be determined by immunofluorescence assays using monoclonal antibodies specific for B-cell surface antigens such as CD20 and CD19. These monoclonal antibodies are often used in conjunction with monoclonal antibodies specific for k and l light chain to determine whether B cells are polyclonal or monoclonal. Although normal peripheral blood does not contain a large population of B cells (usually <10%), flow-cytometric analysis using B cell–specific monoclonal antibodies can be very useful in determining whether the number of circulating B cells is normal in patients with suspected immune deficiency.

Functional tests of humoral immune deficiency also include the evaluation of specific antibody responses after administration of vaccine *in vivo*. Specific antibody responses to tetanus toxoid, *H. influenzae* B toxoid conjugate vaccine, pneumococcal vaccine, and hepatitis B vaccine can be measured by enzyme-linked immunosorbent assay (ELISA). Another functional test is the measurement of *in vitro* immunoglobulin production in response to B-cell mitogens such as pokeweed mitogen. This is primarily a research tool, and is not routinely used in the clinical setting.

Treatment of Acquired B-Cell Deficiency

ACTIVE IMMUNIZATION

In patients with normal T-cell function, active immunization often provides an effective method for enhancing the B-cell response to specific antigens. This is most effective in patients with relatively mild B-cell deficiency and is less effective in patients with profound B-cell abnormalities and in patients with T-cell deficiency. Nevertheless, many patients do not receive adequate vaccination. For example, it was recently estimated that the pneumococcal vaccine has been given to only 21% of those individuals in whom vaccination is indicated (339,340 and 341). This was found despite evidence that there is increasing severity and incidence of pneumococcal infection and increasing frequency of penicillin-resistant pneumococcus. The pneumococcal vaccine contains 23 of the serotypes responsible for more than 88% of the pneumococcal bacteremias in the United States. However, this vaccine provides protection in only 61% of immunocompetent individuals and is less protective in immunocompromised patients. Nevertheless, pneumococcal vaccines are recommended for patients with multiple myeloma, nephrotic syndrome, immunosuppression due to BMT, HIV, and functional or anatomic asplenia due to Hodgkin disease or to other hematologic disorders (sickle cell anemia) (279,342). Other vaccines recommended for this patient population include the influenza virus vaccine as well as the vaccine for *H. influenzae*.

Although vaccination of high-risk patient populations reduces the risk of serious infection, intrinsic defects in humoral immunity limit the ability to develop adequate protective antibody responses. For example, patients with multiple myeloma typically generate an increase in antibody titers after vaccination. However, because preimmunization titers are frequently low, postimmunization titers often fail to reach protective levels (343). In addition, postimmunization antibody levels decrease rapidly, in part because of increased catabolism of immunoglobulins. Because these patients frequently receive repeated cycles of immunosuppressive treatment, maintenance of adequate antibody levels to polysaccharide antigens remains difficult despite vaccination. Nonetheless, pneumococcal vaccine is recommended for prevention of serious pneumococcal infections in these patients, and revaccination should be considered in patients with a rapid decline in pneumococcal antibody levels.

Likewise, patients with CLL should receive polyvalent pneumococcal vaccine, although its efficacy in this populations has not been demonstrated unequivocally. These patients also display a suboptimal response to vaccination, which may be related to impaired antibody production, as well as to defects in antigen presentation (344,345). A series of investigators have examined patients with CLL who received typhoid, diphtheria, influenza, and mumps vaccines. These investigators found that antibody responses were poor, whether the patients had normal immunoglobulin levels or were hypogammaglobulinemic.

After allogeneic stem cell transplantation, several studies have assessed the ability to produce specific antibodies in response to a variety of antigens (tetanus toxoid, diphtheria toxoid, poliovirus, hepatitis virus) (346,347 and 348). These studies have shown that patients generally have increased antibody levels when either the donor

or recipient is immunized before BMT (349). Maximal antibody responses are obtained when both donor and recipient are immunized (349). If patients are not immunized after BMT, antibody production is not maintained, and clinically significant antibody levels are no longer detectable 1 year after BMT (350).

Live attenuated viral (measles, mumps, rubella, oral poliovirus, yellow fever) or bacterial [bacille Calmette-Guérin (BCG)] vaccines should not be administered to patients who are immunosuppressed as a result of leukemia, lymphoma, advanced malignancy, symptomatic HIV infection, or who are receiving therapy with alkylating agents, antimetabolites, radiation, or large doses of corticosteroids. The Centers for Disease Control (CDC) recommends that patients with leukemia who are in remission and more than 3 months after completion of therapy may be given live virus vaccines. In BMT recipients, administration of measles/mumps/rubella (MMR) vaccines is safe and efficacious for patients in remission 2 years after transplantation who do not have active GVHD (279).

PASSIVE IMMUNIZATION

Passive immunization involves the administration of preformed antibodies with the goal of replacing qualitatively or quantitatively defective immunoglobulins. Various studies have examined the use of a variety of antibody preparations, including pooled immunoglobulins, immunoglobulins collected from individuals with high titers of specific antibodies (hyperimmune sera), and monoclonal antibodies.

In patients with hypogammaglobulinemia not associated with malignancy or cytotoxic treatment, administration of pooled immunoglobulins has been effective in preventing infections due to encapsulated bacteria and in the treatment of certain enteroviral infections (351,352,353 and 354). In patients with malignancy, this benefit is not as clear (355). In patients with plasma cell dyscrasias, immunoglobulin supplementation is thought to be of little help, given the increased clearance of antibody (356). In CLL, prophylaxis with IVIG has been shown to decrease but not to eliminate respiratory infections in high-risk patients. In a randomized placebo-controlled multicenter study, the course of 81 CLL patients who were either hypogammaglobulinemic or had a history of infection was monitored (357). Those that received immunoglobulin replacement (400 mg/m^2/kg every 3 weeks) had significantly fewer bacterial infections (23 infections in 41 patients) than did those who received placebo (42 infections in 40 patients). In particular, infections caused by *S. pneumoniae* and *H. influenzae* but not gram-negative organisms were less common in the patients who received immunoglobulin. In addition, patients who received IVIG remained free of serious bacterial infections for a longer period than did control patients, but the benefit was limited to moderate or minor infections and not to a decrease in major or life-threatening infections. There was no difference in the incidence of viral or fungal infections in the two groups. Because of the high cost of therapy (358) and its limited ability to prevent serious bacterial infections, monthly IVIG should be considered only in patients with hypogammaglobulinemia who experience repeated bacterial infections. A potential limitation of prophylactic IVIG therapy is that although IgG is replaced, the most common immunoglobulin deficiencies present in CLL are often either IgM or IgA, which are not replaced by this therapy.

After allogeneic stem cell transplantation, bacterial, fungal, and viral infections (especially CMV) are a major cause of mortality and morbidity. Patients who are at highest risk for CMV disease are those individuals who are CMV seropositive before transplant or who have received marrow from a CMV-seropositive donor. In this group, the rates of CMV infection approach 70% after transplant in some studies (359). Thus IVIG has been evaluated as a source of passive immunity against CMV, bacterial and fungal infections, as well as an immunomodulator to decrease the development of acute GVHD. Numerous studies have been conducted to study the efficacy of IVIG or CMV hyperimmune globulin in preventing posttransplant complications from CMV infections, other infections, and GVHD (360,361 and 362). Nevertheless, the results of these studies have not been conclusive, because there are substantial differences in patient characteristics, the chemotherapy and radiation therapy regimens, GVHD prophylaxis regimens, supportive care procedures, and differing antibody titers of IVIG products that were used. Most studies have shown benefit in CMV-seronegative recipients undergoing BMT (362). CMV hyperimmune globulin has been shown to be effective prophylaxis against symptomatic CMV infection in seronegative recipients undergoing renal transplantation (363). In BMT patients, CMV hyperimmune sera and IVIG have been used in conjunction with ganciclovir in the prevention and treatment of CMV-associated disease (364,365). In general, combined approaches using passive immunization with specific antiviral drug therapy have shown greater efficacy in selected studies (366). Because IVIG remains a very expensive therapy, the use of IVIG should be reevaluated as newer and more effective antiviral agents become available for clinical use.

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82 AGING AND THE IMMUNE SYSTEM

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“The concept of age as a factor determining serologic response is comparatively new in immunologic thought.” Leona Baugartner, 1934 (1)

Biologic aging is the consequence of time on living organisms. In general terms, an organism changes constantly throughout life. *Development* refers to that constant change starting when biologic function increases and becomes more adaptive, and ending with *senescence*, when biologic function decreases and becomes less adaptive. Thus senescence is one part of development. During senescence, physiologic variance increases as homeostatic reserve diminishes. This process may be aptly termed *homeostenosis*.

Immune senescence is associated with a greater frequency of infectious and neoplastic diseases. Epidemiologic research suggests that infectious disease ranks eighth among the most common causes of all deaths in the United States but rises to rank fourth in persons older than 65 years. Cancer incidence is known to increase exponentially after age 30 years. Cigarette smoking offers probably the most important avoidable risk of cancer. However, aging appears to be an even greater risk factor. The extent to which immune senescence and the decline in “immune surveillance” contribute to the age-associated increase in cancer is debated. However, most agree that age-associated changes in the immune system, such as the appearance of monoclonal immunoglobulins and clonal lymphocyte expansions, usually precede the appearance of lymphoid neoplasms including chronic lymphocytic leukemia, multiple myeloma, and lymphoma.

Thymic involution is the most striking change associated with aging and has been known for more than 100 years. This gross anatomic change in the thymus gland was recognized long before its immune function was discovered. The principal function of the thymus gland is now recognized to be the production of a naive and diverse population of T lymphocytes. As the production of naive T lymphocytes decreases, cell-mediated and humoral immunity that depends on T lymphocytes declines. The production of B lymphocytes by the bone marrow in mammals also is reported to decline with age. It appears that the rate at which the bone marrow produces B-lymphocyte precursors is regulated by $CD8^+$ T lymphocytes. Thus an age-associated decline in the production of both T and B lymphocytes may be expected to occur as a result of thymic involution. It is generally believed that thymic involution is the pacesetter of immune senescence.

Because of the gradual involution of the central organs of lymphocyte development, immune senescence does not lead to the type of immune deficiency associated with congenital absence of T or B lymphocytes. This is not surprising, as the lymphocyte repertoire is well established before the onset of thymic involution. Thus immune senescence is associated with neither a decline in the number of blood lymphocytes nor the concentration of serum immunoglobulins. Interestingly, some cytokines produced by T lymphocytes increase, while other decrease with age. Interleukin (IL)-2 production by T lymphocytes decreases with age although T-lymphocyte production of other cytokines, such as IL-4 and IL-6, increases with age. This is discussed further later. Immune senescence is more precisely characterized by shifts in the number, distribution, and activity of lymphocyte subsets, antibody specificities, and cytokines with age. For this reason, immune senescence is best described as a process leading to a state of immune dysregulation.

CELL-MEDIATED IMMUNITY

Effect of Age on Thymocyte Development and T-Lymphocyte Development

The involution of the thymus begins at the time of sexual maturity and is completed by midlife, about age 50 years in humans, although a striking decrease in thymocyte size can be seen associated with illness (2). The decreasing mass of the thymus reflects a progressive decline in the number of thymocytes, leading to a decrease in overall production of T cells. Hormone release also influences the mass of the thymus (3,4). Thus, elevated thymic involution is reported to be slowed by thyroid hormone levels or depressed sex hormone levels. Factors intrinsic to the immune system that have been reported to contribute to thymic involution include decreased migration of thymocyte precursors from the bone marrow to the thymus, decreased entry of precursors into the thymus, and decreased differentiation of precursors within the thymus. Surprisingly, it is reported that the number of the thymocytes in early development is comparable in old and young individuals. It is concluded that the production of precursors and their entry into the thymus are not rate limiting. However, the number of thymocytes that have rearranged their T-cell antigen receptor (TCR) b-chain genes and that therefore are at a later stage in development declines progressively during aging. This suggests that impaired rearrangement of the TCR b-chain gene complex may underlie the age-associated decrease in thymocyte number associated with thymic involution. Consistent with this idea is the report that a mouse line, expressing rearranged a- and b-chain TCR transgenes, does not undergo thymic involution (3). However, we have found that this is not a general finding. Three other TCR transgenic mouse lines demonstrate normal thymic involution, suggesting that the expression of rearranged TCR transgenes is not sufficient, in itself, to prevent thymic involution (4). Decreased production of IL-7 also has been suggested to contribute to thymic involution. This cytokine is known to stimulate the proliferation of T-lymphocyte precursors within the thymus. Preliminary studies suggest that the repeated injections of IL-7 increase the number of T-lymphocyte precursors within the thymus of old mice (5), but whether this is the major factor that controls thymic involution is unknown.

Effect of Age on Peripheral T Lymphocytes

As discussed earlier, impaired thymocyte development during aging is associated with decreased production of T lymphocytes (6). Despite the decreasing number of T lymphocytes exported from the thymus during aging, the number of peripheral T lymphocytes does not decline. There appear to be two reasons for this: (a) long life span of T lymphocytes, and (b) their capacity for self-renewal. During early life, immune reactions to environmental antigens lead to clonal expansion of memory T cells, and a decrease in the number of naive T cells generated by the thymus. These cells represent the diverse T-cell repertoire. At all times of life, clonal expansion is followed by clonal regression. Early in life, naive lymphocytes from the thymus maintain the number and diversity of the T-lymphocyte repertoire after clonal regression. Later in life, fewer naive cells and more memory T cells are circulating. Clonal expansion and involution continues, but at a reduced rate.

As unstimulated lymphocytes are lost from the repertoire, lymphocytes undergoing clonal expansion or self-renewal take their place. Early in life, holes that develop in the T-cell repertoire as a result of clonal expansion and loss of cells are refilled by naive T cells produced by the thymus gland, However, such holes in the T-cell repertoire can be filled only as long as the thymus gland continues to export a diverse repertoire of naive T cells. Age-associated involution of the thymus results in a decreasing diversity of the peripheral T-lymphocyte repertoire. Two observations support this idea: (a) the greater age-associated depression in the primary as compared with the secondary immune response: these responses reflect the naive and memory lymphocytes, respectively; and (b) the greater restriction in the T-lymphocyte repertoire regenerated in old compared with young subjects after peripheral T-lymphocyte depletion that may be caused by radiation, chemotherapy, or human immunodeficiency virus (HIV) infection (7). The increased rate of infection and death of elderly individuals after peripheral T-cell depletion by HIV infection is attributable, at least in part, to the impaired rate and limited diversity of T-lymphocyte regeneration (8).

Extrathymic production of T lymphocytes occurs in the intestine. However, there is little evidence that extrathymic production of the g/d T lymphocytes that are produced in the intestine maintains the number or diversity of peripheral T lymphocytes during aging. The number of g/d T lymphocytes expressed as a percentage of blood T lymphocytes does not appear to vary with age, as would be expected if extrathymic production replaced thymic production of naive lymphocytes (9).

In addition to the age-associated decrease in the diversity of the T-lymphocyte repertoire, shifts in the relative number of T-lymphocyte subsets also result from the decreased production of T lymphocytes by the thymus during aging. There is a significant increase in the ratio of $CD4^+$ to $CD8^+$ T lymphocytes during aging (Table 82.1). In both subsets, the shift occurs in the ratio of naive to memory T lymphocytes, which express the CD45RA or CD45RO surface molecules, respectively (10).

	Young Donors	Aged Donor	Statistical Significance (paired *t* test)
CD4[a]	60.2 ± 2.9	73.4 ± 5.5	$p < .0001$
CD8[a]	38.7 ± 3.7	28.6 ± 1.7	$p < .0500$
CD4 + CD45RA[b]	60.3 ± 6.0	44.7 ± 7.3	$p < .0500$
CD4 + CD45RO[b]	46.4 ± 8.1	65.6 ± 8.3	$p < .0500$
CD8 + CD45RA[c]	73.0 ± 8.0	46.6 ± 4.0	$p < .0500$
CD8 + CD45RO[c]	23.2 ± 3.8	62.7 ± 7.2	$p < .0500$

Expression of cell-surface markers was measured by immunofluorescence and analyzed on a Coulter profile flow-cytometry system. Young donors were 20 to 32 years old: mean, 26 years. Aged donors were 55 to 85 years old: mean, 76 years. Six pairs of donor in each group were studied.
[a] Percentage of T cells.
[b] Percentage of CD4+ cells.
[c] Percentage of CD8+ cells.
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TABLE 82.1. $CD4^+$ and $CD8^+$ T-Cell Subsets in Peripheral Blood

The difference between memory and naive T cells requires further discussion. Early in life, the thymus exports a large number of naive T lymphocytes, and immunologic experience is limited. The percentage of naive T lymphocytes exceeds that of memory T lymphocytes. As time passes, exposure to antigens stimulates the activation of naive T lymphocytes and their transformation into memory T lymphocytes. This, combined with the age-associated decrease in the output of naive T lymphocytes from the thymus, leads to the reciprocal shifts in the percentage of naive and memory T lymphocytes. In elderly subjects, the percentage of memory T lymphocytes actually exceeds that of naive T lymphocytes (Table 82.1). Naive T lymphocytes respond poorly if at all to recall antigens. In contrast, a high percentage of memory T lymphocytes respond to specific recall antigens. Naive and memory T lymphocytes differ not only in the molecules required for their activation but also in the cytokines they produce. Naive T lymphocytes produce a T-helper (Th)1 profile of cytokines [e.g., IL-2, interferon (IFN)-g], whereas memory T lymphocytes produce a Th2 profile of cytokines (e.g., IL-4, IL-6). For this reason, the reciprocal change in the naive and memory T-lymphocyte subsets during aging not only limits the response to new antigens but also alters cytokine-dependent immune responses (11). Age-associated alterations in T-lymphocyte expression of CD28 and CD40L also compromise antibody responses to foreign antigens (12). A subset of $CD4^+CD28^-$ T cells has been defined in aging individuals that has been postulated to play a role in the poor proliferative response seen *in vitro*. An abnormality in activity of a nuclear transactivating factor has been suggested as the cause of the defect (13).

Effect of Age on Cell-Mediated Immunity *In Vivo*

The role of the thymus gland in the development of immune competence was demonstrated by neonatal thymectomy of experimental animals (14). Neonatally thymectomized animals are markedly impaired in cell-mediated and T-cell–dependent humoral immunity. If thymectomy is delayed by a month, until after the peripheral T-cell repertoire is established, defects in cell-mediated immunity develop only after a long interval and are less striking. This observation partially explains the long interval between the involution of the thymus at puberty and the appearance of impaired cell-mediated immunity during the second half of life.

The cutaneous delayed-type hypersensitivity (DTH) reaction is a classic manifestation of cell-mediated immunity and the first to be shown to decline during aging (15). Virtually all young adults express DTH to many common environmental antigens. In the elderly, DTH reactions are elicited by few antigens, and the intensity of the DTH reaction is diminished. The loss of DTH with age appears to be yet another risk factor that contributes to shortened survival (16). However, such tests of DTH might be attributable to differences in the interval between sensitization to an environmental antigen and the testing of the DTH reaction in old and young humans. To control for this difference, young and old humans were sensitized to a novel antigen, nitrochlorobenzene, and the DTH reactivity tested after the same interval in all individuals. The results of these better-controlled experiments confirmed an age-associated defect in the DTH response (15). In addition to environmental antigens, infectious agents induce DTH reactions. An age-associated decline in DTH to *Mycobacterium tuberculosis* (MTb) or varicella-zoster virus (VZV) antigens also is observed.

Infection with MTb or VZV usually induces protective immunity that reduces the number of infectious organisms but rarely eliminates organisms from the body. Viable MTb and VZV remain latent for decades in the lungs or nervous system, respectively. As immune senescence develops and the DTH reaction declines, reactivation of tuberculosis or VZV as “shingles” occurs in elderly adults. Thus cell-mediated immunity plays an important role in preventing the dissemination of these microorganisms that results in the reactivation of disease.

As discussed earlier, infection is a major problem in the elderly. Influenza is a classic example. Immune protection from influenza depends on both humoral and cell-mediated immunity to the virus. The T lymphocyte–dependent antibody response to the influenza-specific hemagglutinin (HA) is essential to prevent influenza infection. During aging, the antibody response to influenza HA after infection or vaccination declines, and the virus is cleared more slowly from the circulation (17). The increased severity of influenza in the elderly reflects the decreased generation of influenza-specific cytotoxic T lymphocytes required for the clearance of influenza-infected cells and recovery from the disease (18). Thus age-associated impairment in T-lymphocyte function contributes to the increased susceptibility to and severity of influenza in the elderly.

Effect of Age on the Proliferation of T Lymphocytes *In Vitro*

Age-associated impairment in cell-mediated immunity observed *in vivo* appears to be more complex than that which can be explained by depletion of naive cells and expansion of the memory cell populations. Studies have demonstrated multiple defects in the lymphocytes from aged subjects. It is possible to separate the effects of the “aged” environment on T-lymphocyte function from defects intrinsic to the T lymphocytes and/or antigen-presenting cells by measuring their function *in vitro*, where the effect of the aged environment is eliminated. Initially, *in vitro* studies focused on the proliferation of T lymphocytes in culture, as cell-mediated immunity depends on lymphocyte clonal expansion *in vivo* (19). In virtually all studies, an age-associated decline in the proliferation response of T lymphocytes cultured with mitogens is noted in humans and experimental animals. This proliferative defect is intrinsic to T cells, as the capacity of antigen-presenting cells does not change with age (20).

During a normal life span, T lymphocytes make proliferative responses to the large number of antigens in the environment. Cell division is accompanied by a decreasing length of telomeres, the terminal portions of chromosomes (21). The average length of telomeres on chromosomes in T lymphocytes decreases with age. In addition, at every age, the length of telomeres in memory T lymphocytes is shorter than that in naive T lymphocytes, as antigen-driven proliferation is associated with the transition of naive into memory T lymphocytes. At some point in the proliferative history of the cell, the length of the telomeres becomes too short to permit further division. The causal relationship between telomere length and proliferative capacity was demonstrated in experiments in which the gene coding for the enzyme telomerase, which maintains telomere length, was transfected into fibroblasts, cells with a finite proliferative capacity. Transfected fibroblasts maintained their telomere length and had an increased proliferative capacity compared with untransfected fibroblasts (22).

The reactivation of tuberculosis in the elderly is correlated with a decline in MTb-specific DTH *in vivo* and decreased proliferation of T lymphocytes cultured with MTb antigens. DTH reactivity is essential to contain viable MTb within granulomas formed by monocytes. The granuloma reaction is highly dependent on the production by T lymphocytes of IFN-g and the response of monocytes to this cytokine. Based on *in vitro* studies, the T-lymphocyte production of and response of monocytes to IFN-g have been reported to become impaired during aging, contributing to disease exacerbation (23). An age-associated defect in the proliferative response of T lymphocytes cultured with VZV antigens also has been demonstrated and correlated with decreasing DTH reaction to VZV. The defect in T-lymphocyte proliferation from elderly subjects cultured with VZV antigens is correlated with a low frequency of VZV-responsive T lymphocytes (24). Interestingly, the low frequency of VZV-specific T lymphocytes in elderly humans can be boosted by immunization with an attenuated VZV. Current clinical studies will determine whether immunizing elderly subjects with the live, attenuated, OKA strain of VZV will decrease the frequency of shingles and postherpetic neuralgia in elderly adults by increasing cell-mediated immunity to VZV antigens.

A large body of evidence suggests that T lymphocytes from aged individuals also have defects in signal transduction after interaction of mitogenic molecules with their receptors on the surface of the T cells. This defect contributes to their impaired proliferative response (25). Many age-associated defects in early cytoplasmic steps in T-lymphocyte activation, such as the mobilization of free intracellular calcium and phosphorylation of proteins necessary for gene activation, have been documented. Among the many genes whose activation is necessary for proliferation are protooncogenes. These genes play an important role in the entry of T cells into the cell cycle before cell division. The c-*myc* protooncogene encodes a DNA-binding protein that is essential for the transit of T lymphocytes from the G_0 to G_1 phase of the cell cycle. The steady-state level of c-*myc* mRNA is lower in phytohemagglutinin (PHA)-activated T cells from aged as compared with those from young persons (26). The expression of another protooncogene, c-*jun*, detectable within 30 minutes of T-lymphocyte activation by PHA, which encodes another DNA-binding protein, AP-1 (27), is defective in the lymphocytes of aged subjects. Because the IL-2 enhancer contains an AP-1 binding site, a decline in the expression of AP-1 may play a critical role in the age-associated decline in the secretion of IL-2 by T lymphocytes, as discussed earlier.

Similar abnormalities in the induction of cyclin-dependent kinases important in cell division and in the kinase activity of Raf-1 have been discredited (28,29). Pathways that lead to JNK activation are similarly reported to be age sensitive (30). Some suggest that a defect in Fas-mediated apoptosis is responsible for the expansion in memory T cells.

Aging also is associated not only with the decreased production of IL-2 by activated T lymphocytes, as has already been discussed, but there also is a decreased response to exogenous IL-2 in culture (31). The decreased response to IL-2 of activated T lymphocytes from old donors (32) is due to their failure to express high-affinity IL-2 receptors (IL-2R). The high affinity IL-2R is made up of a three-protein complex. The IL-2R a and b chains bind IL-2 with low or intermediate affinity, respectively. A third, IL-2R g chain does not bind IL-2 but interacts with the other two chains to form a high-affinity complex. The a and b chains of the IL-2R are expressed similarly on activated lymphocytes from old and young donors. However, only 50% of the activated T lymphocytes from elderly donors express high-affinity IL-2R, presumably because the expression or activity of the IL-2R g chain is altered during aging (32).

Not all cytokines produced by activated T lymphocytes in culture decrease with age. There is an age-associated increase in the secretion of Th2 cytokines, IL-4 and IL-6, by activated T lymphocytes in culture (11). As a generalization, it appears that aging is associated with a shift in the production by activated T cells from cytokines with a Th1 profile (signature cytokine, IL-2) seen in the first half of the life span to a Th2 profile (signature cytokine, IL-4) seen in the second half of the life span. This shift in cytokine profile also is observed *in vivo* (Table 82.2) (33). It is known that Th1 and Th2 cytokines can stimulate greater production of their own class of cytokines and inhibit the production of the other class of cytokines. Administration of IL-2 to old mice reversed the age-associated shift in cytokine expression by T lymphocytes. Thus the ratio of IL-2–expressing to IL-4–expressing T lymphocytes is increased 1.5-fold in IL-2–treated mice compared with 0.7-fold in untreated old mice (Table 82.2). A further abnormality is an increase in IL-10 production *in vitro* by lymphocytes of aging individuals after PHA stimulation (34).

Animals	IL-2 Expressing (%)	IL-4 Expressing (%
Young	6.1	5.2
Old	2.0	3.1
Old IL-2 treated	12.2	8.1

[a] Percentage of IL-2 or IL-4 expressing CD25+ T lymphocytes ex vivo. CD25+ T lymphocytes were solated by using magnetic beads and permeabilized. The percentage of permeabilized CD25+ T lymphocytes expressing intracellular cytokine was determined by flow cytometry (29).

TABLE 82.2. Percentage of IL-2 or IL-4 Expressing T Lymphocytes Activated *In Vivo*[a]

In part this shift in cytokine production reflects the age-associated shift from naive to memory T lymphocytes. Both memory and naive T lymphocytes produce IL-2, but memory T lymphocytes are the predominant producers of IL-4 and IL-6. This shift in cytokine secretion may contribute to the altered susceptibility of the elderly to certain infectious diseases and the increasing level of serum Ig. It is possible that it contributes to the increasing frequency of B lymphocytes secreting immunoglobulin (Ig), of monoclonal serum Ig, and autoantibodies, as well as the benign and malignant clonal B-lymphocyte expansions that occur during aging (35).

HUMORAL IMMUNITY

Effect of Age on the Development of B Lymphocytes

As discussed under B-cell development, pro-B cells and other cells in this lineage are produced in the bone marrow. In contrast to the thymus, which is entirely given over to cells of T lineage, B-lineage cells make up only a small proportion of bone marrow cells. The great majority of bone marrow cells are hematopoietic precursors, whose production continues throughout life. This makes it difficult to appreciate a decline or “involution” of B-lineage cells by gross or microscopic examination. However, the study of bone marrow cells by using flow cytometry and antibodies specific for the various stages of B-lymphocyte development has permitted the effect of age on bone marrow B-cell development to be determined. With these tools, we now appreciate that the number of bone marrow B-cell precursors decreases with age. This involution of the bone marrow lymphoid compartment begins after thymic involution (35).

Pre-B lymphocytes are the most numerous of the B-lineage cells in the bone marrow, and their number decreases by 60% to 90% during aging. Interestingly, the number of pro-B lymphocytes does not decrease with age; thus it appears that age-associated defects affect the transition of pro-B to pre-B lymphocytes. The generation of pre-B lymphocytes from pro-B lymphocytes depends on the expression of the pre-B receptor, made up of a three-protein complex: the Ig heavy chain, l5, and VpreB (see Chapter). When Ig rearrangement does not occur, as in RAG-deficient mice, no pre-B cells are produced (36). During aging, the rearrangement of the Ig heavy-chain genes in pro-B lymphocytes is impaired (37). Pre-B lymphocytes without the pre-B receptor are highly susceptible to apoptosis, explaining the increased percentage of apoptotic pre-B cell in the bone marrow. Despite the decreased rate of B-lymphocyte production, the number of peripheral B lymphocytes is maintained during aging in part by an age-associated increase in their life span and their capacity for self-renewal (38). However, when peripheral B lymphocytes are depleted, their regeneration is slower in old mice than in young mice (39), and diversity of the regenerated B-lymphocyte population decreases in elderly mice. The diversity of the B-cell repertoire depends on the generation of naive B lymphocytes in the bone marrow. Therefore the decreased diversity of the B-lymphocyte repertoire in old mice after their peripheral depletion suggests an impaired production of naive B lymphocytes by the bone marrow. In Fig. 82.1, it can be seen that a diverse B-cell repertoire before cyclophosphamide administration is regenerated in young but not in old mice. Furthermore, in young mice with B-cell populations of limited diversity before peripheral B-cell depletion, the B-cell population that is regenerated is more diverse than that originally present, suggesting the generation of naive cells. In contrast, in old mice, the B-cell population of limited diversity does not gain greater diversity after peripheral depletion. These findings are consistent with an age-associated decrease in the production of naive B lymphocytes by the bone marrow.

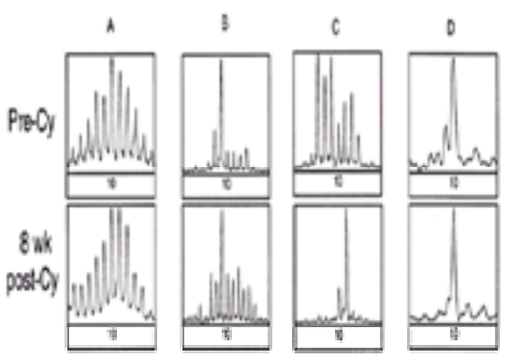

Figure 82.1. Effect of age on the diversity of the B-lymphocyte repertoire spleen cells were obtained from 2-month-old **(A, B)** and 18-month-old **(C, D)** BALB/c mice by partial splenectomy before 4 and 8 weeks after treatment with 200 mg cyclophosphamide/kg. The diversity of the B-cell repertoire with respect to immunoglobulin M CDR3 mRNA size shown before and 8 weeks after cyclophosphamide treatment.

Effect of Age on the Peripheral B-Lymphocyte Subsets

B lymphocytes are divided into two subsets based on the surface expression of the CD5 molecule. Most B lymphocytes do not express surface CD5, and in mice, are generated in the bone marrow. These “conventional” B lymphocytes are the principal source of antibody to foreign antigens. CD5-positive B lymphocytes are generated in the peritoneal cavity of mice. The repertoire of the population of $CD5^+$ B lymphocytes includes many antibody molecules that have not undergone somatic rearrangement and are in the germ-line configuration. The antibody is skewed toward the production of autoantibodies. The percentage of $CD5^+$ B lymphocytes increases during aging, reflecting the continued production of $CD5^+$ B lymphocytes in the peritoneal cavity at a time when the production of $CD5^-$ B lymphocytes in the bone marrow is decreasing (40). This shifting balance of $CD5^-$ to $CD5^+$ B lymphocytes appears to be the cellular basis for the age-associated shift in the specificity of antibodies from foreign to autologous antigens.

Old and young mice were immunized with trinitrophenylated keyhole limpet hemocyanin (TNP-KLH), an antigen that stimulates $CD5^-$ B lymphocytes, or TNP-Ficoll, an

antigen that stimulates $CD5^+$ B lymphocytes. Young mice produced comparable numbers of splenic anti-TNP plaque-forming cells (PFCs) when immunized with TNP conjugated to either carrier. In contrast, old mice produced few anti-TNP PFCs after immunization with TNP-KLH than with TNP-Ficoll. This finding suggests that aging compromises the response of $CD5^-$ but not $CD5^+$ B lymphocytes to foreign antigens. Thus, a greater antibody response to a foreign antigen in older individuals may be achieved by targeting the available $CD5^+$ B lymphocytes.

Effect of Age on Natural and Induced Antibodies to Foreign Antigens

The first report suggesting an effect of age on the immune system was published more than 70 years ago by Thomsen and Kettel (42). These investigators reported that the titer of natural antibodies in human serum to the A and B erythrocyte antigens (isoagglutinins) declines with age. Forty years later, the decline in natural antibody specific for a foreign antigen, in this case, salmonella flagellin, was confirmed (43). It is now recognized that the antibody response of elderly humans to virtually all vaccines, including encephalitis, hepatitis, influenza, and tetanus toxoid, is less than that observed in young individuals (44). In contrast, the magnitude of the antibody response to most T-independent antigens such as pneumococcal polysaccharides is maintained during aging. However, the duration of protective immunity achieved by pneumococcal vaccine is shorter in old compared with young humans (45). For this reason, humans older than 65 years should be revaccinated with pneumococcal vaccine every 5 to 8 years.

The effect of age on the antibody response to a foreign antigen was studied in greater detail in mice immunized with hapten-conjugated protein. Old mice immunized with nitrophenylated-bovine gamma globulin elicited a reduced PFC response with a preferential loss of high-affinity and IgG antibody–producing cells compared with the response of young mice (46). It is known that high-affinity antibody activates complement more efficiently than does low-affinity antibody, and it is interesting that (a) antiphosphorylcholine (PC) antibodies protect a higher percentage of mice from challenge with living pneumococci than do low-affinity anti-PC antibodies from old mice (47).

Production of high-affinity antibody has been demonstrated to occur within germinal centers (GCs). It is here that high-affinity antibodies are generated by somatic mutation, and IgG antibodies are formed after isotype switching. During the initial response to antigen, B cells with affinity for the introduced antigen migrate to GCs, where they undergo clonal expansion, somatic hypermutation, isotype switching, and subsequent selection. It is now recognized that these processes are all compromised in aging. Furthermore, aging is associated with a decrease in the number and size of GCs as a result of impaired Th-cell function (48). Transfer experiments have revealed that defects intrinsic to both B and T lymphocyte from old mice contribute to the decreased affinity maturation seen in aged animals. It is not known whether the loss of GCs during aging is the cause or the consequence of this process.

In humans, the level of all serum Ig except for IgD remains unchanged or increases with age. In mice, the level of IgM, IgG, and IgA increases with age, as does the number of splenic and bone marrow Ig-secreting B lymphocytes (49). If Ig specific for most foreign antigens decreases during aging, what are the specificities of the antibodies that maintain or increase the level of serum Ig?

In contrast to the decreasing production of most antibodies to foreign antigens, the level of autoantibodies increases with age. This age-associated shift in Ig specificities from foreign to autologous antigens was first reported 40 years ago when the level of serum anti–salmonella flagellin antibodies and autoantibodies specific for the nucleus were compared in humans of different ages (Fig. 82.2). It was observed that the titer of antibodies specific for the foreign antigen, salmonella flagellin, decreased with age, whereas the percentage of persons with antinuclear autoantibodies increased with age. Subsequent studies revealed that 60% of healthy elderly persons have autoantibodies specific for one or more of the following autoantigens: nucleoprotein, IgG, or thyroglobulin (50). With the exception of the oldest cohort studied, which had a lower level of autoantibodies than did the rest of the population older than 65 years, the percentage of individuals with autoantibodies increased with age (51). Of interest, centenarians also have lower levels of antithyroglobulin autoantibodies than do younger elderly humans. In contrast, organ-nonspecific autoantibodies increase with age, including the oldest age cohorts. These studies raise the possibility that organ-specific autoantibodies are related to chronic disease and are a factor for shortened survival.

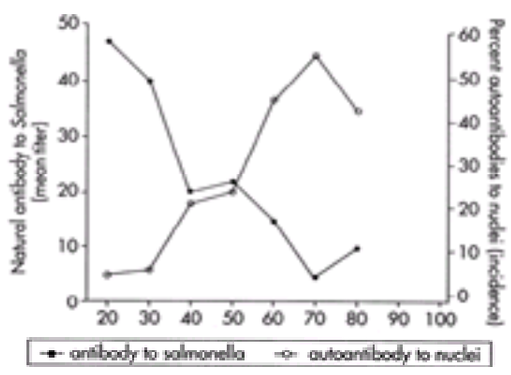

Figure 82.2. Age-associated cross-wiring of the immune system. Serum was obtained from individuals between ages 20 and 80 years. The titer of natural antibody to salmonella flagellin and the percentage of individuals with antinuclear antibody were measured. (Adapted from Rowley MJ, Buchanan H, Mackay JR. Reciprocal change with age in antibody to extrinsic and intrinsic antigens. *Lancet* 1968;2:24, with permission.)

In mice, four factors have been suggested to contribute to the age-associated increase in autoantibodies: (a) an increase in the frequency of autoantibody-producing B lymphocytes detected by limiting dilution analysis (52); (b) polyclonal activation of B lymphocytes in old mice, possibly stimulated by increased IL-4 and/or IL-6. There are 5 to 10 times as many splenic B lymphocytes secreting Ig in unimmunized old compared with young mice (41); (c) age-associated decline in intrathymic negative selection of autoreactive T cells (53); and (d) cell-transfer experiments suggest that the aged environment stimulates autoantibody production (54). Thus more autoantibodies are generated in old compared with young mice given syngeneic bone marrow cells.

The age-associated shift in antibody specificities from foreign to autologous antigens also is observed after immunization with a foreign antigen. Thus immunization of humans with tetanus toxoid stimulates a greater number of autoantibody-producing lymphocytes in the elderly compared with that in the young (55). Similarly, old mice immunized with sheep erythrocytes (SRBCs) produce fewer B lymphocytes secreting anti-SRBC antibodies but many times the number of B lymphocytes secreting antimouse RBC antibodies compared with these responses in young mice (56). The age-associated loss of the exquisite specificity of the immune response to the nominal antigen, we have termed “cross-wiring” of the immune response. Immunization of old mice with a foreign antigen activates more autoantibody-producing lymphocytes than lymphocytes producing antibodies to the nominal antigen. The cellular basis of the cross-wiring of the immune response during aging appears to be due to the decreasing ratio in the number and activity of conventional $CD5^-$ to $CD5^+$ B lymphocytes with age, as discussed earlier.

Although the serum concentration of autoantibodies increases with age, we found no change in the autoantibody repertoire. Thus comparison of the specificities of autoreactive Ig in serum from young and old mice or humans with lysates of normal tissues reveals the same pattern of binding (57). The binding profile of serum Ig to a large number of proteins from normal tissues that have been electrophoretically separated is unchanged during aging.

Despite the increased production of autoantibodies during aging, the incidence of autoimmune disease does not increase with age. Autoimmune diseases usually are seen for the first time during the middle third of the life span. Pernicious anemia and thyroiditis, which occur late in life, are exceptions to this generalization. Similarly in animals, the induction of experimental autoimmune disease is more difficult in old compared with young animals. Old animals are more resistant than young to the induction of autoimmune hemolytic anemia or arthritis by xenogeneic erythrocytes or type II collagen (58).

Among the organ-nonspecific autoantibodies that increase with age are antiidiotypic autoantibodies (59). These autoantibodies are of particular interest as they can suppress the immune response to foreign antigens by reacting with surface Ig on antigen-specific B lymphocytes and inhibiting their secretion of antibody to the foreign antigen. The antiidiotypic autoantibody response is a good example of the cross-wiring of the immune response: old mice produce greater quantities of antiidiotypic autoantibodies and lesser quantities of antibodies to foreign antigens. Thus the appearance of this class of autoantibodies not only reflects the state of immune dysregulation observed during aging but also may contribute to the immune deficiency associated with it.

Transfer studies in mice have been used to identify the cellular basis of the age-associated increase in the antiidiotypic autoantibody response (59). Long-lived splenic T lymphocytes but not bone marrow cells transplanted from old mice to young mice transferred the capacity to produce a strong antiidiotypic autoantibody. T lymphocytes from old mice stimulated antiidiotypic autoantibody production by B cells from young or old mice. Furthermore, depletion of peripheral T cells from old mice decreased the production of antiidiotypic autoantibodies and led to an increase in the production of antibodies to foreign antigen.

Studies in humans suggest a clinical impact of antiidiotypic autoantibody production (60). Elderly humans, previously immunized with tetanus toxoid, have a higher level

of serum antiidiotypic autoantibodies before boosting than do young individuals. The level of antiidiotypic autoantibodies before immunization of old and young individuals with tetanus toxoid is inversely correlated with the level of antitetanus antibodies that develops after immunization. Thus, the elderly subjects with the highest level of antiidiotypic autoantibody made the lowest antitetanus-antibody response. These results confirm the importance of age-associated antiidiotypic autoantibody in the impaired antibody response to vaccines in humans.

Clonal Lymphocyte Expansion during Aging

The immune response becomes less heterogeneous with respect to isotype, antigen-binding affinity, idiotype, and the size and sequence of the Ig heavy-chain variable region during aging (35). This is due, in part, to the decreased production of naive lymphocytes by the thymus and bone marrow and the decreased diversification of the naive B lymphocytes as a result of decreased somatic mutation and isotype switching within the GCs. As the primary sites of lymphocyte production lose their capacity to export a naive repertoire of diverse specificity, the memory lymphocyte population maintains the total number of lymphocytes by peripheral renewal and clonal expansion. The age-associated increase in the incidence of serum monoclonal Igs presumably reflects the increasing frequency of B-cell clonal expansion during aging (35). Direct evidence for age-associated clonal B- and T-lymphocyte expansion has been obtained by cloning and sequencing studies of the CDR3 portion of the mRNA for the TCR and BCR in both humans and mice.

It had been recognized for a long time that clonal lymphocyte expansion is often followed by lymphocyte malignancies. Each year, in 1% of humans with monoclonal serum Ig, multiple myeloma develops. Lymphoma and chronic lymphocytic leukemia also occur late in life after the expansion of clonal B or T lymphocytes. The links between age-associated malignancies and clonal lymphocyte expansions, which precede them, include the expression of the $CD5^+$ surface marker on nearly all expanded B-cell clones and chronic lymphocytic leukemia cells. Furthermore, 50% of chronic lymphocytic leukemic cells produce autoantibodies, as do spontaneously activated B cells in elderly humans and experimental animals. It remains to be proved by “molecular fingerprinting” that the lymphocyte malignancies are derived from the clonal lymphocyte expansions. Studies are now under way to establish whether the nucleic acid sequence of the variable region of the BCR expressed by expanded clonal lymphocytes also is expressed by the lymphocyte malignancies that occur later in life.

Enhancing Protective Immunity in the Elderly

Understanding the mechanisms underlying immune senescence leads to suggestions for ways to maximize the immune response to vaccines of the elderly. First, foreign antigens might be targeted to $CD5^+$ B lymphocytes, whose function is better maintained during aging than is that of $CD5^-$ B lymphocytes. Second, antigens may stimulate a better immune response if delivered to lymphoid compartments whose function is less compromised during aging. The production of antibodies specific for a foreign antigen by mucosal lymphocytes has been reported to be better maintained during aging than that by splenic and lymph node B cells (61). This raises the possibility that antigen presented to mucosal surfaces of the respiratory, digestive, or urinary tract may induce better protective immunity than can be achieved by intramuscular or subcutaneous immunization.

Another factor contributing to the poor response of elderly to conventional vaccines is that higher doses of antigen may be required to achieve a maximal immune response in old compared with young subjects. Live recombinant viral vaccines that express a relevant antigen may offer advantages. Viral proliferation increases the quantity of foreign antigen to the level that best stimulates each recipient, old or young. We have demonstrated the proof of this principle in mice (62). Young mice immunized with a nonproliferating viral vaccine expressing the PR-8 influenza virus hemagglutinin (HA) produced a high level of anti-HA antibodies and anti-HA cytotoxic T lymphocytes (CTLs) and had complete protection from influenza challenge. In contrast, old mice made modest immune responses that protected only 50% of them from influenza challenge. However, immunization with a live recombinant vaccinia vaccine expressing the influenza HA stimulated both old and young mice to produce high antibody and CTL responses to HA, and the mice were completely protected from influenza challenge. In summary, improved protective immunity might be achieved in older humans if antigen dose, composition, and route of antigen delivery were altered to target lymphocytes and the lymphoid compartments where activity is best maintained during aging. Finally, the use of living, attenuated or recombinant vaccines may offer distinct advantages in inducing or maintaining protective immunity in the elderly. In this regard, we await with great interest the results of ongoing studies using the attenuated VZV, now used in children to prevent chickenpox, to maintain immunity to this virus and thus decrease the reactivation of the latent virus in the elderly, leading to shingles and postherpetic neuralgia.

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83 HEMATOPOIETIC STEM CELL TRANSPLANTATION

Michael B. Maris, M.D., and Rainer Storb, M.D.

Hematopoietic stem cell transplantation (HSCT) is an effective and increasingly used modality for the cure of both malignant and nonmalignant diseases. For patients with malignant disorders, much effort has been dedicated over the last 30 years in optimizing the doses of radiation and chemotherapy (referred to as conditioning) used to maximize tumor cell kill and minimize regimen related toxicity (1,2,3 and 4). For those patients with nonmalignant diseases (Table 83.1) such as severe aplastic anemia (SAA) and severe combined immunodeficiency disease (SCID), the goal has been to optimize host immunosuppression for facilitating donor engraftment (5,6,7,8,9 and 10). At the same time, voluntary marrow or infant cord blood registries have been set up to expand the pool of donors beyond immediate family members. The safety of HSCT continues to improve, as exemplified by the reduced incidence of mortality from opportunistic infections. Despite this, substantial challenges remain. In particular, regimen-related toxicity and graft-versus-host disease (GVHD) remain difficult problems for some patients. Older patients have more regimen-related toxicity and mortality due to the combined effects of high-dose chemoradiotherapy and GVHD, and therefore most transplant centers will not offer HSCT for patients older than 50 years (11). A number of groups have recently reported on a variety of nonmyeloablative HSCT programs that use attenuated doses of chemotherapy and/or radiation (12,13,14,15,16 and 17). Preliminary reports have described significant antitumor responses due to immunologically mediated allogeneic effects, otherwise known as the *graft-versus-leukemia* (GVL) effects. Nonmyeloablative HSCT is performed in some centers on an outpatient basis and permits the application of this procedure to elderly and infirm patients who otherwise would not tolerate a conventional HSCT (11).

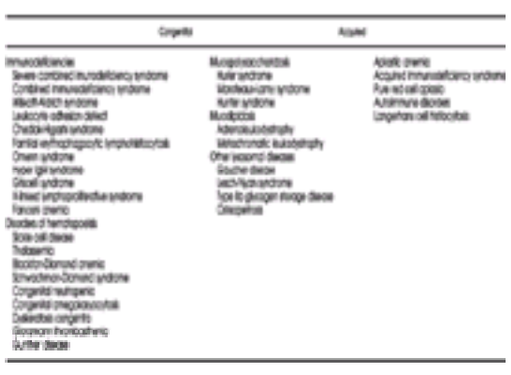

TABLE 83.1. Nonmalignant Diseases Corrected by Hematopoietic Stem Cell Transplantation

The changes occurring in the field of HSCT have been striking since the last edition of this book. As HSCT becomes more widely used and the list of medical indications grows, it will become more common to care for these patients in the general medical community. Therefore, it is prudent for general practitioners and medical subspecialists to have some familiarity with the indications and complications of HSCT. This chapter provides an overview of HSCT, including donor/recipient selection, transplantation techniques, indications, complications, and the future directions of HSCT.

SOURCE OF HEMATOPOIETIC STEM CELLS

An adequate number of stem cells for HSCT can be obtained from the bone marrow, the peripheral blood, or the umbilical cord of newborn infants. Bone marrow contains a high concentration of stem cell and has been the most common stem cell source. Stem cells circulate in the peripheral blood in low numbers, and their number can be greatly increased after exposure to chemotherapy, cytokines, and growth factors. These peripheral blood stem cells (PBSCs) are easily collected with standard apheresis techniques. The use of PBSCs is becoming more common primarily because it negates the risk of general anesthesia required for harvesting of bone marrow. The observation of large quantities of stem cells in the umbilical cord of newborn infants has led to the recent use of this product in allogeneic HSCT.

The stem cells can be transplanted in an autologous, syngeneic, or allogeneic manner. An *autologous* graft is collected from the patient in remission and transplanted at a later time. A *syngeneic* graft is from a monozygous twin who is identical at all genetic loci. *Allogeneic* grafts can be taken from nonsyngeneic siblings or alternative donors. Allogeneic donors who have been used with success include human leukocyte antigens (HLA)-identical siblings, HLA-mismatched relatives, and HLA-matched unrelated donors. Most allogeneic grafts come from genetically different siblings who are nonetheless genetically identical for the HLA coded for genes within the human major histocompatibility complex (MHC). An alternative donor refers to a family member who is genetically HLA identical at one haplotype and phenotypically identical at the other or mismatched for one or more HLA alleles. Acceptable sibling and family donors are found approximately 35% of the time. The identification of phenotypically identical *unrelated donors* is possible through large national and international registries of volunteers such as the National Marrow Donor Program (NMDP), and a donor can be found for approximately 50% to 60% of white patients. The underrepresentation of minorities has made the identification of donors difficult for ethnic groups other than whites. A Cord Blood Registry also is available for donation.

In vitro manipulation of the stem cell graft may be performed either to reduce the number of alloreactive T cells in allogeneic grafts or to reduce the risk of infusion of contaminating tumor cells in autologous grafts. Alloreactive T lymphocytes can be removed by a variety of techniques to reduce the risk of developing GVHD. These techniques are effective at preventing GVHD, but also may lead to increased risks of rejection and relapse that result in no net survival advantage. Often autologous grafts are treated *in vitro* with antibodies with or without complement or alkylating chemicals in attempts to purge the graft of tumor cells. The use of the alkylators perfosfamide and mafosfamide, which are similar to cyclophosphamide (CY) results in delayed myeloid and platelet engraftment; the efficacy of this treatment is yet to be clearly demonstrated in randomized controlled trials.

COLLECTION AND INFUSION OF HEMATOPOIETIC STEM CELLS

The three principal sources of HSCs that are commonly used include bone marrow, PBSCs, and umbilical cord blood (18). The harvesting of bone marrow is performed

while the donor is under anesthesia. Multiple punctures are made through the skin and the cortex of the iliac crest with the aspiration of less than 5 mL of marrow per puncture. This process is continued until 10 mL of marrow per kilogram of donor weight is removed. This will provide 1×10^8 to 8×10^8 nucleated cells per kilogram recipient body weight. The marrow is collected in a heparinized tissue-culture medium and is screened through wire mesh to remove tissue particles before infusion to the recipient.

The collection of PBSCs is usually performed at a local blood bank or the apheresis unit in the hospital. Filgrastim combinant human granulocyte colony-stimulating factor (rhG-CSF)] is the most commonly used agent to mobilize stem cells from the marrow into the peripheral blood. Other agents that can be used to mobilize stem cells include granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage stimulating factor (M-CSF), interleukin 3 (IL-3), and PIXY 321 (a fusion protein of GM-CSF and IL-3). Autologous mobilization typically uses chemotherapy with or without rhG-CSF. Interestingly, growth-factor administration can contribute to the mobilization of hematopoietic and epithelioid tumor cells into the peripheral blood (19). The typical dose of filgrastim is 10 to 16 µg/kg subcutaneously over a 4- to 5-day period. PBSCs are collected by using an apheresis machine. Nine to 12 L of blood can be processed over a 3-hour period. Two apheresis collections are usually performed on consecutive days. The stem cell yield of the PBSC collection is estimated from the number cells that express the cell-surface marker CD34. The $CD34^+$ cells represent both committed and noncommitted progenitor cells.

Life-threatening complications from bone marrow or PBSC collection are rare. Among more that 10,000 marrow harvests performed at our institution, there have been no deaths, although several deaths have been reported worldwide (20). These included one due to cardiac arrest, one to ventricular tachycardia, one to aspiration pneumonitis, and one to *Klebsiella* septicemia. More commonly, donors of bone marrow harvest will experience local pain at the harvest sites that will improve over several days to several weeks (21). Most donors who receive large doses of filgrastim for stem cell mobilization will have pain of hip, spine, and long bones due to proliferation of the myeloid marrow. This pain can be quite debilitating and difficult to control even with narcotic analgesics (22,23). Although G-CSF is generally safe, the long-term effects on hematopoiesis are unknown. The possibility of adverse effects on normal volunteer donors has prompted the NMDP to limit the donation of PBSCs to patients on experimental protocols and to monitor donors periodically over several years for adverse outcomes.

PREPARATION OF THE RECIPIENT FOR TRANSPLANTATION

Role of the Major Histocompatibility Complex in Human Stem Cell Transplantation

The HLA system is an integral portion of the immune system and is the primary barrier to successful HSCT. The HLA genes encode cell-surface molecules or *antigens*, whose function is to bind to T lymphocytes, resulting in immune responses (24). These genes are located in two clusters on the p21.3 band of the short arm of chromosome 6. They encode HLA class I (-A, -B, -C) and class II (-DR, -DQ, -DP) antigens. The genes are passed to siblings as a haplotype. The HLA haplotypes segregate in a classic mendelian fashion, creating a 25% chance of shared HLA identity of the major MHC antigens between siblings with the same parents.

The clinical success of allogeneic HSCT is directly related to the disparity of the HLA between donor and host. The host alloimmune response can cause the rejection of the graft. Conversely, the uncontrolled alloimmune response of the graft can cause life-threatening GVHD. The effector mechanisms that contribute to graft rejection and GVHD are T cells in the HLA-matched and -mismatched setting, whereas natural killer (NK) cells have been implicated in causing rejection in the HLA-mismatched setting (25,26 and 27). Improvements in the understanding of the HLA system and improved donor selection at the molecular level may reduce the complications and improve the results of allogeneic HSCT.

Human Leukocyte Antigen Typing: Serologic, Mixed Culture, and Molecular Techniques

Traditional methods for identifying the major HLA antigens have used serologic typing (24). The clinical importance of HLA antigen disparity has been shown to be important in HSCT outcomes such as overall survival, leukemia free survival, (Fig. 83.1) and engraftment. Mononuclear cells of the peripheral blood are incubated with panels of alloantisera consisting of anti-HLA antibodies. Lysis of cells is measured in a complement-mediated microcytotoxicity assay. A single epitope (private specificity) or multiple epitopes (public specificities) uniquely characterize the serologically defined HLA molecules. A cross-reactive group (*CREG*) refers to HLA antigens of the same subclass (e.g., HLA-A1), recognized by similar serology. Some CREG antigens are molecularly very similar and may differ by only a single amino acid substitution. The HLA-D antigens have traditionally been defined by cellular mixed-culture toxicity assay.

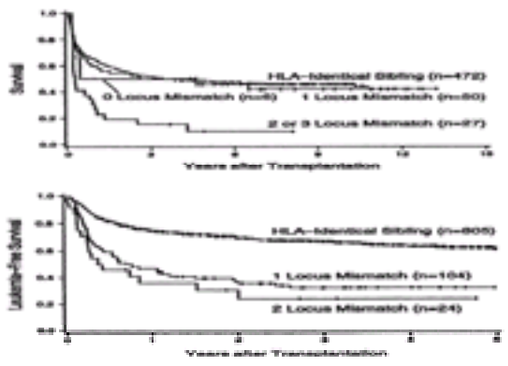

Figure 83.1. Effect of donor and recipient human leukocyte antigen (HLA) disparity on survival after marrow transplantation. Probability of survival in patients with chronic myeloid leukemia in the chronic phase, acute myeloid leukemia in first remission, or acute lymphoblastic leukemia in first or second remission, according to the degree of donor and recipient HLA disparity. Results from a single-center study. B: Probability of survival in patients with chronic myeloid leukemia in the chronic phase, acute myeloid leukemia in first remission, or acute lymphoblastic leukemia in first remission. Results from a study of the International Bone Marrow Transplant Registry. (A: reproduced from Anasetti C, Beatty PG, Storb R, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *Hum Immunol* 1990;29:47–91, with permission. B: Reproduced from Szydlo R, Goldman JM, Klein JP, et al. Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. *J Clin Oncol* 1997;15:1767–1777, with permission.)

More recently several groups have advanced to molecular typing of the genes that encode HLA antigens of the MHC (28,29,30,31 and 32). Studies have determined that serologically defined specificities are broad and may contain multiple alleles. These molecular techniques use polymerase chain reaction technology. The different techniques include sequence-specific primers (SSPs), sequence-specific oligonucleotide probe hybridization (SSOP), or direct sequencing. Most groups at this time perform at least molecular typing at HLA-DRB1 for all patients.

Significance of Human Leukocyte Antigen Disparity in Clinical Outcomes

Most alternative-donor and unrelated-donor HSCTs have been performed with serologic matching at the HLA-A, -B, and -DR. HLA differences even at the allelic level can effect HSCT outcomes as was conclusively shown in one patient with graft rejection (25) in whom a single amino acid difference in HLA B44 led to graft rejection of a donor marrow that was serologically identical to that of the recipient. In a series of retrospective studies, Petersdorf et al. (28,29,30,31 and 32) evaluated HLA disparity at the molecular level with clinical outcome in patients who have undergone HSCT for hematologic malignancies (28,29,30,31 and 32). In a study evaluating class I allele disparity in 21 patients who rejected an unrelated donor allograft, 71% were found to have allele mismatch at HLA-C alone or in combination with a one-allele HLA-A or -B mismatch (28). These combinations were identified in only 33% of controls. Multiple allele disparity at HLA-A and -B also was associated with graft failure. In a multivariable analysis, HLA-C mismatch was identified as an independent risk factor for graft rejection (28).

The importance of the class II system antigens HLA-DRB1 and -DRQ as being independent risk factors for the development of GVHD for recipients of unrelated-donor HSCT was identified in two retrospective studies (29,30). The first study evaluated DRB1 disparity in 364 patients that received grafts from HLA-A, -B, and -DR serologically matched unrelated-donor graft for hematologic malignancies. Disparities were identified by SSOP evaluation at the DRB1 alleles in 59 pairs. In multivariable analysis, patients with a one-allele DRB1 disparity were found to have increased risks of both GVHD and transplant-related mortality. The second study evaluated 449 recipients of HLA-A, -B, and -DR serologically matched unrelated-donor grafts for hematologic malignancies. Most of the donor/recipient pairs (75%) were matched at DRB1 and DQB1. The relative risks of grades III and IV GVHD were 0.42 for patients matched at both alleles, 0.55 for single-locus DRB1 mismatches, 0.61 for single-locus DRQ mismatches, and 0.71 for combined DRB1 and DQB1 mismatches. A similarly designed Japanese retrospective study found quite different results; class I HLA mismatching was highly correlated with GVHD and death, whereas no association with GVHD or mortality was demonstrated with class II mismatching. These differences are as yet unexplained, but the frequencies of the allele distribution between patients in the two studies are quite different (Petersdorf, personal communication). Nevertheless, both studies strongly suggest the biologic relevance of HLA class I and II alleles and the importance of allele-level

typing on outcome of HSCT.

Design of Conditioning Regimens

CONVENTIONAL CONDITIONING REGIMENS

The design and use of preparative regimens or *conditioning* must take into account the patient's underlying disease. In patients with nonmalignant diseases, the conditioning regimen's role is to provide host immunosuppression; in those transplanted for malignant diseases, the conditioning regimen serves the dual purpose of immunosuppression and the eradication of cancer. The underlying disease process has influenced the choice of the chemotherapy and/or radiation therapy used. Obviously, in nonmalignant disorders, no advantage exists to escalating the doses of chemotherapy and/or radiation therapy to those that are maximally tolerated. In some severe disorders of immune function, such as SCID or leukocyte functional antigen-1 (LFA-1) deficiency, the underlying dysfunction of host immunity may be so severe that the use of conditioning for host immunosuppression may not be required or may be relatively mild to obtain sustained engraftment (33). However, in other nonmalignant diseases of hematopoiesis (for example, AA or thalassemia), host immunosuppression is required to prevent graft rejection. Finally, in patients with hematologic malignancy, despite the intensity of the conditioning regimens, relapse of the underlying malignancy remains a problem (33).

Preparative regimens for SAA commonly use CY. A regimen developed in Seattle used CY at 50 mg per day on 4 consecutive days followed by stem cell infusion 36 hours after the last dose. Unfortunately, a high rate of graft rejection occurred in those patients previously transfused with unirradiated blood and, therefore, allosensitized to non-HLA antigens. Efforts to intensify the conditioning regimen to reduce the risk of graft rejection have included the addition of 3 to 10 gray (Gy) of total body irradiation (TBI) with or without lung shielding, thoracoabdominal irradiation (TAI), and total lymphoid irradiation (TLI) (33). Other chemotherapeutic agents also have been added to CY alone or CY and irradiation. The addition of viable buffy-coat infusions to the marrow grafts improved engraftment, but resulted in increased chronic GVHD (33). The successful use of antithymocyte globulin (ATG) with CY to improve host immunosuppression for second transplant after rejection of an initial allograft has resulted in ATG being incorporated in most current front-line SAA regimens (33).

In patients with genetically determined nonmalignant disorders (e.g., Wiskott-Aldrich, thalassemia major) who have normal or greater than normal bone marrow cellularity, CY (120 to 200 mg/kg) and busulfan (BU; 0.5 to 4.0 mg/kg/day for 4 days, taken orally) or dimethylbusulfan (DMBU; mg/kg intravenously) have been successfully used (34,35 and 36). Another approach has been to combine procarbazine and ATG with TBI to prepare patients with Wiskott-Aldrich syndrome (37).

Until recently, it was widely believed that patients with hematologic malignancies could be cured only with large doses of radiation and chemotherapy. Early studies in animal models demonstrated the feasibility of marrow grafting from one animal to another after the administration of myeloablative doses of TBI. Most early conditioning regimens included TBI because of the extensive preclinical experience using that modality. In principle, TBI is an effective technique for killing malignant cells; it penetrates into privileged sites such as the central nervous system (CNS) not accessible to chemotherapy because of the blood–brain barrier. Initial attempts using a single dose of TBI at 10 Gy as a single modality resulted in one of six patients with relapsed acute lymphoblastic leukemia (ALL) entering a durable remission. Thomas in 1977 (38) published a series of 100 patients demonstrating success with 10 Gy of single-dose TBI combined with CY. Because of the high incidence of interstitial pneumonia (IP) noted with single-dose TBI, investigations into fractionated schedules of TBI demonstrated efficacy with reduced incidence of IP (39).

Other groups evaluated the efficacy of chemotherapy-only regimens. Fatal cardiac toxicity was reported with very-high-dose CY (60 mg/kg/day for 4 days). Even with lower doses of CY, all patients who survived conditioning relapsed. BU (16 mg/kg) in combination with CY (200 mg/kg) was introduced by the Johns Hopkins group (40). The Ohio State transplant group and others have demonstrated the efficacy of the BuCy2 regimen, consisting of BU (16 mg/kg) with reduced-dose CY (60 mg/kg/day for 2 days) (41,42). Because of the variable metabolism and the association of therapeutic serum BU levels with prevention of leukemia relapse, some groups are now targeting BU doses to maintain therapeutic serum levels (43). Over the last decade, several groups have effectively combined etoposide with BU/CY or CY/TBI for patients with advanced hematologic malignancies to reduce the relapse rate (44,45 and 46).

NONMYELOABLATIVE CONDITIONING REGIMENS

The toxicities of conventional HSCT combined with the significant risk of relapse led many investigators to explore the use of less intensive conditioning regimens to reduce regimen-related toxicities and to rely primarily on the immunologically mediated graft-versus-tumor effect. The nonmyeloablative approaches taken by different groups can be divided into (a) reduced intensity of conventional cytotoxic regimens, and (b) pre- and posttransplantation immunosuppression to control both host and donor immune reactions, thereby preventing both rejection and controlling GVHD (17). In both approaches, the establishment of mixed or full donor chimerism provides a platform for further adoptive immunotherapy using donor lymphocyte infusions (DLIs) to eradicate residual malignant cells. Examples of investigators who used attenuated doses of conventional HSCT programs include the transplant teams of Boston, Houston, Bethesda, Geneva, and Jerusalem groups (12,13,14,15,16 and 17). The Seattle group has used a different approach based on preclinical studies in a random-bred dog model. The combination of mycophenolate mofetil and cyclosporine (CSP) was compared with other immunosuppressive medications at sublethal doses of radiation to determine if mixed hematopoietic chimerism could be obtained (47). With this combination of drugs, mixed-chimerism was obtained by using a nonmyeloablative dose of only 200 cGy of TBI, but all dogs rejected with CSP alone, and four of six dogs rejected with the combination of CSP and methotrexate (MTX) (47). With this model, the combination of mycophenolate mofetil and CSP was shown to be an effective synergistic postgrafting immunosuppressive at preventing GVHD and enhancing engraftment (47,48). The initial nonmyeloablative regimen used in Seattle for patients with HLA-matched sibling donors used pretransplant immunosuppression of 200 cGy delivered as a single fraction at 7 to 8 cGy per minute, and postgrafting immunosuppression with CSP, 6.25 mg/kg twice daily orally from day –3 to day 35, and mycophenolate mofetil, 15 mg/kg twice daily orally from day 0 to day 27 (49,50). PBSCs from HLA-identical sibling donors were infused on day 0. Donor/host chimerism in peripheral blood T cells was evaluated on days 28 and 56 by using either microsatellite markers or, for sex-mismatched donor/recipient pairs, fluorescence *in situ* hybridization of X and Y chromosomes. Recently, fludarabine, 30 mg/m^2, given on 3 consecutive days before transplant, has proven effective in reducing the small risk of rejection seen in the first 45 patients and improving the degree of donor chimerism after transplant (11).

SUPPORTIVE CARE AND OPPORTUNISTIC INFECTIONS DURING THE EARLY POSTGRAFTING PERIOD

The patient undergoing a conventional HSCT first has pancytopenia and then profound impairment of immune function due to transplant conditioning and underlying disease at the same time that primary host defenses, such as mucocutaneous integrity, are breached because of conditioning toxicity. Patients will maintain some residual immune function for approximately 2 to 7 weeks after transplant. After a conventional HSCT, the patient typically will be neutropenic for 7 to 24 days, with earlier engraftment being seen in recipients of PBSCs (Fig. 83.2) (51,52,53 and 54). After initial engraftment, severe immunologic impairment will last at least 4 months, and serious bacterial, fungal, and viral infections often occur (36,55,56,57 and 58). Immunologic impairment may persist for 6 to 12 months, and can last for years in those patients who have chronic GVHD. In general, for those patients undergoing an autologous HSCT or recipients of autologous or allogeneic PBSCs, the degree of immunologic impairment is not so profound, and immunologic recovery occurs more quickly (53,59,60).

Bacterial infections are the most common pathogens in the early granulocytopenic period. Approximately 5% of HSCT recipients may die of bacterial infections during this period. Most centers will use prophylactic broad-spectrum antibiotics at the onset of neutropenia. Fevers develop in almost all HSCT patients, but only in approximately 50% of cases will pathogens (usually bacterial) be isolated. Growth factors are not commonly used in allogeneic HSCT because they have not been demonstrated to improve outcome despite accelerated granulocytic recovery of ~6 days (61,62). High-dose intravenous immunoglobulin (IVIG) appeared to be effective in reducing the incidence of infectious complications in two randomized controlled trials but is not commonly used because of prohibitive cost (63). Laminar airflow rooms, to prevent exposure to bacterial and fungal pathogens, are no longer commonly used because of the expense, and improved efficacy could not be clearly demonstrated compared with that in rooms with filtered air (64). Not surprisingly, thorough hand washing is probably the most effective strategy to prevent nosocomial infections in HSCT patients (65). The earlier recovery of granulocytes and enhanced immune reconstitution associated with the use of PBSCs results in less regimen-related toxicity during the early posttransplant period (53,59,66).

Fungal infections continue to be a significant problem in HSCT. In approximately 10% to 20% of patients, an invasive fungal infection may develop, usually with an *Aspergillus* sp. (67). The mortality rate approaches 75% in HSCT patients with *Aspergillus* pneumonitis and is 100% for multiple organ or brain involvement (68). In the persistently febrile HSCT patient, aggressive evaluation and empiric treatment with antifungal antibiotics such as amphotericin B is warranted (67). New lysosomal preparations of amphotericin and new antifungal antibiotics show promise in being more effective and less toxic than standard amphotericin. The use of prophylactic fluconazole during the first 4 to 8 weeks after transplantation has been very effective at preventing systemic candidiasis and improving survival (67).

Viral infections are among the most important infections during the first 3 to 4 months after HSCT (55,69). Acyclovir is effective at preventing both the reactivation of the varicella-zoster virus and the herpes simplex virus 1 (the cause of the common cold sore). The reactivation or primary infection of cytomegalovirus (CMV) is the most important post-HSCT infection. Prior CMV infection and evidence of viral latency is demonstrated in 75% of individuals by detection of specific antibodies to CMV (55,70,71). The patient is typically asymptomatic during the early reactivation of CMV. Before the use of effective antiviral medications, the incidence of CMV pneumonitis was 15%, with a case fatality rate of 50% to 85% (55). Patients who are CMV seronegative before transplant and who receive a CMV-negative graft can be protected from primary CMV infection by the transfusion of CMV-seronegative blood products or blood products rendered leukocyte poor by filtration (72). Ganciclovir (dihydroxyethylethoxymethoguanine) combined with CMV immunoglobulin has demonstrated efficacy for established infections (73). High-dose ganciclovir results in reduction but not complete elimination of CMV infections and pneumonia in seropositive patients (74). Two randomized placebo-controlled studies have shown that ganciclovir can prevent fatal CMV pneumonias (73,75). Prophylaxis with ganciclovir in CMV-seropositive patients is effective while receiving therapy;

however, there appears to be an increased incidence of late CMV disease, possibly caused by delayed reconstitution of HLA-restricted cytotoxic T lymphocytes (CTLs) to CMV antigens (76). The major complication of ganciclovir therapy has been marrow suppression. Because of these concerns, a strategy used for CMV-seropositive patients after HSCT is to monitor for evidence of reactivation by searching for inclusion bodies in peripheral blood mononuclear cells (PBMCs) (76). Ganciclovir is then used at "induction doses" for 1 week, and the dose is decreased by 50% thereafter. In this way, only patients with CMV reactivation at risk for progression are exposed to ganciclovir, with the associated risk of neutropenia and delayed CMV-restricted CTL reconstitution. These strategies have greatly reduced the risk of mortality due to CMV. Other strategies being developed include the *ex vivo* expansion of donor-derived, CMV-specific cytotoxic T cells to be used when there is evidence of CMV reactivation or infection (77).

Other potential opportunistic infections such as *Toxoplasma gondii* and *Pneumocystis carinii* pneumonia are rarely clinical problems now in HSCT because of the effective use of antibiotic prophylaxis with trimethoprim or trimethoprim/sulfamethoxazole.

The toxicities due to conventional conditioning regimens, the complications of HSCT, and the severe immunologic impairment that follows necessitate the performance of HSCT in highly specialized facilities with experienced physicians, nurses, and support staff. Special attention should be directed at symptoms that suggest infections, primarily because of the rapidity in which certain infectious complications can become life threatening. In those patients with chronic GVHD, daily antibiotic prophylaxis with penicillin should be given to reduce the incidence of infections with encapsulated organisms. This has been shown to improve patient survival (78). The supportive care for HSCT patients has improved over the last 30 years and probably is a contributing factor in the better results seen with time.

GRAFT FAILURE

In the setting of a conventional allogeneic HSCT, graft failure is a significant complication due to the associated high case mortality rate (79,80 and 81). Graft failure usually occurs either as the persistence of the conditioning regimen–induced pancytopenia with no evidence of donor-derived cell recovery or the decrease in the donor-derived cell counts after initial recovery, resulting in aplasia. The incidence of graft failure is low from an HLA-identical sibling (<5%), but the risk increases proportional to the degree of HLA disparity. For example, the rate of graft failure increases from 7% to 10% for phenotypically HLA-identical pairs to 15% to 25% for two and three HLA loci–mismatched pairs. There also is a significant risk of graft failure after T-cell depletion of the graft, even though a recent study suggested that T-cell depletion of the host could abrogate this problem (82). Donor T cells are presumably needed to destroy host effector cells capable of rejecting the graft.

There are two types of graft failure: early (<1 month) and late (>1 month). The mechanisms that underlie these types of graft failure depend on (a) the intensity of the conditioning regimen, (b) the degree of HLA mismatch, (c) the T-cell content if the graft; and (d) the nature of the postgrafting immunosuppression. A much higher incidence of graft failure occurs in those patients with AA who received previous transfusions of red cells and platelets (83,84 and 85). This pretransplant sensitization to alloantigens (allosensitization) is mediated through memory T lymphocytes and is clearly involved in graft rejection (85). T lymphocytes are clearly associated with rejection, and NK cells have been implicated as mediators of rejection in allogeneic HSCT (25,26 and 27).

In humans the incidence of graft rejection can be decreased by intensifying the conditioning regimen, but this usually results in an unacceptable increase in transplant-related mortality (86). Recently, groups have explored using the relatively T cell–specific purine analog, fludarabine, in conditioning regimens and have demonstrated significant immunosuppression with a minimal degree of toxicity (11,12,87). Another promising method is targeting antibodies to cell-surface molecules of the host necessary for cell adhesion or effector functions to improve immunosuppression (anti–LFA-1, anti-CD44) (88). Selective depletion of T cells to decrease the risk of GVHD, but permit engraftment and GVL, are being explored. Finally, improved postgrafting immunosuppression with new combinations of drugs, such as mycophenolate mofetil and CSP, also may function to suppress host effector cells to facilitate engraftment (47,89).

GRAFT-VERSUS-HOST DISEASE

Incidence and Pathophysiology

Theoretically, acute GVHD can be expected to occur in all cases except for syngeneic and autologous transplants. This result would be anticipated because of highly polymorphic minor histocompatibility antigen differences even between HLA-identical siblings (90,91 and 92). T lymphocytes of the donor graft recognize antigens of the host as foreign, become sensitized, proliferate, and attack host tissue, thereby producing the clinical symptoms of GVHD (91,93). However, syndromes that appear clinically identical to GVHD have occurred in recipients of syngeneic and autologous transplants (94,95). This finding illustrates that the pathophysiology of GVHD is complicated and may also be related to autologous T-cell reactivity in the course of reestablishment of the immune system.

GVHD was first described in lethally irradiated mice that were then infused with allogeneic marrow cells. These animals developed *secondary disease,* a syndrome of diarrhea, skin changes, and liver abnormalities later defined as GVHD. In 1966, Billingham (96) described the three requirements for GVHD to occur: (a) the inclusion of immunologically reactive cells in the graft, (b) the expression of tissue antigens in the host not present in the donor, and (c) the inability of the host to reject the graft immunologically (96). Subsequently, the immune-reactive cells were identified as T cells, and the tissue antigens expressed by the host cells were identified as major and minor HLA antigens. The pathophysiology of GVHD has been reviewed (90,91,93,97,98 and 99). The initial step in GVHD is believed to be an afferent phase in which resting mature T cells respond to antigens presented by host dendritic antigen-presenting cells (100). A second signal from IL-1 leads to the activation of T cells and expression of the IL-2 receptor on their surface. Autocrine IL-2 secretion results in T-cell clonal proliferation. The efferent phase may include cytokine dysregulation, which could contribute to further tissue and target-cell damage (97). Animal studies suggest that tissue damage is mediated by secondary effector cells that include cytotoxic T cells, macrophages, and dendritic cells (91). Some investigators are evaluating the role of cytokine production in initiating, perpetuating, and amplifying T-cell allogeneic reactivity (97).

Acute GVHD occurs in approximately 10% to 80% of allogeneic transplant recipients. The incidence and severity depends on the degree of HLA disparity between donor and host, the gender of donor and host, depletion of T cells, the pretransplant conditioning regimen (TBI vs. no TBI), and the posttransplant GVHD prophylaxis regimen (99). The primary targets of GVHD include the skin, gut, liver, and hematopoietic system (101,102,103,104 and 105). The clinical manifestations include a dermatitis that can range from a macular papular rash to severe desquamation with bullae formation. Gastrointestinal mucosal involvement can result in large volumes of stool that sometimes can be bloody. Symptoms include anorexia, nausea, vomiting, crampy abdominal pain, and diarrhea. Liver involvement rarely results in a right upper abdominal pain and tenderness. Laboratory results can demonstrate hyperbilirubinemia, elevated transaminases, and elevated alkaline phosphatase. Profound deficiencies of immune function result directly from the adverse effects of GVHD compounded by the immune-suppressive therapy used to control GVHD (99).

The diagnosis of GVHD is made clinically and usually confirmed pathologically by evidence of apoptotic bodies at the tips of the rete ridges in the skin and the base of the crypts of the gut epithelium (106). Grading systems of GVHD rely on clinical parameters that are readily measurable and correlate well with patient prognosis (Fig. 83.3) (101). Whereas lower grades of GVHD are correlated with low morbidity and mortality, higher grades are highly predictive of mortality (101,107,108).

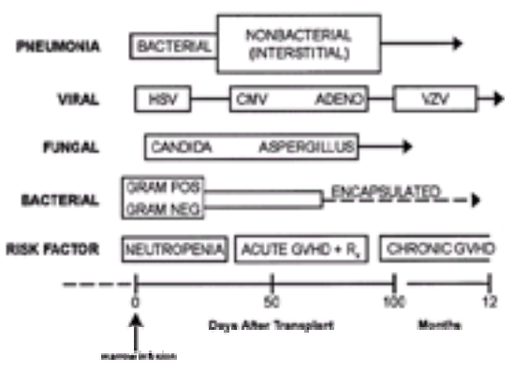

Figure 83.2. Schematic of infectious syndromes at various times after human stem cell transplantation. *HSV*, herpes simplex virus; *CMV*, cytomegalovirus; *VZV,* varicella-zoster virus; *GVHD*, graft-versus-host disease. (Reproduced from Meyers JF. Infections in marrow recipients. In: Mandell GL, Douglas RG, Bennett JE, eds. *Principles and practice of infectious diseases.* 2nd ed. New York: Wiley, 1985:1674–1676, with permission.)

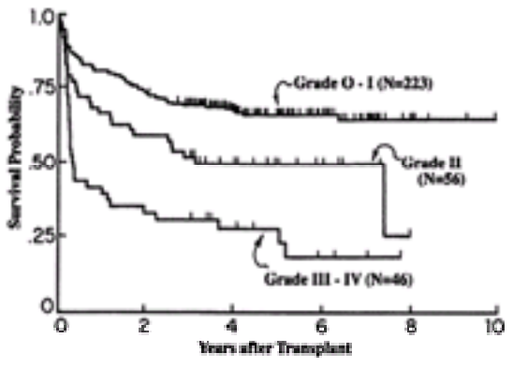

Figure 83.3. Kaplan-Meier estimates of survival based on the clinical grade of acute graft-versus-host disease. For this analysis, 325 patients without advanced malignancy were studied. (Reproduced from Nash RA, Pepe MS, Storb R, et al. Acute graft-versus-host disease: analysis of risk factors after allogeneic marrow transplantation and prophylaxis with cyclosporine and methotrexate. *Blood* 1992;80:1838–1845.)

Prophylaxis for and Treatment of Acute GVHD

Before the discovery of CSP, initial strategies to control GVHD centered on the use of the antimetabolite, MTX, and chemotherapy after allogeneic transplants (105,109). MTX schedules were continued weekly up to 3 months. An unacceptably high incidence of acute GVHD resulted from omitting immunosuppressive drugs in patients given unmodified allogeneic marrow (110). MTX and CSP were shown in controlled prospective trials to be equivalent at reducing the severity of GVHD (111,112). Results of these randomized studies have been corroborated by retrospective analyses performed by the marrow transplant registries (113,114). Although a large randomized trial failed to show differences (112), a smaller trial suggested a higher leukemic relapse recurrence rate in patients treated with CSP (115), similar to findings made in a retrospective analyses of registry data (116,117).

When CSP is combined with a short course of intermittent MTX (given over the course of 11 days), prevention of acute GVHD was significantly improved compared with treatment with either drug alone (118,119). Survival advantages have been clearly realized in patients given transplants for SAA (120), myelodysplastic syndrome (121), and chronic myeloid leukemia (119). In patients given transplants for acute leukemia in first remission, the survival advantage from prevention from GVHD was offset by a higher leukemic relapse rate (122), but a retrospective review by the IBMTR demonstrated a decreased relapse rate. The MTX and CSP combination, which reduces the incidence of acute GVHD in HLA-identical siblings to 30% to 40% in North American patients, is reported to be even more effective in patients of Japanese origin. Morishima et al. (123) reported only 5% incidence of acute GVHD and attributed this result to a lesser degree of genetic diversity among Japanese patients. At this time, the combination of methotrexate with CSP for acute GVHD prophylaxis is used in ~75% of the transplant centers worldwide. Recent prospective multiinstitutional trials of CSP and MTX compared with tacrolimus and MTX demonstrated decreased acute GVHD in the tacrolimus arm in recipients of both HLA-matched sibling and matched unrelated-donor HSCT, but no survival advantage in either patient group (124,125 and 126). In a randomized study, the use of prophylactic corticosteroids combined with CSP was found to be no better than CSP in preventing acute GVHD and possibly in contributing to more chronic GVHD (127). The City of Hope team in a randomized trial compared CSP and MTX with CSP and prednisone. The incidence of acute GVHD was less in the CSP/prednisone arm, 47% to 28%, respectively (128). A randomized trial of CSP and short-course MTX compared with CSP and prednisone showed less acute GVHD (9% to 23%) in the former arm (129). A randomized trial of an anti–IL-2–receptor antibody did not demonstrate efficacy (130). Some newer immunosuppressive drugs that show promise in acute GVHD prophylaxis and treatment include mycophenolate mofetil combined with CSP, fusion proteins or antibodies to the T-cell receptor costimulatory molecule CD28, and others (48,131,132).

Depleting the HSC graft of T lymphocytes by immunologic or mechanical means is another way to reduce the risk of acute GVHD (133,134,135 and 136). Various techniques have produced reductions in the number of T lymphocytes infused by 1 to 3 logs. With the removal of T cells, most of the differentiated lymphocytes that cause GVHD are presumed eliminated, and the immune system is expected to return to an early prenatal state (137). It is presumed that newly formed allogeneic T cells become tolerant to the host and recognize the host as self. Nearly all clinical studies have shown a significant reduction in acute GVHD when T-cell depletion is used (133, 135 and 136). However, the reduction of GVHD in these studies is almost always accompanied by substantial increases in the risk of graft rejection and leukemic relapse (134,135). The net effect usually is no discernible improvement in overall survival (134). Attempts to overcome graft rejection and relapse by selectively depleting only $CD8^+$ lymphocytes produced equivocal results (138). Similarly, the use of an anti–LFA-1 monoclonal antibody to prevent rejection of T cell–depleted grafts has not been convincingly successful (139,140). Favorable results of T-cell depletion have been reported by Roy et al. (141) and Soiffer et al. (142), who depleted T cells with anti-CD6 monoclonal antibody and rabbit complement; by Schattenberg et al. (143), who used the elutriation technique; by Young et al. (144), who used a two-step soybean lectin agglutination and sheep red blood cell rosette procedure; and Hale et al. (82), who used a rat monoclonal IgM antibody CAMPATH-1G to deplete the graft and host T cells. Reports from the first group indicate a degree of mixed chimerism of donor and host cells after marrow grafting that has resulted in a stable equilibrium without a higher risk of relapse (145).

To date, T lymphocyte depletion of HSCT has not produced an improved event-free survival because of the high risk of graft rejection and leukemic relapse. However, the impressive decrease in acute GVHD suggests that T-cell depletion may yet prove to be promising, provided that graft rejection and relapse risks can be reduced. Improvements in conditioning regimens, newer immunosuppressive medications, targeted antibody therapy, and possibly genetic manipulation of donor T cells may facilitate achieving this goal.

Treatment

The mainstays for treatment for acute GVHD are corticosteroids, ATG, and CSP. Prednisone is usually initiated at doses of 1 to 2 mg/kg/day. The response rate in limited single-organ acute GVHD is approximately 50% to 60%, but decreases to 30% in patients with severe multisystem acute GVHD. In one randomized study, the addition of corticosteroids after transplant on day 15 did not decrease the incidence of acute GVHD or change outcome (124,146,147). In a recent study, early treatment with very large doses of methylprednisolone (10 mg/kg/day) was found to be no more efficacious than standard-dose methylprednisolone (148). For those patients for whom front-line therapy fails, the therapeutic options and success at achieving control of GVHD are limited. Approximately 25% to 40% of patients can be salvaged with ATG (149,150). Monoclonal antibodies to block cytokines responsible for T-cell proliferation have demonstrated promise in a recent trial (151). Oral psoralen to enhance the effect of ultraviolet light (PUVA therapy) is effective in some patients at controlling steroid-resistant acute GVHD of the skin and may improve survival (152,153).

CHRONIC GRAFT-VERSUS-HOST DISEASE

Chronic GVHD occurs in 25% to 50% of recipients of allogeneic HSCTs at 3 to 18 months after transplantation (reviews in 104; 93,154,155). The clinical manifestations resemble collagen vascular disease, including skin lesions, keratoconjunctivitis, buccal mucositis, esophageal and vaginal strictures, intestinal involvement, chronic liver disease, generalized wasting, pancytopenia, immunologic dysfunction, and obstructive bronchiolitis. Risk factors for the development of chronic GVHD include HLA disparity, patient age, and previous acute GVHD. If chronic GVHD is left untreated, most patients become disabled or die of infection. Prednisone given alone or combined with CSP is the most effective therapy for chronic GVHD (156). With therapy, approximately 50% of patients survive with Karnofsky performance scores of 100%, and another 25% with scores of 80% to 90%. In half of the patients, therapy can be discontinued after 9 to 12 months. Newer agents that have shown responses in small groups of patients with chronic GVHD include thalidomide (157,158 and 159), tacrolimus (160,161), and mycophenolate mofetil (131,162,163). Nevertheless, the management of patients with chronic GVHD remains unsatisfactory.

GRAFT-VERSUS-TUMOR

One reason for the success of allogeneic transplantation for malignancy is the alloimmune responses mediated against the host tumor cells. Early animal studies by Barnes et al. (164,165) demonstrated the eradication of leukemia from mice receiving allogeneic marrow compared with those given syngeneic marrow after myeloablative irradiation. Mathé et al. (166) coined the term “adoptive immunotherapy” to describe the antitumor response of allogeneic cells. In 1973, Bortin et al. (167) described the GVL effect (167). Weiden et al. (168,169), in a series of retrospective studies later confirmed by the International Bone Marrow Transplant Registry (IBMTR), described the decreased incidence of leukemia relapse in recipients of allogeneic transplants in whom GVHD developed, presumed to be due to a GVL effect. Supporting this observation of a GVL effect in allogeneic HSCT is the observed increase in incidence of relapse after T-lymphocyte depletion of the donor graft (134,135). Kolb et al. (170) provided direct evidence of GVL by using DLIs to achieve complete remissions of leukemia in allogeneic transplant recipients in whom relapse of chronic myelogenous leukemia developed. Finally, success has been demonstrated in attenuated transplant regimens (see end of chapter) that rely primarily on an allogeneic effect of the graft on the tumor (12,171,172 and 173). Antitumor responses from either GVHD or response to DLI has been demonstrated for Hodgkin disease, non-Hodgkin lymphoma, multiple myeloma, chronic myelogenous leukemia, acute myelogenous leukemia, acute lymphocytic leukemia, and renal cell

carcinoma (172,174,175,176,177 and 178).

Clinical studies to augment the GVL effect by manipulating postgrafting immunosuppression (or withholding it completely) have been largely unsuccessful (110,179). These studies demonstrated increased transplant mortality due to the detrimental effect of worsened GVHD and no change in the incidence of leukemia relapse. The use of DLI (add back) after T lymphocyte–depleted HSCT to decrease the risk of relapse is being prospectively studied. Studies that use cytokines, such as IL-2, to augment a GVL response are currently in progress.

INCIDENCE AND ORIGIN OF RECURRENT MALIGNANT DISEASE

The recurrence of malignant disease remains a major cause of failure of HSCT (105,180,181,182,183,184 and 185). In more than 95% of cases, the original malignant cell population reappears, as shown by blood genetic markers. Such reappearance demonstrates the inability of current pretransplant-conditioning regimens to eradicate all clonogenic cells in every patient. Donor-type cells have been implicated as the cause of recurrent leukemia in fewer than 5% of cases. The escape from immune surveillance probably plays a major role in relapse after allogeneic HSCT (186), which may be correlated to a low absolute lymphocyte count after HSCT and slow lymphocyte recovery (187). The treatment of relapsed disease is difficult and the options limited (see previous section). Another complication is the development of Epstein-Barr virus lymphoproliferative disorder (EBV-LPD), which occurs in 12% to 25% of patients after T cell–depleted HSCT (188). The EBV-LPD arises from poor T-cell immune surveillance that allows the uncontrolled reactivation of EBV in previously infected B lymphocytes of donor origin. EBV-LPD/lymphoma has had a high case mortality rate. Therapies have included generation of cytotoxic lymphocytes before transplant, unmodified DLI, and a recent study demonstrated a response with rituximab, a monoclonal antibody to CD20 (189,190).

HEMATOPOIETIC AND IMMUNOLOGIC RECOVERIES

Hematopoiesis after conventional HSCT is stable, as exemplified by patients with stable hematopoiesis who are more than 20 years after transplant. Nevertheless, certain functional impairments may remain for prolonged periods, including poor granulocyte chemotaxis, reduced clonogenic precursors in the marrow, and diminished immunologic function (57,191). Clonal dominance suggestive of reconstitution of hematopoiesis from a small number of pluripotent hematopoietic stem cells has been reported in several experimental situations and in two patients (192). However, studies by our group using methylation site polymorphisms on the inactivated X chromosome in more than 20 allogeneic recipients of female marrow grafts, monitored for more than 20 years after transplant, failed to show evidence of clonal dominance and are consistent with polyclonal reconstitution (193,194). Mixed hematopoietic chimerism seen after some T cell–depleted HSCTs may restrict T-cell–repertoire diversity through interactions of host and donor cells (195).

After transplant, the HSCT recipient's immune system consists of three components, each of which can provide some protection from infection (57). First, traces of the host immune system may be operative, as documented by transient production of host-type isohemagglutinin titers in ABO-incompatible recipients for as long as 12 months. Second, immune cells from the donor are transplanted along with the stem cells and convey “instant” immune function to the recipient. Transfer to the recipient of donor immunity to a number of recall and neoantigens has been documented in 85% of patients without GVHD and in 45% of patients with chronic GVHD. Finally, the most important component is regeneration of the immune system originating from the grafted stem cells. All recipients, regardless of the kind of transplant, have within 30 days a return of normal monocyte/macrophage function, cytotoxic effector cell–mediating natural killing, antibody-dependent killing, and lectin-dependent killing (191). Beyond 4 to 5 months, most patients without GVHD show a return to normal of most other immunologic parameters. Nevertheless, even among healthy, long-term survivors, a few fail to produce the appropriate humoral antibodies, and 30% to 40% of these patients show persistent *in vitro* B-cell defects and decreased T-cell activity, which are even more pronounced in patients who have chronic GVHD (63).

The differences in the recovery of the immune function between children and adults implicate the importance of the thymus in T-cell immunologic reconstitution (137,191). Attempts to accelerate immune recovery by administering cytokines, grafting thymic tissue or thymic epithelial monolayers, and injection of thymosin fraction V or thymopoietin, however, have been unsuccessful (196,197 and 198).

LATE EFFECTS

Growth and Development

Regimens that incorporate TBI carry a greater risk of causing endocrine dysfunction compared with chemotherapy-based regimens, especially in children (199,200 and 201). These abnormalities include hypothyroidism, growth hormone deficiency, delayed puberty, and primary ovarian failure. Hypothyroidism that requires hormone replacement occurs in 5% to 25% of children treated with TBI. Growth hormone deficiency also is common, occurring in more than 50% of patients treated with TBI. Many children have an abnormal growth velocity that is typically multifactorial and includes factors related to irradiation and chemotherapy, including lesions of cartilage, bone, and the epiphyseal growth plate; hypothyroidism; gonadal damage; and delayed or precocious puberty (202). Children with abnormal growth velocity and growth-hormone deficiency have not demonstrated a consistent response to growth-hormone replacement therapy (202). After TBI, many prepubertal females will have delayed onset of menses or primary ovarian failure. High-dose cyclophosphamide (Cytoxan) has been implicated in causing amenorrhea in women older than 40 years (203). However, there is a disproportionately higher incidence of children born to women that have received a chemotherapy rather than a TBI regimen. Other problems that can afflict survivors after allogeneic HSCT include chronic obstructive pulmonary disease (204), hepatic problems (205), depression, and chronic pain (206), but overall the health and performance are typically very good, and most patients are able to be employed (206,207).

CLINICAL RESULTS

Aplastic Anemia

Pancytopenia and a hypocellular marrow characterize severe aplastic anemia (SAA). SAA can result as an idiosyncratic effect from drug exposure and has occurred in association with hepatitis, drug abuse, and pregnancy, but in the majority of the cases, no inciting etiologic factor is identified. The response of patients to immunosuppressive therapy, high-dose CY, and failed allogeneic HSCT suggests an immunologically mediated mechanism directed at the hematopoietic stem cells (208). Other factors such as defective marrow stem cells and impaired or defective marrow environment also may play a role in some individuals. Cellular mechanisms that suppress hematopoiesis are likely important in some patients with SAA, as demonstrated by the increased levels of cytokines interferon (IFN)-g and tumor necrosis factor that inhibit both hematopoietic progenitor cells and stem cells (209). However, no correlation of increased IFN-g level or suppressing activity could be detected in an earlier study of SAA patients (210). The etiologic role of CD4 cells was suggested by the overrepresentation of HLA-DR2 in white patients with SAA (208), but this was not confirmed in a much larger group of Seattle patients (R. Storb, unpublished data). In its severe form, AA has a mortality of 80% to 90% despite supportive care; most patients die within 6 months of diagnosis. During the early 1970s, the observation that acquired AA could be corrected by the infusion of syngeneic marrow led to the treatment of this disease by allogeneic marrow transplantation from HLA-identical sibling donors (192,211). HSCT is considered front-line therapy in those patients with an HLA-identical donor. For those patients without an HLA-identical donor, a trial of immunosuppression with a combination of ATG, methylprednisolone, and CSP is usually successful in achieving recovery of autologous hematopoiesis in 30% to 65% of patients (84,212). Unfortunately, the risk of relapse is significant, and mortality for nonresponders is high (84). The use of an unrelated HSC donor may be necessary for any of the surviving 50% to 60% of patients who have not responded or relapsed after immunosuppressive therapy and do not have an HLA-identical sibling or alternative donor (213,214 and 215).

By the mid-1970s, three major problems were identified that limited the success of allogeneic HLA-identical marrow transplantation: graft rejection, acute GVHD, and chronic GVHD. The incidence and severity of these complications have diminished since then.

GRAFT REJECTION

HSCT recipients are treated before transplant with intensive immunosuppressive therapy to prevent graft rejection. In most regimens, CY is given either alone or in combination with TBI, TLI, TAI, or ATG. It has been demonstrated in animal models that previous blood transfusions will increase the risk of rejection despite otherwise adequate immune suppression, presumably because of sensitization of the host to minor histocompatibility antigens present on donor cells (83,84 and 85). During the early 1970s, graft rejection was the major cause of failure of marrow transplants after CY, with rejection rates ranging from 30% to 70%, and survival on the order of 40% to 45% (216,217). Avoiding transfusions, the standard use of irradiated and/or leukocyte-reduced transfusions and the introduction of the CY/ATG regimen have reduced the risk of graft rejection over time and have improved survival (Fig. 83.5, Fig. 83.6, and Fig. 83.7). Predictive factors for rejection among multiply transfused patients given CY were a low marrow cell dose (<3 × 10^8 marrow cells/kg body weight) and positive *in vitro* tests for cell-mediated immunity of recipient against donor cells, consistent with the concept of transfusion-induced sensitization (85).

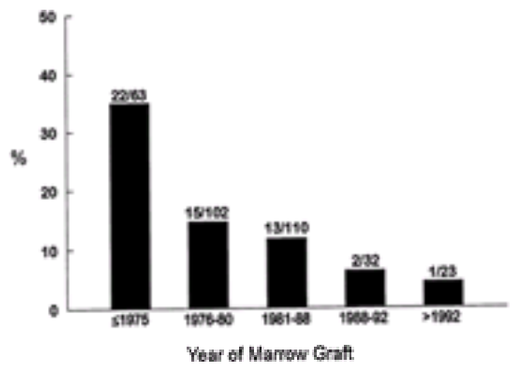

Figure 83.5. The incidence of graft rejection versus year of transplantation for patients in Seattle receiving marrow from human leukocyte antigen (HLA)-identical siblings. All patients were conditioned with cyclophosphamide-containing regimens. The numbers above the bars indicate the number of rejections per number of patients who received transplants. The data are current as of May 1997. (Reproduced from Wagner JL, Storb R. Allogeneic transplantation for aplastic anemia. In: Thomas ED, Blume KG, Forman SJ, eds. *Hematopoietic cell transplantation.* 2nd ed. Boston: Blackwell Science, 1999:791–806, with permission.)

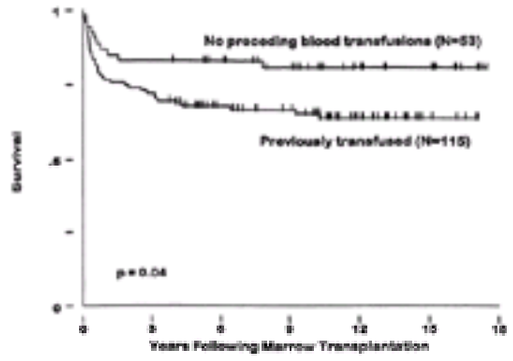

Figure 83.6. The effect of transfusion status on actuarial survival*. Tick marks* denote censoring times of surviving patients. The data are current as of January 1996. The *p* values were calculated by using the log-rank test and are two sided. (Reproduced from Doney K, Leisenring W, Storb R, Appelbaum FR, for the Seattle Bone Marrow Transplant Team. Primary treatment of acquired aplastic anemia: outcomes with bone marrow transplantation and immunosuppressive therapy. *Ann Intern Med* 1997;126:107–115.)

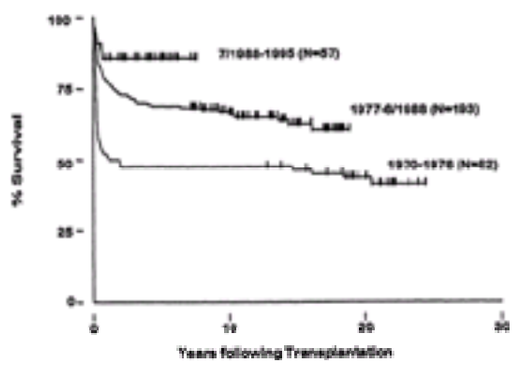

Figure 83.7. Overall survival rates by transplant year for marrow grafts from human leukocyte antigen–identical family members after cyclophosphamide conditioning. The combination of methotrexate and cyclosporine was used for graft-versus-host disease prophylaxis in some patients since 1981 and in all patients since 1985. Most patients who received transplants since July 1988 were conditioned with cyclophosphamide and antithymocyte globulin without supplemental buffy-coat cell infusions. *Tick marks* indicate surviving patients as of April 1996. (Reproduced from Wagner JL, Storb R. Allogeneic transplantation for aplastic anemia. In: Thomas ED, Blume KG, Forman SJ, eds. *Hematopoietic cell transplantation*. 2nd ed. Boston: Blackwell Science, 1999:791–806.)

Two forms of graft rejection may occur. Primary rejection occurs when there is no sign of hematologic function of the graft. Late rejection after initial recovery of hematopoiesis may be seen weeks to months after the transplant. The outlook was poor for patients with primary graft rejection, although more recent data showed that patients can be rescued with second transplants (218,219). Conditioning regimens for second transplants include those that use CY combined with TBI or, alternatively, a combination of CY and antithymocyte globulin (218). The latter regimen resulted in the successful second transplant in 15 to 19 patients and nine survivors in one of our studies (220).

A number of strategies were attempted to decrease the risk of graft rejection. The results of these trials are presented in Table 83.2. A comparative trial between 1976 and the mid-1980s evaluated the use of buffy-coat infusions after transplantation in multiply transfused SAA patients (85,221). The buffy-coat cells were given to improve the number of stem cells given and to provide lymphoid cells believed to be important in enhancing engraftment. A decrease in graft rejection was seen, but at the price of an increased incidence of *de novo* chronic GVHD (Fig. 83.8), a complication that did require prolonged immunosuppressive therapy (222). The survival of patients given buffy-coat infusions was improved compared with that of those patients not given infusions, a finding not confirmed in a retrospective analysis by the IBMTR (223). CY was combined with limited TBI or limited field irradiation by other transplant teams to increase the intensity of pretransplant immune suppression (224,225 and 226). This technique also decreased the risk of graft rejection, although the intensity of the limited-field radiation needed was high, on the order of 600 to 750 cGy, to be effective. The use of irradiation, especially limited-field irradiation, has been associated with a significant risk of solid tumors of epithelial origin compared with that in regimens using only CY (207,227).

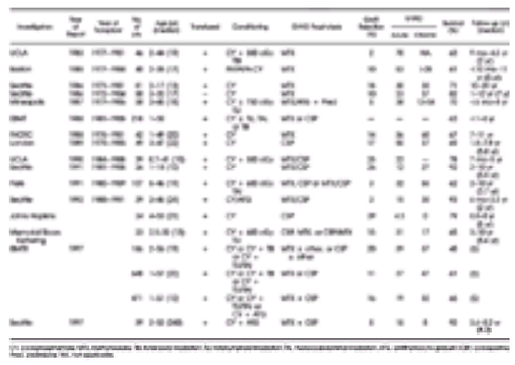

TABLE 83.2. Results of HLA-Identical Marrow Grafts for Aplastic Anemia

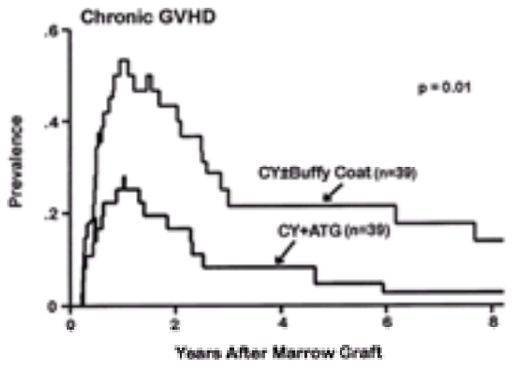

Figure 83.8. The prevalence of chronic graft-versus-host disease (GVHD) among patients with severe aplastic anemia conditioned with cyclophosphamide and given either antithymocyte globulin (ATG) or buffy-coat infusion. There was a statistically significant lower incidence of chronic GVHD among patients who were conditioned with cyclophosphamide (CY) and ATG compared with those who received CY for conditioning with or without buffy-coat infusions. The *p* values were calculated by using the log-rank test. The data are current as of January 1997. (Adapted from Storb R, Leisenring W, Anasetti C, et al. Long-term follow-up of allogeneic marrow transplants in patients with aplastic anemia conditioned by cyclophosphamide combined with antithymocyte globulin [Letter]. *Blood* 1997; 89:3890–3891, with permission.

Over the last 30 years, the incidence of graft rejection has decreased significantly (Fig. 83.5) (145,218,228). The year of transplant has emerged as an independent significant factor affecting rejection over time significantly (Fig. 83.7) (218,228). The reason for this is multifactorial and includes changes in conditioning regimens, improved GVHD prophylaxis, and better supportive care. At our institution we have found a marked decrease in rejection to less than 5% and improved survival greater than 90% when ATG is combined with CY. Furthermore, the 10-year survival for second transplants has increased from 5% to 83%, which on multivariate analysis was found to be due to GVHD prophylaxis with MTX/CSP (Fig.83.9) (218). The importance of GVHD prophylaxis with CSP was identified by the IBMTR in a retrospective analysis to be the most significant factor for improved survival in patients transplanted throughout the world (228).

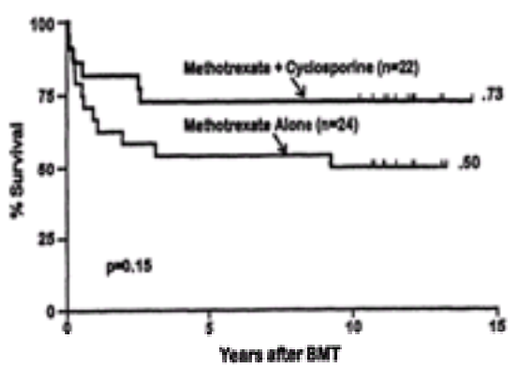

Figure 83.9. Survival of severe aplastic anemia patients given grafts from human leukocyte antigen (HLA)-identical siblings after conditioning with cyclophosphamide. Shown are the results of a randomized prospective trial comparing methotrexate (MTX) alone versus MTX and cyclosporine for graft-versus-host disease prophylaxis. *Tick marks* indicate survival as of April 1996. *BMT*, bone marrow transplantation.

SURVIVAL

The survival of patients treated more recently has improved markedly over the last 30 years (Fig. 83.7). As described earlier, improvement is due to the reduction in the incidence of graft rejection and acute GVHD. Results at our institution with patients who received CY conditioning and MTX as GVHD prophylaxis show an overall survival of 67% for multiply transfused patients at 7 to 11 years, 82% for untransfused patients observed for 1 to 12 years, and 71% for pediatric patients observed for 10 to 20 years. Pediatric patients given CY followed by MTX and CSP as GVHD prophylaxis have survivals on the order of 92%, with observation time ranging from 2 to 10 years. The survival for both pediatric and adult patients given CY/ATG before and MTX/CSP after transplant is at 93%, with an observation time of 14 years (218,229). Figure 83.7 demonstrates the improved survival over time in our patients. A recent retrospective analysis from our institution compared the outcomes of recipients of HSCT from genotypically or phenotypically identical donors with those patients treated with immunosuppressive therapy and demonstrated significant improvement of survival in all patients younger than 40 years given an HLA-identical BMT (84).

The transplant team at UCLA (230) used CY and 300 cGy of TBI, an approach that virtually eliminated the problem of rejection, but led to significant increase in acute GVHD (Table 83.2). Survival at 9 months to 4.5 years was 63%. An attempt by this group to use CY and 300 cGy of TLI followed by CSP/MTX resulted in a rejection rate of 23%, acute GVHD in 22%, and survival in 78% at 7 months to 5 years. This group concluded that 300 cGy of TLI was not sufficient to suppress rejection. The Minneapolis group (224,231) used CY combined with 750 cGy of total lymphoid irradiation along with a combination of MTX, ATG, and prednisone for GVHD prevention. Rejection was virtually eliminated; the rate of acute GVHD was 38%; and survival at 6 months to 8 years was 70%. The Boston team (232) reported rejection of 10% and survival of 61% at 10 months to 11 years, using a combination of procarbazine, ATG, and CY. The Hammersmith Hospital group (233) used CY followed by CSP after grafting. In 1989, they reported rejection of 17%, acute GVHD at 50%, and survival at 69%; observation ranged from 1.8 to 7.8 years (233). The group in Paris (234) used CY and 600 cGy thoracoabdominal irradiation followed by MTX, CSP, or a combination of the two. In 1991, they reported rejection of 3%, acute GVHD of 32%, and chronic GVHD of 62% in patients monitored from 1 to 10 years. The Johns Hopkins team (235) used CY followed by CSP and found a rejection rate of 31%, acute GVHD of 4.5%, and survival of 79% at 8.5 years. The use of alternative-donor and unrelated-donor SCT for SAA has been much less successful, with reported survival of only 30% (236). The most significant problem in alternative and unrelated HSCT is GVHD. Margolis et al. (237) reported significantly improved survival of 55% for those patients that received a T cell–depleted unrelated allograft for SAA.

A review by the European Bone Marrow Transplant (EBMT) group in 1988 (238) combined the results in patients receiving CY with or without limited-field irradiation or TBI, and given postgrafting immunosuppression with either CSP or MTX, showed an overall survival of 63% over 1 to 6 years. The rejection rate with CY conditioning regimens alone was high, but the incidence decreased when CY was combined with some form of irradiation. The incidence of acute GVHD was 40% with a variety of postgrafting immunosuppression regimens; chronic GVHD was 45%; and survival was 63% at more than 7 years after transplant. Generally CSP-treated patients did better than MTX-treated patients. Another more recent retrospective review from the IBMTR (228) demonstrated improved patient survival from 48% in years 1976 to 1980 to 66% in years 1988 to 1992. This improved survival was due to decreased early mortality from GVHD and interstitial pneumonia, and interestingly, the risk of graft failure was not found to be different. CSP was again identified as a significant factor that improved survival (228). In a study at our institution that evaluated the long-term outcomes of SAA patients that survived longer than 2 years after a BMT, it was found that a majority of patients had a return to normal function (207). However, those patients in whom chronic GVHD developed were at a much higher risk for complications that included dermatologic in 14%, cataracts in 12%, lung disease in 24%, bone and joint in 18%, solid tumor in 12%, and depression in 19% (207). The risk of solid tumors after allogeneic transplants for SAA was confirmed in a combined study of our group with the Paris group, who found that irradiation and immunosuppression with azathioprine for chronic GVHD conferred an increased risk of 5.4 and 8.3, respectively, for developing a solid-tumor malignancy (227).

In conclusion, the survival of HSCT patients with SAA has improved dramatically for patients with an HLA-identical donor and is considered the treatment of choice at this time (239). For those patients without an HLA-identical donor, a trial of immunosuppression is recommended, and if not successful, an alternative donor or unrelated HSCT should be considered. The improved survival seen over the last 30 years is directly related to better transplant conditioning, posttransplant immunosuppression, and supportive care, which have reduced the incidence of rejection, acute and chronic GVHD, and transplant complications (84,218,228,240). These improvements have resulted in long-term disease-free survivals (DFSs) of more than 90% for patients with an HLA-identical donor (218,229). Chronic GVHD continues to be a problem that directly affects the rate of posttransplant morbidity and mortality (207). The complications after transplant including secondary malignancies must be better characterized (207,227). The higher probability of secondary tumors makes radiation-based regimens inadvisable in HLA- identical HSCT recipients (207). Improvements must be made in the use of alternative and unrelated HSCT programs to decrease the complications that affect survival. Finally, nonmyeloablative approaches may be helpful in reducing the transplant-related toxicity of HSCT for SAA, but strategies will most likely be developed to overcome the rejection risk from prior allosensitization.

Nonmalignant Disorders of Hematopoiesis

Table 83.1 lists the genetic diseases that are successfully treated with HSCT. Since the first report in 1968, HSCT has been the treatment of choice for SCID, a rare fatal syndrome due to a variety of genetic abnormalities that results in the abnormal function of lymphocytes. Disease-free long-term survival after allogeneic HSCT is greater than 55% (241,242 and 243). Failures are due to poor immunologic status after grafting and involve infections, primarily interstitial pneumonia. The longest survivor is more than 32 years after his transplant. The EBMT retrospectively analyzed 214 HLA-nonidentical T cell–depleted transplants for SCID. An improved DFS was noted for SCID variants with absence of functional T and B cells (B-SCID) compared with variants deficient in T cells but with present B cells (DFS of 35% vs. 60%, respectively). The median follow-up was less than 5 years. Other factors associated with poor survival included lung infection before HSCT, and the use of monoclonal antibodies for T-cell depletion. Patients with B-SCID had increased incidence of death due to early infection, slower rate of engraftment, increased incidence of GVHD, and slower T- and B-cell reconstitution. Patients who received a BU and CY conditioning regimen appeared to have improved outcomes, although this was not statistically significant compared with other regimens (243). The Duke University group (244) used *in vitro* T-cell depletion with soybean lectin and sheep erythrocyte rosetting as GVHD prophylaxis for 89 infants with SCID, using no pretransplant conditioning and no postgrafting immunosuppression except in two of the three cord blood recipients (244). There was a significant degree of HLA nonidentity with 77 patients given HLA-haploidentical parental marrow, 12 patients given HLA-identical marrow from a related donor, and three patients given unrelated placental cord blood. The overall survival was 81% at a follow-up between 3 months and 16.5 years, specifically, of 78% for haploidentical transplant recipients survived compared with 100% for HLA-matched sibling transplant recipients. The incidence of acute GVHD was very low. There was a return of normal T-cell function in all but four of 72 surviving patients, but the B-cell compartment was mostly composed of recipient cells with abnormal production of antibodies, requiring the use of IVIG replacement therapy in 45 patients (244). Filipovich et al. (245) reported the results of eight infants with SCID given non–T-cell–depleted marrow transplants from phenotypically HLA-matched donors using various GVHD prophylaxis regimens (245). Engraftment was found to be 100%, with survival of 75% at 1 to 3 years. Preexisting opportunistic infections were the cause of death in the two patients who died during the study (245).

Wiskott-Aldrich syndrome is an X-linked primary immunodeficiency characterized by a clinical triad consisting of thrombocytopenia with small platelets, eczema, and immunodeficiency, usually reflected by recurrent otitis media. Most reports on transplants are from single institutions that have used a variety of different regimens. TBI has been used to create “marrow space” for the new marrow to grow. The Boston group has used ATG and procarbazine for additional immunosuppression. Because of concerns about the late effects of irradiation, many groups use chemotherapy-based regimens containing CY and either BU or DMBU (246,247), with survival for recipients of HLA-identical sibling grafts of greater than 85%. Ozsahin et al. (248) performed transplants using CY/BU conditioning in ten patients with HLA-identical grafts, and 16 patients with HLA partially matched, related T-cell–depleted grafts. Survival was 80% for HLA genotypically identical recipients and 60% for HLA partially matched related-donor recipients. Unfortunately, seven patients died of viral infectious complications, primarily EBV-LPD (248). Successful transplants using HLA-matched and -mismatched unrelated donors were demonstrated with GVHD prophylaxis consisting of CSP/prednisone or CSP/ATG or anti-CD 5 ricin A chain immunotoxin without T-cell depletion (245). In this study, engraftment was found to be 100% with 83% survival at 1 to 3 years (245). The Sloan Kettering Memorial group (249) performed BMT for 17 patients with Wiskott-Aldrich syndrome using BU/CY for HLA-identical sibling marrow recipients and BU/CY and TBI for those patients given HLA-disparate grafts from their parent. Ten of the 11 patients with HLA-identical or phenotypically HLA-matched parent grafts survive. However, only one of the six patients with HLA-disparate parental grafts survives, with others dying of transplant-related causes.

Fanconi anemia is a genetic disorder associated with diverse congenital abnormalities, chromosomal fragility, progressive bone marrow failure, and an increased risk of leukemia and other cancers. A unique feature of Fanconi anemia is the impaired DNA repair mechanisms that make patients susceptible to developing malignancies and unusually sensitive to conventional HSCT chemotherapy-conditioning programs (242). Fanconi anemia patients, therefore, have an increased incidence of regimen-related morbidity and mortality and, after transplant, are very sensitive to secondary malignancies (207,227). Older reports have achieved long-term DFS of approximately 60% (242,250). More recent studies using attenuated conditioning doses of CY with or without irradiation using GVHD prophylaxis with CSP and either MTX or prednisone have reported improved survivals between 89% and 100% (251,252 and 253). The IBMTR (254) reviewed the results of HLA-matched (n = 151) and alternative or URD (n = 48) HSCT using a variety of attenuated conditioning regimens for Fanconi anemia patients. The 2-year probability of survival was 66% for recipients of HLA-matched sibling and 29% for alternative or unrelated donor transplants, respectively. Factors associated with improved survival of patients in this study included: younger age; higher platelet count before transplant; the use of ATG with conditioning; a lower-dose CY with limited field irradiation (instead of TBI); HLA-matched sibling transplant instead of alternative or unrelated transplant; and CSP for GVHD prophylaxis (254). Unfortunately, even less toxic strategies are still required to decrease the significant early mortality associated with alternative and unrelated HSCT for patients with Fanconi anemia (254,255). A multiinstitutional clinical study evaluating the role of the nonmyeloablative unrelated HSCT approach for Fanconi anemia patients is currently under way.

Other diseases of marrow failure (pure red cell aplasia, Blackfan-Diamond Langerhans cell histiocytosis, lymphohistiocytosis) (256,257,258,259,260,261 and 262), hemoglobinopathies (b-thalassemia, sickle cell disease) (263,264), enzyme deficiencies that result in toxic build-up of metabolic substances (Gaucher disease, mucopolysaccharidosis, Hurler syndrome) (265,266), and other disorders (267,268) have been cured with HSCT.

In thalassemia major, DFS has ranged between 53% and 87% (269,270 and 271). Good results also have been obtained after HSCT for sickle cell disease with event-free survival of 93%, overall survival of 93%, and rejection or recurrence of disease of 14% (263). The use of HSCT is somewhat controversial because of the risk of treatment-related mortality. For that reason, only high-risk patients with HLA-identical siblings are usually selected for HSCT (263). Unfortunately, complications stemming from iron overload will increase the risk of death associated with this procedure (264). With both diseases, prevention through genetic counseling should be given high priority. Gene therapy to correct the defects that cause thalassemia and sickle cell disease are possible in principle; however, the efficiency of transfection vectors and fidelity of transduction of stem cells must be vastly improved before this becomes a therapeutic option (272).

Autoimmune Disease

Multiple sclerosis, systemic lupus erythematosus, and scleroderma are immune-mediated diseases that typically afflict young, otherwise healthy individuals and can on occasion have rapid and debilitating courses (8). The usual treatments for these disorders include immunosuppressive medications including corticosteroids, and on occasion, pulsed CY. There have been case reports of patients achieving durable remissions of autoimmune disorders after HSCT for concurrent hematopoietic malignancies (273,274). HSCT has been demonstrated to cure animals afflicted with several experimentally derived autoimmune disorders (275,276). Recently, several groups have developed HSCT programs targeting severe autoimmune diseases with poor prognostic features (9,10). The rationale for this approach is that ablation of the immune system with high-dose chemotherapy will arrest the underlying autoimmune process by removal of effector lymphocytes. The reconstitution of the immune system would ideally occur without the production of pathologic effector cells. The mechanisms by which the autoimmune process could be arrested or cured include the following: (a) deletion of autoimmune effector lymphocytes; (b) recapitulation of lymphocyte ontogeny without the antigenic signal that triggered the autoimmune process; (c) change of the ratio of suppresser and effector cells, resulting in suppression or tolerance; or (d) the complete replacement of host cells by allogeneic cells, resulting in a new lymphocyte repertoire (9). An obvious concern is that the use of autologous stem cells will result in reinfusion of contaminating effector cells mediating the autoimmune process. $CD34^+$ selected stem cells are being used in some centers to reduce the risk of contamination with immune effector cells. Most conditioning regimens use CY and ATG with or without G-CSF (10,277). Preliminary results from our institution and the Northwestern Hospital transplant group indicate improvement or stabilization of disease in the majority of patients treated (10,277,278).

Malignant Disorders

In 1975 Thomas et al. (105) presented results on the recipients of allogeneic HLA-identical sibling transplants for patients with refractory leukemia. Approximately 10% to 15% of these patients were found to be long-term survivors. Improved outcomes were subsequently demonstrated in acute leukemia patients who were in first or second complete remission compared with conventional chemotherapy. This led to the use of HSCT in a variety of other malignant disorders. Depending on the stage of malignancy when HSCT is carried out, recurrent disease may account for 20% to 75% of treatment failures. For allogeneic HSCT, significant acute GVHD is diagnosed in 30% to 75% of patients, depending on the level of genetic disparity (Fig. 83.4) (79,279,280). Acute GVHD has a case fatality rate of 5% to 50% and thus may account for 10% to 25% of treatment failures. Conditioning regimen–related toxicity and infections during the early pancytopenic period may result in 5% to 20% of deaths. The incidence of interstitial pneumonia has decreased in recent years because of improved prophylaxis and treatment of CMV reactivation. Failure of the marrow graft usually only occurs in the setting of T-cell depletion. Myelodysplasia and secondary acute myeloid leukemia (AML) is an increasingly recognized problem in autologous HSCT, especially after “purging” of the graft with chemotherapy (281,282 and 283).

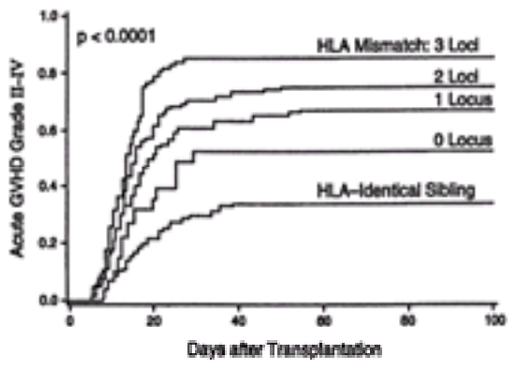

Figure 83.4. Effect of donor and recipient human leukocyte antigen (HLA) disparity on acute graft-versus-host disease (GVHD). Probability of grade II to IV acute GVDH according to the degree of mismatching for HLA-A, -B, and -D/DRB1 in patients who received transplants from a haploidentical relative or an HLA genotypically identical sibling. All patients received unmodified marrow and methotrexate plus cyclosporine for GVHD prophylaxis. (Reproduced from Anasetti C. Hematopoietic cell transplantation from HLA partially matched related donors. In: Thomas ED, Blume KG, Forman SJ, eds. *Hematopoietic cell transplantation.* 2nd ed. Boston: Blackwell Science, 1999:904–914, with permission.)

The exposure of tumor cells to chemotherapy results in the development of resistant clones by a variety of mechanisms. The use of very high doses of chemotherapy followed by HSCT is a way to overcome marrow toxicity and, ideally, to overwhelm any tumor-resistance mechanism. Unfortunately, even with the extremely high doses of chemotherapy used in HSCT, a significant proportion of patients will not be cured (4,284,285 and 286). The problem of relapse is much less for recipients of allogeneic recipients of HSCT because of the GVL effect. There are clearly cures with the use of autologous transplantation, but again, the relapse rate is high. Most recent strategies have focused on decreasing the risk of GVHD in allogeneic HSCT and reducing the risk of relapse in autologous HSCT, but the absolute success of any one technique such as T-cell depletion or tumor cell purging has not been conclusively demonstrated (148,287,288).

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a disease that results in a loss of control of proliferation of early myeloid cells in the bone marrow and an arrest in myeloid development with associated marrow-production defects usually manifested by granulocytopenia, thrombocytopenia, and anemia. The standard of care over the last 15 to 20 years has been to offer allogeneic HSCT to patients with a HLA-identical sibling donors, and it has been demonstrated to cure 50% to 78% of patients (2,3 and 4,289,290). Approximately 3,500 allogeneic SCTs are carried out each year for AML (291). Of the HSCTs performed from HLA-identical sibling donors, 60% are performed as consolidation in first complete response (CR), 10% for patients who never achieve a CR, 10% to 15% in second CR, and less than 5% for more advanced disease (291). Interestingly, although the majority of patients with AML are older, the median age of transplantation is 30 years, and fewer than 5% of patients receiving HSCT are older than 50 years because of the poorer outcomes in this group (11).

The most common conditioning programs for HLA-identical sibling SCT for AML in first CR use CY/TBI or BU/CY (3,292,293 and 294). A retrospective analysis by the French Bone Marrow Transplant Group (293,294) comparing CY/TBI with BU/CY showed improved DFS, decreased relapse, and reduced transplant mortality for the CY/TBI group. However, an excess of transplant-related mortality in the BU/CY group was seen, related primarily to GVHD. These results were not confirmed in a retrospective multivariate analysis by the EGBMT (294), which showed equivalent transplant-related mortality of approximately 23%, relapse rate of approximately 26%, and DFS of approximately 54%. However, a report from the Nordic transplant group (292,295), recently updated, confirmed the increase in transplant-related mortality in patients who receive BU conditioning that was related to increased incidence of GVHD, as well as of hepatic and pulmonary complications. Attempts to improve tumor eradication by intensifying pretransplant chemotherapy or irradiation in the transplant conditioning regimen have resulted in a reduced incidence of relapse at the expense of worsened regimen-related toxicity and mortality, negating any benefit in improved survival (Fig. 83.10 and Fig. 83.11 (3,296,297 and 298). The addition of etoposide to HSCT conditioning regimens appears to be well tolerated, and there appears to be improved DFS in small nonrandomized studies (299,300). GVHD prophylaxis with CSP/MTX used in later AML HSCT studies probably accounts for some of the recently observed improved DFS (301). In contrast, a retrospective analysis by Carlens found that CSP/MTX prophylaxis was associated with worsened outcome because of increased relapse of leukemia, presumed to be due to less acute GVHD (286). A recently published randomized trial compared different doses of intravenous CSP during the peritransplant period in pediatric patients with acute leukemia and showed that an increased dose of CSP resulted in decreased acute GVHD and statistically increased leukemia relapse compared with a lower dose (302). In a large retrospective analysis, Frassoni (285) reported improved DFS due to lower incidence of transplant-related mortality over time for patients receiving HSCT for AML that could not be correlated to any one factor, but was hypothesized to be due to overall better supportive care (285). Factors shown to affect adversely the outcome of HLA-identical HSCT for AML include (a) female donor and male recipient pair, (b) intermediate and high-risk disease, (c) disease beyond first CR, (d) lower number of stem cells infused, (e) poor-prognosis cytogenetics, (f) older age of patient, and (g) increased time from diagnosis to SCT (286,303).

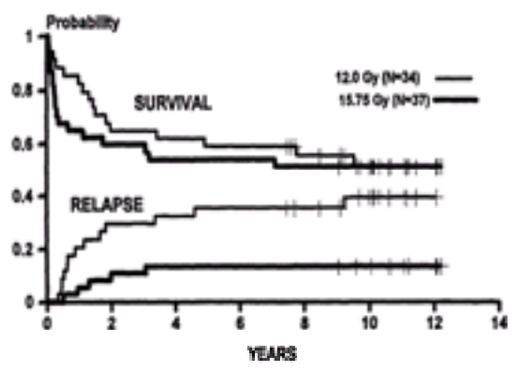

Figure 83.10. Kaplan-Meier estimates of survival and cumulative incidence of relapse for patients conditioned for human leukocyte antigen (HLA)-identical marrow transplantation by 120 mg/kg cyclophosphamide and 12.0 Gy or 15.75 Gy of fractionated total-body irradiation. Results are updated to February 1998. (Reproduced from Clift RA, Buckner CD, Appelbaum FR, et al. Long-term follow-up of a randomized trial of two irradiation regimens for patients receiving allogeneic marrow transplants during first remission of acute myeloid leukemia [Letter]. *Blood* 1998;92:1455–1456, with permission.)

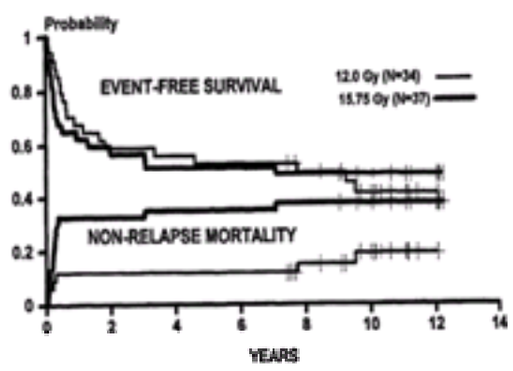

Figure 83.11. Kaplan-Meier estimates of surviving event-free (relapse and death were identified as events) and of dying of causes other than relapse for patients conditioned for human leukocyte antigen–identical marrow transplantation by 120 mg/kg cyclophosphamide and 12.0 Gy or 15.75 Gy of fractionated total-body irradiation. Results are updated to February 1998. (Reproduced from Clift RA, Buckner CD, Appelbaum FR, et al. Long-term follow-up of a randomized trial of two irradiation regimens for patients receiving allogeneic marrow transplants during first remission of acute myeloid leukemia [Letter]. *Blood* 1998;92:1455–1456, with permission.)

Over the last 15 years, autologous HSCT has become an important therapy for the treatment of AML. Single institutional studies demonstrated a DFS of 45% to 55%, but most of this survival advantage could be ascribed to selection bias. A major concern of autologous HSCT, especially in diseases like AML, is the infusion of tumor cells along with HSCs. Many groups have attempted to "purge" the marrow *ex vivo* with chemotherapy (e.g., mafosfamide, perfosfamide) to reduce the risk of tumor contamination (304). The European Cooperative Group for BMT presented a retrospective study that demonstrated significantly decreased relapse and improved leukemia-free survival for recipients of mafosfamide-purged autologous HSCT (305). Other groups have not demonstrated that these strategies have improved outcome primarily due to delayed granulocytic and megakaryocytic engraftment in treated patients (300,306). Another concern about autologous HSCT, especially in those patients who have received *ex vivo* purging, is the increasingly well-recognized high incidence of myelodysplasia and secondary AML due to infusion of HSCs previously exposed to chemotherapeutic agents (307,308). The role for purging must be addressed in randomized trials.

The superiority of allogeneic HSCT compared with conventional chemotherapy has been shown in retrospective studies (309) and some older prospective randomized studies (289), but not others (310). Autologous HSCT has been shown to be better than chemotherapy in some studies (298,300) and equivalent in others (306,311). Most large comparative studies use "genetic" randomization of patients with HLA-identical donors to the allogeneic HSCT arm of the trial and then randomize those patients without an allogeneic donor to the autologous HSCT or chemotherapy arms. These studies have been difficult to perform, primarily because of the attrition of patients from their assigned treatment arms (297,312). Nevertheless, the studies performed to date have demonstrated either superiority of the allogeneic arm, especially in high-risk individuals (285,313), or rough equivalency in regard to survival and DFS compared with autologous HSCT or chemotherapy (Fig. 83.12) (297,312).

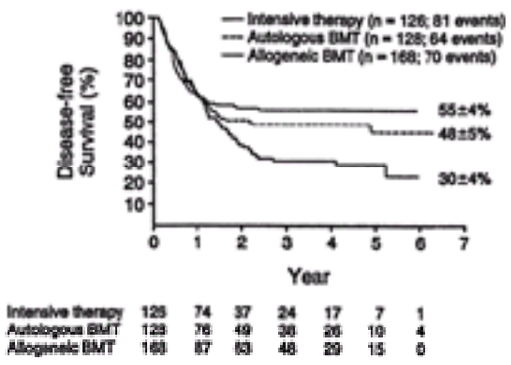

Figure 83.12. Kaplan-Meier estimates of disease-free survival for patients assigned to allogeneic bone marrow transplantation (BMT; *dotted line, n* = 168), autologous BMT (*dashed line, n* = 128), or intensive consolidation chemotherapy (*solid line, n* = 126). Percentages represent estimated disease-free survival rates ± standard error of the mean. The number of patients at risk is shown below each time point. (Reproduced from Zittoun RA, Mandelli F, Willemze R, et al. Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia: European Organization for Research and Treatment of Cancer (EORTC) and the Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) Leukemia Cooperative Groups. *N Engl J Med* 1995;332:217–223, with permission.)

In those patients with AML who have refractory or advanced disease beyond first CR, the probability of obtaining another CR with chemotherapy is low. During early first relapse, it was found to be advantageous to proceed directly to HSCT rather than to attempt reinduction chemotherapy for a second CR (Fig. 83.13) (314). For patients with advanced disease, a variety of agents such as etoposide, melphalan, and cytarabine have been used in addition to CY/TBI to intensify the conditioning regimen (44,315,316 and 317). For these patients, there is a 30% chance of long-term survival after autologous or allogeneic HSCT (287). The use of PBSCs has been shown to reduce the transplant-related mortality in advanced-stage hematologic malignancies by improving the rate of engraftment and hematopoietic reconstitution (53,54,59,66,318). This effect may be due to an increased number of SCs and committed progenitors transplanted (319).

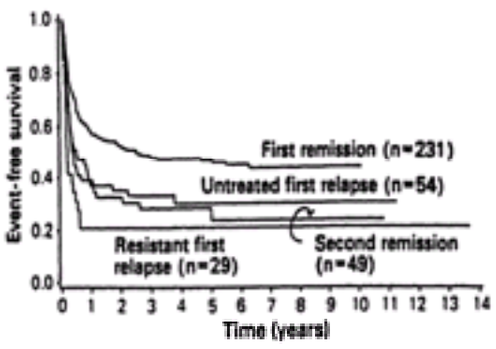

Figure 83.13. Effect of remission status at transplantation on disease-free survival after allogeneic bone marrow transplantation for patients with acute myelogenous leukemia. (Reproduced from Clift RA, Buckner CD, Thomas ED, et al. The treatment of acute non-lymphoblastic leukemia by allogeneic marrow transplantation. *Bone Marrow Transplant* 1987;2:243–258, with permission.)

As induction and consolidation regimens for AML have improved, there has been concern about exposing "cured" patients to otherwise unneeded toxicities of HSCT. It is obviously not possible to predict which patients will relapse after chemotherapy, but certain clinical and laboratory findings have been identified as prognostic indicators of relapse. A low-risk younger patient has a 30% to 40% chance of relapsing after achieving a CR, and the probability of entering a second CR is less than that for a achieving a first CR; this probability decreases with age. Some investigators, therefore, have recommended HLA-identical HSCT or autologous HSCT for patients in first CR who have poor prognostic factors or for those beyond first CR (288,320), but others have argued for allogeneic HSCT as "consolidation" therapy (304,321). For those patients without an HLA-identical sibling donor who relapse after first CR or with poor prognostic features, an alternative or unrelated HSCT should be considered (322).

ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia (ALL) is characterized by arrest of growth and uncontrolled accumulation of lymphoblasts. The probability of obtaining a complete remission by using combination chemotherapy in adults is 70% to 85% and 95% in children. Most, but not all, children who enter a CR will eventually be cured with further chemotherapy (185). Adults and infants, on the other hand, have an incidence of relapse of 40% to 60% and have a much lower probability of cure, attributable to the higher incidence of high-risk features in adults (323). Poor prognostic features used to decide between HSCT and chemotherapy include older age, high white blood cell count at diagnosis, a mediastinal mass, and certain morphologic and cytogenetic features.

The majority of phase II studies evaluating allogeneic HSCT for ALL in adults have demonstrated a leukemia-free survival of 21% to 64%, with a relapse probability of between 10% and 50% (324,325 and 326). A retrospective analysis by the IBMTR that compared allogeneic HSCT results with the results of chemotherapy from two German cooperative groups did not demonstrate an advantage to transplantation (327,328). A prospective trial comparing allo-HSCT with chemotherapy appeared to confirm this finding, because no difference was found in DFS (45% vs. 31%; $p = .1$) (329). However, in a subset analysis of high-risk ALL patients, a statistical difference was found in favor of allogeneic HSCT for high-risk patients in first CR. Two studies evaluated HSCT compared with chemotherapy for children beyond first remission, one a prospective study by Torres et al. (330) in patients in first relapse and the other a retrospective study by the IBMTR (331). Both studies showed significant benefit of allogeneic HSCT in terms of DFS and relapse. Another retrospective review by the IBMTR evaluating CY/TBI versus BU/CY as pretransplant conditioning demonstrated highly significant improved survival and decreased treatment failure (leukemia relapse) in those patients that received CY/TBI (332).

A subtype of ALL that contains the karyotypic abnormality termed the Philadelphia chromosome (Ph^+) carries a particularly poor prognosis and is rarely cured with chemotherapy (333). A retrospective analysis by the IBMTR of allogeneic SCT for Ph^+ ALL in first CR has shown a 2-year DFS of 38% (334). The City of Hope and Stanford groups reported better results with 3-year relapse-free survivals of 50% to 68% and probability of relapse of 12% (326). A report from our group indicated that acceptable results for unrelated HSCT for Ph^+ ALL can be obtained with a DFS at 2 years of approximately 49% (335). However, delays in initiating an unrelated donor

search for high-risk ALL continues to be a problem (336).

Autologous HSCT remains an option for patients without HLA-matched siblings. In comparative trials, superior DFS results have been found for HLA-matched sibling HSCT compared with autologous HSCT (326), but equivalent results were found in a smaller older study (337). Weisdorf et al. (338) compared unrelated HSCT and autologous HSCT for ALL and found DFS results that were equivalent for patients younger than 18 years, but improved DFS rates were found for recipients of unrelated HSCT in disease beyond first CR (338). In the future, molecular markers of minimal residual disease detected by polymerase chain reaction technology may become a useful tool in predicting the relapse of “good-risk” ALL patients and determining which patients should proceed immediately to HSCT.

CHRONIC MYELOGENOUS LEUKEMIA

The median survival of patients with CML is 45 months after diagnosis, but occasional patients have survived up to 20 years (339). No patient has been cured of CML by conventional means, and retrospective evaluations have not demonstrated survival advantages in using chemotherapy (340,341). IFN therapy has been demonstrated to produce a hematologic disease response in the majority of patients treated (340,341). A modest increase in survival advantage of 20 months, however, has been demonstrated only when interferon is combined with chemotherapy (342,343). The cost and side effects of IFN therapy are substantial (340,341). A recent report from Druker et al. (344,345) reported excellent disease responses by using a tyrosine kinase inhibitor in a group of patients with CML in chronic phase and blast crisis for whom IFN therapy had failed. No significant side effects were reported. Although these initial results are unprecedented and quite encouraging, the durability of the disease responses is yet to be determined.

Allogeneic HSCT is the only known cure for CML currently available (86,86,346,347,348 and 349). However, transplant-related complications may shorten the lives of some patients, and there is controversy over the up-front mortality of allogeneic HSCT and the improbability of cure with interferon with or without chemotherapy (341,343). In addition, there is evidence that exposure of CML chronic-phase patients to BU and interferon, and delaying HSCT beyond 2 years will negatively affect the probability of success of allogeneic HSCT (343,350,351 and 352). In experienced transplant centers, patients with CML chronic phase less than 1 year from diagnosis have 1-year and 5-year probabilities of survival after HLA-identical sibling HSCT of 90% and 81%, respectively (Fig. 83.14) (350). For recipients of HLA-identical sibling HSCT, the estimated survivals and event-free survivals were equivalent after conditioning with CY and TBI as compared to BU and CY, but there was significantly more regimen-related toxicity in the CY/TBI arm (Fig. 83.14) (346). The expected survivals after progression to accelerated and blast crisis are clearly worse and reported to be at 28% to 40% and 15%, respectively (Fig. 83.15) (348,349). The primary problem is leukemic relapse, which approaches 50% to 75% in advanced-stage CML patients (Fig. 83.16) (348,349). Support for unrelated HSCT comes from a retrospective analysis from the Hammersmith Hospital, which demonstrated a 2-year survival of 73% and a 2-year leukemia-free survival of 73% (353). At our institution, DFSs at 5 years of 74% for patients younger than 50 years have been observed (354).

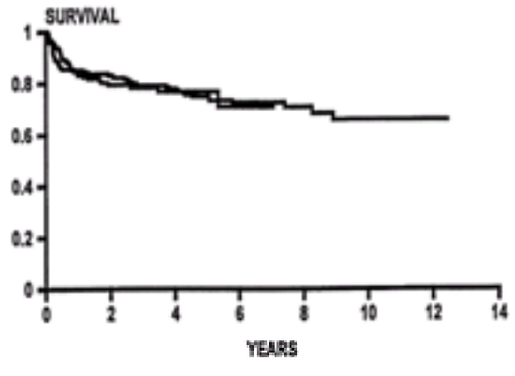

Figure 83.14. Kaplan-Meier probabilities of survival of 336 patients transplanted for chronic myeloid leukemia in chronic phase (CP). Patients were categorized by transplant regimen. Patients in the group with the shorter period of follow-up were treated with the busulfan + cyclophosphamide (CY) regimen, and the other group received the cyclophosphamide (CY) + total body irradiation regimen. The lines are not labeled because there is clearly no significant difference between the statistics they represent. (Reproduced from Clift RA, Buckner CD, Thomas ED, et al. The treatment of acute non-lymphoblastic leukemia by allogeneic marrow transplantation. *Bone Marrow Transplant* 1987;2:243–258, with permission.)

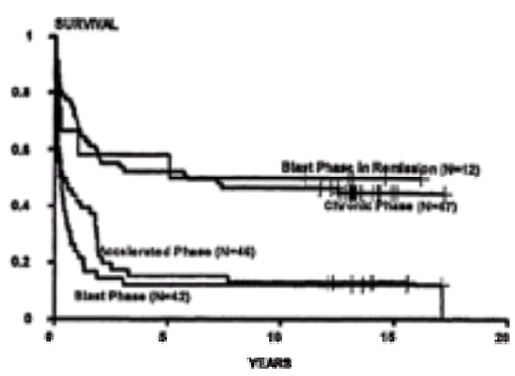

Figure 83.15. Kaplan-Meier probabilities of survival by phase at the time of transplantation for 155 patients with chronic myeloid leukemia who received transplants through 1983 from human leukocyte antigen–identical siblings, first published in 1986. The results are updated as of June 1997. (Reproduced from Thomas ED, Clift RA. Allogeneic transplantation for chronic myeloid leukemia. In: Thomas ED, Blume KG, Forman SJ, eds. *Hematopoietic cell transplantation.* 2nd ed. Boston: Blackwell Science, 1999:807–816, with permission.)

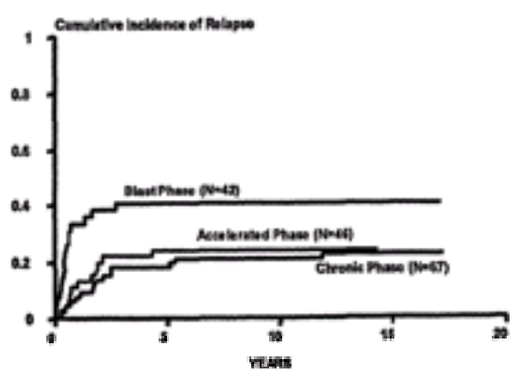

Figure 83.16. Cumulative incidence of cytogenetic relapse by phase at the time of transplantation for 155 patients with chronic myeloid leukemia who received transplants through 1983 from human leukocyte antigen–identical siblings, first published in 1986. The results are updated as of June 1997. (Reproduced from Thomas ED, Clift RA. Allogeneic transplantation for chronic myeloid leukemia. In: Thomas ED, Blume KG, Forman SJ, eds. *Hematopoietic cell transplantation.* 2nd ed. Boston: Blackwell Science, 1999:807–816, with permission.)

MYELODYSPLASTIC SYNDROME

The myelodysplastic syndromes (MDSs) are clonal hematopoietic disorders characterized by ineffective hematopoiesis, progressive cytopenias, and, in some affected individuals, a predisposition to developing leukemia (355,356 and 357). There is no satisfactory conventional therapy for MDSs (341,355). Cytotoxic therapy has resulted in CR in some people, but these remissions have been short. HSCT has been the only curative treatment for this heterogeneous disease (121,358,359). Best results with HSCT have been in MDS patients without excess blasts, in which event-free survivals have ranged from 41% to 56% (121,358,359). Chemotherapy-based

conditioning regimens appear to be effective for MDSs (359,360). Risk factors that adversely affect the outcome of HLA-matched sibling HSCT include older patient age, a higher percentage of marrow blasts, and previous cytoreductive chemotherapy (361). Autologous HSCT is an option for some patients without an HLA-identical sibling donor, but is imperfect because of difficulty mobilizing stem cells in some patients and the high risk of relapse after transplant (362). The use of unrelated HSCT is an option for patients without an HLA-identical donor (363). In general, therapy for treatment-related MDS and secondary AML arising from exposure to alkylating agents and radiation before autologous SCT is problematic (307,308,364). This therapy-related MDS is rarely cured with chemotherapy and is more difficult to treat with HSCT, primarily because of the higher incidence of regimen-related toxicity and relapse (365,366).

B-CELL MALIGNANCIES AND HODGKIN DISEASE

The treatment of advanced stage B-cell malignancies, chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma (NHL), and Hodgkin disease by allogeneic or autologous HSCT has met with limited success, but generally with superior results to those with salvage chemotherapy (367,368,369,370,371,372,373,374,375,376 and 377). Typically most patients with these diseases are older, have received a significant amount of chemotherapy before HSCT is considered, and, therefore, have poor bone marrow reserve (371,378,379 and 380). These factors are believed to contribute to the significant regimen-related toxicities and poor survival that occur after allogeneic HSCT. Most retrospective series and the few prospective studies have shown generally worse survival after allogeneic HSCT compared with autologous HSCT (372,373,379,380,381,382 and 383). Recent small, single-institution trials have demonstrated better results with allo-HSCT, with DFS at 2 years approaching 70% (384,385 and 386). However, autologous HSCT remains the initial therapy of choice in advanced stage, B-cell malignancies. The significant risk of relapse combined with the risk of developing therapy-related myelodysplasia and secondary AML after autologous HSCT for B-cell malignancies reflects the imperfection of this therapy (307,364,385,387).

MULTIPLE MYELOMA

Multiple myeloma (MM) is a malignancy of plasma cells that usually results in the accumulation of paraproteins, and is characterized clinically by lytic bony lesions, renal failure, anemia, and hypercalcemia (388). MM is considered incurable with conventional therapy, and median survival is typically 3 years (388). The use of allogeneic HSCT is limited in the therapy of MM primarily because of the older age of patients and the high degree of transplant-related mortality of approximately 40% to 45% (389,390,391 and 392). However, prolonged DFS, a much lower relapse rate, and molecular CRs in those patients who survive an allo-HSCT compared with recipients of autologous-HSCT suggest that a graft-versus-myeloma effect exists (390,391,392 and 393). Autologous HSCT is associated with much less transplant-related mortality than is allo-HSCT and has shown relatively equivalent DFS in younger patients (392,394,395). Autologous HSCT has been demonstrated to have better CR rates and improved event-free survival and survival compared with chemotherapy alone (396). Recently Barlogie et al. (397) showed encouraging results with an aggressive program using early-after-diagnosis tandem autologous HSCT to improve the probability of achieving a CR, followed by IFN as immunotherapy (397). The early use of autologous HSCT in the disease course of MM may be beneficial because of lessened exposure of the hematopoietic stem cells to alkylator chemotherapy that would improve the ability to mobilize stem cells and possibly reduce the risk of developing therapy-related myelodysplasia (1,307,389). In both autologous and allogeneic HSCT studies, factors that predict poor response and poor survival include elevated b_2-microglobulin, karyotypic abnormalities, prolonged exposure to chemotherapy, and in most studies, chemotherapy-unresponsive disease (390,394,397,398). Obviously, less toxic conditioning regimens are needed for allogeneic HSCT, and more effective methods are required for autologous HSCT to improve the survival of MM patients.

OTHER HEMATOLOGIC DISEASES

Allogeneic HSCT has been successful in more rare myeloproliferative and stem cell disorders such as myelofibrosis, hairy cell leukemia, and histiocytic medullary reticulosis (399,400 and 401).

SOLID TUMORS

Many centers have investigated the use of autologous HSCT for a variety of solid tumors (402,403,404,405 and 406). The use of autologous HSCT for breast cancer increased dramatically after the publication of encouraging phase II studies in the early 1990s. More recently, early analysis of all of the randomized controlled trials did not demonstrate survival benefits for women with high-risk or grossly metastatic breast cancer (407,408), except for one study that, unfortunately, was found to be falsified (409). Whether autologous HSCT is useful in certain subsets of patients with breast cancer, such as high-risk stage III, chemotherapy-responsive stage IV, or in solid tumors in general, are valid questions that must be addressed under the auspices of well-designed randomized controlled trials.

TARGETED THERAPY

Monoclonal antibodies directed at cell-surface antigens of malignant cells provide an ideal way of directing therapy to the appropriate cells. Antibodies directed at specific antigens have been found to be efficacious in providing tumor responses in CLL and breast cancer, although the mechanism(s) of action remains uncertain. However, not all directed antibodies provide disease responses. Recently, humanized monoclonal antibodies conjugated to cytotoxic antibiotics or radioactive isotopes have been used to treat non-Hodgkin lymphoma and AML (410,411 and 412). These techniques also have been used in conjunction with autologous and allogeneic HSCT (413,414).

Coupling radioactive isotypes to bone-seeking substances such as holmium ethylenediaminetetramethylene phosphonic acid (^{166}Ho-EDTMP) provides another approach of localizing therapy to the bone marrow and reducing systemic toxicities. This approach has obvious utility in patients with MM or leukemia. Canine studies have shown that ^{166}Ho-EDTMP was distributed to bone, and after 24 hours, the concentration of isotope is 200-fold that in any other organ (415). Marrow suppression was achieved with this technique because the marrow is never farther from the bone than 200 to 400 µm and the -emitting isotope has a range of several millimeters. Increasing doses of the path length of the ^{166}Ho-EDTMP led to increasingly prolonged and severe myelosuppression, but complete myeloablation was not achieved. It was reasoned that repopulation of marrow was achieved by circulatory hematopoietic stem cells sequestered in the spleen. This hypothesis was supported by the complete marrow aplasia that occurred when splenectomy was performed before the administration of ^{166}Ho-EDTMP.

ALLOGENEIC STEM CELL TRANSPLANTATION FROM ALTERNATIVE DONORS AND HAPLOIDENTICAL FAMILY MEMBERS

Genotypically HLA-identical sibling HSCT donors can be found only in less than one third of all patients. Extended searches of patients' families will identify a phenotypically identical or one HLA antigen–mismatched family member at frequency of 10% to 11%. Results of 281 HLA-haploidentical transplants compared with 967 concurrent genotypically HLA-identical marrow grafts were analyzed by Anasetti et al. (79), who found significant increase in graft failure in the recipients of haploidentical grafts (79). Graft failure was seen in 2% of genotypically HLA-identical siblings, 7% of phenotypically HLA-identical recipients, 9% of one-HLA-locus–mismatched recipients, and 21% of two-HLA-locus–mismatched recipients (180). The risk of acute GVHD is 2.16 times higher with a haploidentical HSCT than with HLA-identical HSCT. However, the HLA-nonidentical recipients have a reduced risk of leukemic relapse, presumably because of a greater graft-versus-leukemia effect. As a result, survival of HLA-haploidentical recipients who differ at only one locus on the nonshared haplotype is identical to that of HLA-identical siblings (Fig. 83.1). Because of the sharply increased transplant-related mortality, survival for two- and three-HLA-loci–mismatched patients is significantly worse. Similar results have been reported by the IBMTR (416).

ALLOGENEIC HUMAN STEM CELL TRANSPLANTATION FROM UNRELATED DONORS

The incidences of acute and chronic GVHD are much higher in recipients of unmodified unrelated HSCT compared with recipients of HLA-identical sibling grafts (182). Similar to the HLA-nonidentical family member setting, this adverse effect is somewhat offset by a lower relapse rate. Survival is similar for CML patients younger than 50 years receiving fully phenotypically HLA-matched grafts compared with HLA genotypically identical HSCT at our institution (354). In those patients given an unrelated HSCT for leukemia, event-free survival is similar to that with HLA-identical siblings, probably because of the greater graft-versus-leukemia effect (182). In contrast, in patients with CML in chronic phase, survival of those given an unrelated HSCT is expected to be 45% to 50% for all patients compared with 70% to 75% among similarly treated patients given HLA-identical sibling grafts (279,354,417). This survival difference is due to the higher incidence of transplant-related complications generally attributable to GVHD. It is clear that improved GVHD prevention and management remains the major hurdle in providing safe alternative and unrelated HSCT. Allele-level typing may result in better matching of unrelated donors, which may result in less GVHD and a smaller pool of “ideally” matched donors (30,31,418). However, a major focus in the future will be to determine permissive HLA mismatches that will provide donors for most if not all patients (181,280,418,419).

Nonmyeloablative Human Stem Cell Transplantation

Almost simultaneously over the last 6 years, there has been interest in the development of nonmyeloablative transplant regimens by several groups (12,13,47,87,173,420). The rationale by which these regimens operate is that engraftment, usually mixed hematopoietic chimerism or simply mixed chimerism, can be obtained from reduced doses of radiation or chemotherapy used to condition patients. This state of mixed chimerism has been seen in some cases after standard allogeneic transplant (421,422 and 423), but is more common after the depletion of T lymphocytes in the graft or with less intensive transplant-conditioning regimens (87,173,422,424). Most nonmyeloablative HSCT programs attempt to use postgrafting DLI to convert mixed chimeras to full donor chimeras and as immunologic therapy to eradicate the underlying malignancy or hematopoietic defect. It has recently been shown in the dog model that long-term stable mixed chimerism is due to

mutual host/donor tolerance, which could be disturbed only by DLIs from donors sensitized to minor host histocompatibility antigens induced by repeated skin grafting from the host (425).

Most nonmyeloablative HSCT programs only attenuate current standard conditioning regimens and, therefore, must still be carried out in the hospital setting. The regimen currently used in Seattle is performed in the outpatient setting. The conditioning regimen uses three doses of fludarabine (30 mg/m^2/day for 3 days) followed by a single dose of 200 cGy of TBI on the day of HSCT. Postgrafting immunosuppression with mycophenolate mofetil and CSP, initially demonstrated to be synergistic in the dog model (47), provided control of host-versus-graft and GVH reactions (420). Of 29 patients who were too old or medically infirm for a conventional HSCT given a nonmyeloablative HSCT at our center, all had mixed donor/host chimerism at day 28, with a higher degree of chimerism seen in patients with significant previous exposure to chemotherapy (11). Mixed donor/host chimerism appeared to be less stable in these older patients treated thus far compared with that seen in the young dog, and most patients went on to full donor engraftment or, in fewer than 16%, to rejecting the allografts (50,426). The rejection of a graft after a nonmyeloablative transplant was nonfatal and accompanied by autologous hematopoietic recovery in all cases (426). Since the addition of fludarabine as part of the pretransplant immunosuppression, no patient has rejected his or her grafts and, in general, higher degrees of donor chimerism have been observed (11). GVHD of grade II to III has occurred in more than 50% of patients with engraftment, and this correlates to a higher degree of donor chimerism (426). Antitumor responses and complete remissions after nonmyeloablative HSCT have been observed in patients with CML, CLL, multiple myeloma, and Hodgkin disease, among others, mediated by the allogeneic effect on the tumor cells (50,427). DLI has been used infrequently in this initial cohort. DLI does not appear to prevent rejection, but when administered for persistent or progressive disease, tumor responses and complete remissions have been observed (427).

CONCLUSIONS

In the last 30 years, the efficacy of HSCT at curing a variety of diseases has been established. Allogeneic HSCT from an HLA-matched sibling is the treatment of choice for SAA, SCID, CML, MDS, a number of fatal genetic diseases, and high-risk or advanced-stage AML and ALL. Autologous HSCT is efficacious for a variety of hematologic malignancies, and its role is being defined for solid tumors and autoimmune diseases. The role of allogeneic HSCT for malignant diseases other than CML, MDS, and high-risk or advanced-stage AML and ALL is less clearly defined and should be investigated through well-designed, randomized controlled trials. The major limitations of allogeneic HSCT, in particular donor procurement, toxicities related to high-dose chemotherapy, and GVHD, continue to be significant problems that limit the application of this procedure to a generally younger subset of afflicted patients. The development of less toxic nonmyeloablative conditioning programs that rely on the *allogeneic effect* rather than high-dose therapy to eradicate cancer cells, coupled with improved HLA typing, larger donor registries, and better supportive care provide optimism that allogeneic HSCT will become a safer and more widely applied curative modality for a variety of malignant and nonmalignant diseases.

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84 TISSUE AND SOLID ORGAN ALLOGRAFT REJECTION

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Human organ transplantation, a combined achievement of medicine, pharmacology, and immunology, provides life-saving organs to patients with end-stage organ failure who have exhausted all other therapeutic options. The complexity of the transplant process arises from the immune response to the transplanted organ (rejection), when this organ is derived from another genetically nonidentical individual of the same species (*allograft*). Allograft rejection is a unique immunologic disease because the onset (*transplant procedure*) and many of the target antigens (*donor antigens*) can be easily identified. Despite these opportunities, the most basic aspects of the disease still are not well understood: the nature of the immunogenic peptides (epitopes), the identity of the effector immune mechanisms, and the factors responsible for recipient differences in the immune response to allografts. Therefore, although important advances have been made in the prevention, diagnosis, and treatment protocols, allograft rejection still occurs frequently, is incompletely controlled by current treatment protocols, and represents a major treatment cost.

In the United States, the United Network of Organ Sharing (UNOS) develops policies to allocate cadaveric organs fairly among the different transplant centers. UNOS also tabulates data on the results of transplantation (1). The most frequently transplanted organs include kidney, liver, pancreas, heart, and lung. Table 84.1 lists the rejection rates of different solid organ allografts, assuming immunosuppression with cyclosporine/FK506, azathioprine, and prednisone. One-year graft failure rates were between 10.6% (for kidney allografts) and 36.2% (for intestine allografts), and the 5-year rates were less promising, between 31.5% (for heart allografts) and 62.6% (for intestine allografts). Kidney transplants, particularly the living-related ones, have the lowest rates of graft failure (about 6%) (1).

Type of Organ	1-Year Graft Failure (%)	5-Year Graft Failure (%)
Kidney (cadaveric)	10.6 ± 0.3	35.3 ± 0.2
Kidney (living-related)	5.5 ± 0.3	21.6 ± 0.3
Liver	18.6 ± 0.5	33.9 ± 0.3
Pancreas	23.8 ± 2.4	58.4 ± 2.4
Intestine	36.2 ± 7.5	62.6 ± 6.2
Heart	14.9 ± 0.6	31.5 ± 0.4
Lung	23.7 ± 1.1	58 ± 0.8
Heart-lung	41.8 ± 5.3	59.5 ± 2.5

Data derived from the 2000 Annual Report by UNOS.

TABLE 84.1. One- and Five-Year Graft Failure Rates of Solid-Organ Allografts

Five-year graft failure rates of living-related kidney allografts are given in Table 84.2 (1). Of note, a six-antigen mismatch from a living related donor does as well as a perfect match from a cadaveric donor (2). Transplant surgeons have pointed to cold ischemia time to explain this phenomena. After all, living-related donation is scheduled electively. The organ is often harvested from an adjacent room. With minimal ischemia time (less than 1 hour), the kidney may face less reperfusion injury (3,4). No satisfactory hypothesis has been offered to explain why a six–human leukocyte antigen (HLA) mismatch from a living donor is not as vigorously rejected.

No. of HLA Mismatches	Cadaveric Allografts Graft Failure Rate (%)	Living-Related Allografts Graft Failure Rate (%)
0	28.4 ± 0.8	13 ± 0.6
1	31.3 ± 1.2	21.5 ± 1.1
2	33.5 ± 0.7	23.3 ± 0.7
3	34.9 ± 0.5	24.4 ± 0.6
4	36.5 ± 0.5	21.6 ± 1.4
5	38.3 ± 0.6	25 ± 1.6
6	40.7 ± 1.1	29.2 ± 2.7

HLA, human leukocyte antigen.Data derived from the 2000 Annual Report by United Network of Organ Sharing (UNOS).

TABLE 84.2. Influence of HLA Matching on 5-Year Graft Failure of Cadaveric Versus Living-Related Kidney Allografts

Still, as described subsequently, HLA matching is the standard for allocating cadaveric kidney allografts. A harvested kidney may be preserved up to 48 hours, until HLA matching can be done. Furthermore, recipients can be sustained with dialysis until they can receive the graft (3). For practical reasons, prospective HLA matching is not done for heart or lung allografts. First, the organs are difficult to preserve. Thus, there is not enough time to define the HLA antigens before transplantation. Thus, organs are allocated based on the UNOS waiting list without consideration of HLA matching (4). While waiting for a graft, heart recipients can receive artificial mechanical hearts or left-ventricular assist devices. A lung recipient could, on a short-term basis, exist on extracorporeal membrane oxygenation, or ECMO (3). Matching for HLA between donor and recipients is not routinely done for liver allografts. Whereas livers can be preserved for a short time, for various reasons, recipients do not mount a strong immune response. Even livers with a different ABO blood type can be accepted, assuming standard immunosuppression (4).

LABORATORY METHODS IN CLINICAL TRANSPLANTATION

Identification of HLA Phenotypes

The HLA class I antigens (A, B, and C) are expressed on all nucleated cells and are recognized by $CD8^+$ T cells. On the other hand, the HLA class II antigens (DR, DQ, and DP) have a selective tissue distribution, are expressed by antigen presenting cells (APCs), and are recognized by $CD4^+$ T cells. Also, such HLA class II antigens can be expressed by a variety of nonimmune cell types upon stimulation with interferon (IFN)-g. Every individual inherits six HLA antigens (three class I, three class II) from each parent. All such antigens are codominantly expressed. The entire set of HLA-A, B, C, DR, DQ, and DP antigens located on chromosome 6 is called a

haplotype. Because HLA differences lead to rejection, transplant physicians attempt to match donor and recipient HLA antigens. For practical reasons, only HLA-A, B, and DR are considered before transplantation (5). Hence, every individual has six alleles to type and match. As indicated in Table 84.2, better-matched grafts have better survival.

It is necessary to determine the HLA antigen composition in prospective donor–recipient pairs before kidney transplantation. Two methods have been used in clinical transplantation to determine a person's HLA phenotype: (a) Traditional tissue typing is based on a complement-dependent cytotoxicity (CDC) test, in which patient lymphocytes are incubated with specific anti-HLA antisera and complement. A positive reaction is indicated by lysis of the lymphocytes. The HLA type then is determined based on the lysis of cells by specific antisera. The alloantisera used in the CDC assays are limited in quantity and are often not specific for a single HLA allele. Production of anti-HLA monoclonal antibodies has encountered many difficulties, including reactivity to framework HLA antigenic determinants present in all alleles. In addition, the CDC assays require millions of viable lymphocytes, which are often difficult to obtain from children and from any patients with corticosteroid therapy, leukemia, or end-stage renal disease. In addition, in cadaveric donors, HLA class II typing is often difficult using peripheral blood lymphocytes and is usually done using lymphocytes isolated from spleen or lymph nodes (6). This may make organ retrieval more time-consuming, leading to a longer cold ischemia time. (b) One of the important technical improvements in the field of tissue typing has been the development of molecular methods for typing the alleles of HLA class I and II genes (6). With new molecular techniques based on the nucleotide sequence of the HLA alleles, such alleles can be typed more accurately using fewer cells. Therefore, a serologically defined HLA type may be divided further into several subtypes at the DNA level. To differentiate between allelic differences, sequence-specific DNA primers (SSPs) are used. If the SSPs can anneal to a fully complementary strand, the corresponding allelic fragment can be amplified by means of the polymerase chain reaction (PCR) technique and directly visualized on an agarose gel. In the case where no complementary strand exists, no amplification occurs (6). Another approach to DNA typing is hybridization of PCR-amplified, biotin-labeled genomic DNA with sequence-specific oligonucleotide probes (SSOP) prebound to a nylon membrane. These SSOPs also bind only to a fully complementary sequence in the amplified DNA. The SSOP-bound allelic DNA then can be visualized by the addition of an avidin–peroxidase conjugate that binds to the biotin-DNA complex. The addition of the appropriate substrate causes the complex of the biotinylated-DNA and SSOP to turn blue in the nylon membrane (6). Although molecular techniques are superior to standard CDC methods, several continuing problems are encountered by the new molecular techniques of HLA typing. As new HLA alleles are discovered and defined, increasing numbers of SSPs and SSOPs are required for more accurate typing. Routine clinical SSP and SSOP testing usually lacks the ability to detect new alleles, unless unexpected cross-reaction patterns are observed. Nevertheless, these molecular approaches have dramatically improved histocompatibility testing for clinical transplantation and also have improved histocompatibility laboratory operating accuracy and efficiency. Previous studies have shown that 12% of kidney allografts are lost during the first year posttransplant because of acute rejection, despite complete HLA matching by conventional methods (7). This observation indicates the presence of undetected HLA mismatches in these recipient–donor pairs that play an important role in allograft rejection. With the use of new emerging techniques such as sequence-based tissue typing, it should be possible to detect a true HLA match from a list of potential related or unrelated donors in both solid organ as well as bone marrow transplantation. The result should be better graft survival, less graft-versus-host disease (GVHD), and reduced immunosuppression following transplantation.

Detection of Prior Sensitization to HLA Antigens

Even more important than the determination of the HLA phenotypes of both donor and recipient are the evaluation and identification of the anti-HLA antibodies in the serum of recipients before transplantation. A major risk factor for graft survival is host presensitization by means of prior transplants, pregnancies, or blood transfusions. Transplanted allografts into a presensitized host with circulating complement-binding antibodies can be lost within minutes to hours because of hyperacute rejection (8,9 and 10). Noncomplement-binding antibodies acting in parallel with a secondary cell-mediated immune response also can induce accelerated rejection within 72 to 96 hours posttransplantation (11). Prior sensitization to HLA antigens in prospective recipients is detected by cross-match techniques in which donor target cells, usually lymphocytes, are incubated with recipient serum in the presence of rabbit complement. Next, eosin dye is added to the serum-treated donor cells; dead targets fail to exclude the eosin dye and stain darkly, whereas viable targets exclude the eosin dye and appear as unstained refractile cells. Several modifications of this technique have improved the sensitivity of this visual method: (a) the addition of antihuman serum amplifies the detection of small but clinically relevant levels of antidonor immunoglobulin G (IgG) that otherwise would remain undetected. (b) Low levels of antibodies bound to donor cells can also be detected by means of the fluorescence-activated cell sorter (FACS), which measures the binding of a second labeled antihuman IgG antibody (6).

NATURE OF THE TARGET ALLOANTIGENS

Major Histocompatibility Antigens

The most important of the antigens expressed by the allograft are the HLAs encoded within the major histocompatibility complex (MHC) on chromosome 6. In normal immune responses, T cells recognize foreign antigens when presented as peptides in association with self-MHC molecules. Details of MHC gene structure and function are given in Chapter 3. In the transplant response, the precise molecular nature of the recognition of the foreign MHC alloantigen is less clear. Most likely, nonself-MHC alloantigens can be directly recognized, with or without peptide (12). Despite these fundamental differences, the cascade of events that occur during the process of allograft rejection appears to be essentially the same as that which occurs against foreign antigens such as bacteria or viruses. These processes lead to the recognition and destruction of the invader, in this case the allograft.

Based on basic immunology research and the transplant experience, better HLA matches correlate with better graft survival and less rejection. Table 84.2 lists the 5-year survival rates for kidney allografts. For the cadaveric organs, the six-HLA match has a 28.4% graft failure rate. The worst match, that is, six mismatches, has a much higher graft failure rate of 40.7% (1). These rates fit with the theory that MHC determines histocompatibility. The more HLAs that are matched, the more histocompatible the graft is. Yet, HLAs are not the only antigens important to histocompatibility. Again, even “perfect” kidney allografts with six HLA matches have at least a 28.4% rejection rate at 5 years (1). In addition, several studies have reported that 10% to 20% of kidney allografts are lost as a result of acute rejection, regardless of a complete HLA match and negative T- and B-cell cross-matches (7). As described above, allograft rejection is mediated largely by the recipient T cells against the highly polymorphic MHC molecules expressed on the donor cells. Other non-MHC antigens exist, however, that are fully capable of triggering graft rejection.

Minor Histocompatibility Antigens

As mentioned, an allograft transplanted between a donor and recipient matched at the MHC genes has a higher acceptance rate; however, allograft rejection still can occur. Such rejection has been caused by the disparities in polymorphic gene products called minor *histocompatibility antigens* (MiHAs) (13,14,15 and 16). It is estimated that the murine genome may contain up to 720 MiHA genes. In humans, this number is estimated to be even larger. Most of these loci are located on autosomal chromosomes, but a few also map to mitochondrial DNA and the Y chromosome (17). Because of the great number of MiHA genes, only identical twins can share all their transplantation antigens, both MHCs and MiHAs. Siblings, even if they are MHC identical, generally match for only about half the total number of MiHAs. The importance of MiHAs in transplantation is manifested by several clinical findings. First, in MHC-matched solid organ transplantations, the disparities in MiHAs often lead to graft rejection (18,19). The second involvement of MiHAs is related to the graft-versus-host disease in MHC-matched bone marrow transplantations (18,19). Third, MiHAs are also involved in the graft-versus-leukemia effect in bone marrow transplantation. The graft-versus-leukemia effect can lead to the eradication of residual host leukemic cells by the engrafted bone marrow transplant (20). The MiHAs also can cause the insidious clinical complications of chronic rejection of MHC-matched solid tissue and organ grafts.

The immune responses to single MiHAs are much less potent than responses to allogeneic MHC molecules because the frequency of responding T cells to a given MiHA is significantly lower. Indeed, MiHA-specific T cells need to be primed *in vivo* before they can be detected *in vitro* in a mixed lymphocyte reaction. The response to MiHAs is similar to the response to viral infection. All cells in the graft express the MiHA, and so the entire graft is destroyed in such responses, just as the analogous responses to tissue-specific peptides can destroy an entire tissue in autoimmunity.

Both $CD4^+$ and $CD8^+$ T-cell cooperation is required for MiHA-mediated graft rejection. Both cells recognize their specific MiHAs in a MHC-restricted manner. This cooperation was implicit from early studies on responses to H-Y antigens in which permissive and nonpermissive MHC class I and class II alleles could be identified that affected $CD8^+$ cytotoxic T-cell generation and skin graft rejection (17,21). The mouse strains expressing permissive alleles allow the generation of both $CD4^+$ T cells and $CD8^+$ cytotoxic T-effector cells; thus, the H-Y antigen can elicit a functional immune response. Whereas the mouse strains expressing nonpermissive alleles have the precursors for the $CD8^+$ cytotoxic T-effector cells, no *in vivo* responses to H-Y are generated because of the lack of appropriate $CD4^+$ T-helper function.

The major features that distinguish MiHAs from other transplantation antigens are that MiHAs are recognized by T cells but not by antibodies and that MiHAs are recognized in a MHC-restricted manner. It is now clear that MiHAs are peptides derived from polymorphic proteins that are presented by MHC molecules on the allograft (22,23). The fact that the MiHAs are peptides recognized in association with MHC molecules explains many of the features of these antigens that were known but poorly understood for a long time. First, it is difficult, if not impossible, to detect humoral responses to MiHAs, probably because most MiHAs come from intracellular proteins; thus, even if an antibody response were to occur, it could not be detected at the cell surface. Second, MiHAs do not stimulate a primary *in vitro* cell-mediated response, whereas MHC antigens evoke a powerful primary response in both mixed lymphocyte reaction and cell-mediated lympholysis assays. This is in agreement with the general difficulty in detecting *in vitro* T-cell responses to peptides of nominal protein antigens unless they have been primed *in vivo*. Third, recognition of MiHAs is MHC-restricted; that is, secondary responses require that the MiHAs be presented in association with the same MHC molecules as during the primary exposure.

These MiHA-specific T-cell clones have been used for the identification of the sequence of these peptides. Because MiHAs are recognized by T cells in the context of

MHC antigens, an obvious approach was to isolate the peptide antigens from their respective MHC molecules. After isolation of the peptides by means of high-pressure liquid chromatography and mass spectroscopy, it has been possible to identify the chemical nature of several human MiHAs presented by HLA-A2.1 and HLA-B7 molecules (11,24,25 and 26). The clinical potential of MiHAs remains to be assessed. Some areas are worth mentioning, however. GVHD is one of the most feared complications of allogeneic bone marrow transplantation (20). In HLA-identical bone marrow transplantation, disparities in MiHAs between the donor and the recipient, with the MiHA being presented by the recipient cells, can induce GVHD (19). In bone marrow recipients with severe GVHD, cytotoxic T cells specific for recipient MiHAs can be isolated from peripheral blood leukocytes (14). A retrospective study showed an association between mismatched MiHAs and the occurrence of GVHD in recipients of bone marrow from HLA-identical donors (18). The high immunogenicity of some of the MiHAs together with the recently identified amino acid composition of the peptides is the foundation for attempts at immunomodulation of GVHD. MiHA peptide analogs may function as MHC or T-cell receptor antagonists that might block the harmful T cells reactive to host MiHAs after HLA-identical bone marrow transplantation. The role of MiHAs in solid organ transplantation is still not well understood, but some recent studies indicated that MiHAs may play a role in renal allograft rejection. Cytotoxic T-lymphocytes restricted to self-MHC and reactive to MiHA can be isolated during the rejection of transplants between HLA identical donor–recipient combinations (27,28).

Tissue-Specific Antigens

A primary target in the immunopathogenesis of renal allograft rejection is the endothelium of vessels within the graft. In fact, immunopathologic studies of hyperacute, acute, and chronic rejection of kidney allografts show evidence of vascular endothelial cell damage (29). This finding suggests that vascular endothelium may be the sensitizing immunogen in a transplanted kidney because it expresses high levels of ABO, HLA, and endothelium-specific, non-HLA antigens (30,31). In fact, Moraes and Stastny (32) showed that 96% of patients who experienced acute kidney allograft rejection also developed antibodies to vascular endothelial cells and 53% showed no cytotoxicity to donor T and B cells. These results were confirmed by Cerilli et al. (33), who also found that 96% of kidney allograft recipients who rejected their transplanted organs had antibodies that reacted with vascular endothelial cells; up to 50% of these patients had no reactivity to T and B cells. These reports indicate that the antibodies detected in these patients were directed against an antigenic system that is independent of HLA and ABO antigenic systems and that is expressed on vascular endothelial cells but not on lymphocytes. In fact, a non-HLA antigenic system expressed on human vascular endothelial cells was first reported by Moraes and Stastny (32) and was shown to be of importance in the rejection process of kidney allografts (34). These studies showed that 60% of cadaveric renal allograft recipients who experienced graft rejection within 7 weeks posttransplantation had antivascular endothelial cell antibodies compared with only 2% of patients with no rejection episodes within this period of time. Moreover, in related studies, our laboratory demonstrated non-HLA complement-fixing antibodies against kidney epithelial cells in up to 78% of rejected renal allografts eluates (35,36). These eluates also reacted with monocytes but not with matched T or B cells (36). These observations strongly suggest that the presence of specific complement-binding antidonor vascular endothelial cell antibodies is highly detrimental to kidney allograft survival and that such antibodies can develop in the absence of anti-HLA antibodies.

In addition to antibodies, T cells also recognize tissue-specific antigens. Manca et al. (37) first reported that lymphocytes from a rejected human kidney allograft, expanded *in vitro*, lyse donor kidney fibroblasts but not donor leukocytes. Given the current understanding of allorecognition (Fig. 84.1), kidney fibroblasts process an intracytoplasmic, kidney-specific protein, present the peptide on class I MHC, and are recognized by $CD8^+$ T cells. Previous studies from our laboratory showed that such tissue-specific peptides, recognized by tissue-specific clones derived *in vitro*, can be isolated and identified (38). Yard et al. (39,40) also isolated kidney-specific T-cell clones from graft infiltrating lymphocytes. These tissue-specific T cells from a single cloning experiment constitute 50% of the total number of clones isolated and were all $CD8^+$ cytotoxic T cells. The high number of tissue-specific clones is consistent with the cytolytic activity of the parent graft-infiltrating T-cell line. This line recognized donor kidney epithelial cells but not donor lymphocytes. Poindexter et al. (41) also reported that more than 10% of the T-cell clones isolated from rejecting biopsies were cytotoxic only to kidney epithelial cell lines but not to HLA-matched lymphoid cells. This finding suggests a role for these cytotoxic T cells in allograft rejection. These results strengthen the hypothesis that tissue-specific $CD8^+$ cytotoxic T cells are a significant component of graft-infiltrating T-cell populations and may play an important role in allograft rejection. The same group also identified a kidney-specific nanomer peptide that is recognized by cytotoxic T-cell clones in an HLA-A3 restricted manner (38). This suggests that tissue-specific peptides may behave similar to minor histocompatibility antigens.

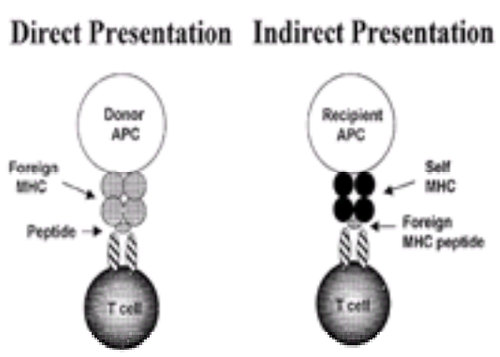

Figure 84.1. Mechanism of allorecognition. Direct allorecognition involves recognition of foreign major histocompatibility complex (MHC) class I molecules with a variety of peptides present in the antigen presentation groove. Indirect allorecognition involves recognition of peptides derived from foreign MHC molecules and presented by self-MHC class II molecules.

MECHANISMS OF ALLORECOGNITION

Various hypotheses have been proposed to explain allograft rejection. In 1955, Billingham et al. (42,43) reported that isografts (organs transplanted between members of the same inbred mouse strain) were not rejected; however, allografts were rapidly rejected in 8 to 10 days. They also noted that if a recipient receives a second skin allograft from the same donor, the second allograft is rejected more rapidly. If the second skin allograft is from a different donor strain, this rejection is not accelerated. Hence, these results were suggestive of specific immunologic recognition and memory in the rejection process. The cellular basis was then elucidated when adoptively transferred lymphocytes transferred the ability to reject the allograft. When an organ from one individual is transplanted into another nonidentical individual of the same species, several cellular and molecular immunologic processes are activated, which, if left untreated, result in rejection of the allograft. This allogeneic immune response consists of a variety of mechanisms directed against the nonself-foreign antigens expressed by the graft. Two models of alloantigen recognition have been proposed (Fig. 84.1) (8,12,17,44). In direct alloantigen recognition, the recipient's T cells recognize foreign MHC molecules directly, without processing by self-APCs (17,44). Recognition is probably due to the inherent natural affinity of the T-cell receptor for MHC molecules. This process is explained in the context of negative and positive selection in the thymus, where T cells are educated to distinguish self from nonself. T cells with too high an affinity for self-MHC molecules plus self-peptides are deleted (*negative selection*), whereas T cells with low affinity for self-MHC molecules plus self-peptides are selected for maturation and exit the thymus for the peripheral immune organs (*positive selection*). This process ensures the deletion of high-affinity autoreactive T cells and the presence in the periphery of low-affinity autoreactive but nonpathogenic T cells with the potential to recognize self-MHC molecules plus foreign peptides with high affinity. Because developing T cells with a high affinity for nonself-MHC molecules would not have been eliminated in the thymus, they should be present in the periphery. These T cells recognize foreign MHC molecules expressed by parenchymal cells of the allograft (Fig. 84.1). For more details on T-cell development, see Chapter 5.

In indirect alloantigen recognition, soluble foreign MHC molecules, possibly shed by apoptotic or necrotic parenchymal cells in the allograft, are phagocytosed and processed by self-APCs infiltrating the allograft during the rejection process. Subsequently, peptides derived from these foreign MHC molecules are recognized by $CD4^+$ T cells as epitopes presented by self-MHC class II molecules expressed on self-APCs (Fig.84.1) (8,12). This mechanism is the same as that involved in the recognition of natural foreign peptides. Several studies showed that indirect allorecognition occurs and is relevant to the process of allograft rejection (45,46 and 47). In addition, a growing body of evidence indicates that during rejection, this pathway of alloantigen recognition by $CD4^+$ T cells has a level of importance comparable to the direct pathway by $CD8^+$ cytotoxic T cells. Further, $CD4^+$T cells may be more important in chronic rejection.

APOPTOSIS IN ALLOGRAFT REJECTION

Several studies demonstrated that programmed cell death, or *apoptosis*, occurs during both acute and chronic solid organ allograft rejection (48,49). The role of apoptosis has been examined in rejection of cardiac, hepatic, and renal allografts. Studies in both animal models and humans detected apoptosis both in allograft parenchymal cells as well as in the inflammatory cell infiltrate. Lagueris et al. (50) showed that apoptosis also occurs in human cardiac allografts. In this study, the incidence of apoptosis in myocytes, capillary endothelial cells, and connective tissue cells was associated with the histologic grade of acute rejection. Two studies have provided evidence for apoptosis in chronic rejection of murine cardiac allografts (48,49). In a heterotopic murine cardiac transplant model, Bergese et al. (48) and White et al. (49) demonstrated a significant incidence of apoptosis in the periarterial cellular infiltrates associated with sclerotic changes consistent with chronic rejection. These two studies, however, did not distinguish between apoptosis in parenchymal cells and graft-infiltrating inflammatory cells.

Krams and Martinez (51) used the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-based *in situ* assay to determine the presence

of apoptosis in a rat model of orthotopic liver allograft rejection. Their studies indicated that an increased incidence of TUNEL-positive cells is observed during the first 7 days posttransplantation of liver allografts but not in syngeneic liver grafts. This increased incidence of apoptosis was associated with indicators of rejection, such as liver enzyme elevation and positive histopathology. Afford et al. (52) examined the role of apoptosis in human liver allograft rejection using the *in situ* TUNEL assay. Their studies demonstrated that hepatocyte apoptosis was common in areas of perivenular hepatocyte dropout. Apoptosis also was observed in the inflammatory mononuclear cell infiltrate in acutely rejecting allografts.

In a rat model of renal transplantation, Wang et al. (26) showed that the expression of Fas-ligand (FasL) was increased in allografts undergoing acute rejection but not in normal kidneys. In contrast, Fas expression was constitutively expressed in allografts as well as in normal kidneys. In biopsies of human kidney allografts, apoptotic cells were detected during rejection episodes (53). There is some controversy about the appearance of such cells during acute versus chronic rejection. Although apoptosis of kidney epithelial cells was preferentially observed during acute rejection in some studies, it was primarily observed during chronic kidney allograft rejection in other studies (54). Apoptosis also has been associated with acute lung allograft rejection, both in humans and in animal models (55). In addition, apoptosis-inducing Fas–FasL interactions have been shown during the development of chronic rejection in a rat lung allograft transplant model (56).

Taken together, evidence is emerging that apoptosis occurs during allograft rejection episodes. Because apoptotic cells are rapidly cleared *in vivo* by infiltrating macrophages (57), the demonstration of apoptotic cells *in situ* by TUNEL-based assays at any given time point actually might underestimate the extent of apoptosis during allograft rejection. See Chapter 12 for a more extensive discussion of apoptosis.

T-HELPER CELL TYPES 1 AND 2 CYTOKINES IN ALLOGRAFT REJECTION

Two functionally different $CD4^+$T helper (Th) cell subsets, characterized by divergent profiles of cytokine production, were identified originally by analysis of murine, and later of human, Th-cell clones (58,59). Interleukin (IL)-2, IFN-g, and tumor necrosis factor (TNF)-b are produced by murine Th1 cells, whereas the murine Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Although human Th1 and Th2 cells also produce distinct profiles of cytokines, the synthesis of IL-2, IL-6, IL-10, and IL-13 is less restricted to a single subset compared with the murine Th cell system (60). Th1 and Th2 cells also were demonstrated *in vivo,* and their presence may correlate with ongoing cell-mediated and humoral immune responses, respectively (61). Th1 cells induce activation of delayed-type hypersensitivity (DTH) reactions as well as cytotoxic and inflammatory immune responses that eliminate intracellular pathogens, whereas Th2 cells induce B-cell responses that eliminate extracellular pathogens.

A large body of evidence suggests that activation of Th1 responses is strongly associated with the allograft rejection process (62). Both IL-2 and IFN-g are readily detected in several types of organ allografts undergoing rejection (63,64 and 65). In addition, the expression of these Th1 cytokines during allograft rejection is associated with the appearance of proinflammatory mediators. Furthermore, adoptively transferred $CD4^+$T cells were sufficient to induce rejection of both MHC class I or II mismatched skin allografts in T cell-deficient mice (66). *In situ* cytokine analysis from such skin allografts demonstrated the upregulation of Th1 cytokines (IL-2 and IFN-g) but not Th2 cytokines (IL-4). In contrast, mice rejecting orthotopic liver allografts showed *in situ* upregulation of Th1 (IL-2 and IFN-g) as well as Th2 (IL-4 and IL-6) cytokine mRNA (67). In addition, both Th1 (IFN-g) and Th2 (IL-4) cytokines have been elevated during acute lung allograft rejection (68). Therefore, multiple cytokine profiles seem to be expressed *in situ* during the acute rejection process. Therefore, it is likely that Th1 effector cells synergize with Th2 effector cells during the process of allograft rejection. In this regard, both IL-4 and IL-10 have been identified as cytotoxic $CD8^+$ cell growth and differentiation factors (69,70). Taken together, these data suggest that acute allograft rejection is mediated by a mixture of Th1 and Th2 cells or by Th0 cells capable of expressing a mixture of Th1/Th2 cytokines.

Although Th2 cells directly activate B-cell proliferation and differentiation into antibody-secreting cells, which are important in chronic allograft rejection, recent studies suggest that Th2 cytokines may be involved in the *in situ* expression of genes with “protective” functions (71). These include molecules such as antiapoptotic proteins (bcl-2 and bcl-xL), proteins protective against oxidative stress (HO), as well as proteins that block the activation of the NFkB gene. Therefore, Th2 cytokines may protect organ allografts from failure associated with chronic allograft rejection.

Therefore, in view of conflicting observations, the relevance of Th1 and Th2 cells to the complex process of allograft rejection continues to be debated. Although Th2 cells often are associated with long-term graft survival, it has not been proven that they indeed mediate organ allograft tolerance. The redundant and pleiotropic nature of different cytokines *in vivo* imply that the Th1/Th2 paradigm may not be sufficient to understand the complex immune mechanisms underlying the allograft rejection process. Indeed, Th1/Th2 paradigm may not be applicable to allograft rejection because the recipient microenvironment, the type of organ allograft, and the therapeutic strategy applied all may influence the intragraft cytokine profile produced during the rejection process. See Chapter 10 and Chapter 11 for a more extensive discussion of Th1 and Th2 cells, respectively.

TYPES OF ALLOGRAFT REJECTION

There are four main types of allograft rejection: hyperacute, accelerated, acute, and chronic. The first two types of rejection represent memory responses, where antibodies were already in circulation or immediately activated on transplantation. The last two types of rejection represent primary immune responses, that is, cell-mediated immune responses (5,72,73).

As mentioned, hyperacute rejection occurs within minutes to 48 hours. Complement-fixing antibodies bind to the vascular endothelium and active complement. Such complement activation may induce thrombosis by attracting polymorphonuclear leukocytes and platelets and activating the coagulation cascade (8,9 and 10). Initially, this type of rejection was the first obstacle to successful transplantation. This condition is not treatable and demands emergency removal of the kidney allograft. Histopathologic examination can differentiate the immunologic etiology of the condition, in contrast to thrombosis from a surgical technical error (74). Antibodies reactive to incompatible blood ABO antigens or donor HLA antigens can cause hyperacute rejection. Therefore, all allografts typically are matched for the blood group antigens. Furthermore, as mentioned, a cross-match is performed before transplantation to rule out prior sensitization to donor HLA antigens. As a result of blood group matching and the cross-match test, hyperacute rejection is rarely seen in clinical renal allograft transplantation (74). The liver is an interesting exception to the preceding discussion. At least in the short term, livers have been successfully transplanted despite a blood group mismatch (3,4), possibly attributable to better immunosuppression, the decreased relative immunogenicity of the liver, or the capacity of the liver to regenerate.

Accelerated rejection occurs within 3 to 5 days posttransplantation and can be due to the binding of both complement-fixing and noncomplement-fixing antibodies. These act through antibody-dependent cellular cytotoxicity (ADCC) and natural killer (NK) cells or monocytes to produce vascular disruption. Because of the involvement of the cellular immune response, antilymphocyte reagents have some efficacy. Frequently, this condition cannot be permanently reversed, and it recurs with more severe rejection episodes until allograft removal becomes the only therapeutic alternative (3,4).

The next barrier to successful transplantation is acute rejection, which occurs between 6 and 90 days posttransplantation, but it also may occur later, during the posttransplant period, as result of treatment noncompliance (3,4). This condition is characterized histologically by cellular infiltrates with $CD8^+$ cytotoxic T cells and an inflammatory response produced by *in situ* cytokine production by $CD4^+$ Th1 cells activated through the indirect pathway of antigen presentation by self-APCs (5,12,72,73). See Table 84.3 for a comparison of acute rejection in different organs. Many animal models, later correlated with human histology, point to the importance of the $CD8^+$ cytotoxic T cell. Although a perivascular lymphocyte infiltrate characteristic of DTH reactions is frequently present, the characteristic acute rejection lesion is thought to be mediated by a direct interaction of lymphocytes with parenchymal cells. Once activated, these cells damage both the vascular endothelium and the graft parenchyma. Allograft dysfunction and failure would inevitably ensue without more immunosuppression. Of note, NK cells also may play a role. These large lymphocytes come from a similar precursor as T cells; however, NK cells do not express T-cell receptors. NK cells kill cells that do not express self-MHC class I molecules. Therefore, because the allograft does not express recipient MHC molecules, the recipient's NK cells will attack the graft. See Chapter 19 for a more extensive discussion of NK cells.

Transplant	Acute Rejection	Chronic Rejection
Kidney	Lymphocytosis, tubulitis	Fibrosis of interstitium, concentric arteriosclerosis
Liver	Portal triad lymphocytosis, inflammation of bile ducts and subendothelium	>50% bile duct loss, foamy cell obstruction of blood vessels
Heart	Lymphocytosis, myocyte necrosis	Concentric arteriosclerosis
Lung	Lymphocytes and plasma cells around blood vessels	Fibrosis narrowing airways, concentric arteriosclerosis

TABLE 84.3. Pathology of Acute and Chronic Rejection

Antibodies do not play an important role in the first episode of acute rejection. Subsequent severe episodes of acute rejection, however, frequently include an antibody-mediated immune response that induces endovasculitis with binding of lymphoid cells on swollen, ischemic endothelial cells (74). For the development of these antibodies, the $CD4^+$ T-cell response must develop first. Acute cellular rejection may therefore have both cellular and humoral components.

Chronic rejection usually appears after 60 to 90 days posttransplantation and induces the typical obliterative disease (3,4). Clinically, patients have a slow but progressive course of allograft deterioration over a period of several months to years. This condition is not responsive to available immunosuppressive strategies. Risk factors for kidney allograft rejection include several acute rejection episodes within the first 90 days posttransplantation and the presence of medium to high levels of anti-HLA antibodies (3,4). Despite the common characteristic of fibrosis, the pathology of chronic rejection differs slightly between the different organ allografts. In the kidney and heart, pathologists note increasing arteriosclerosis in glomeruli and coronary arteries, respectively. In the lung, the fibrosis centers on the airways (4), which suggests an important role of the lung epithelium as an immunologic target during the chronic rejection of the lung allograft.

The etiology and pathogenesis of acute rejection are well understood, but chronic rejection is still being elucidated. Several hypotheses have been proposed. The fibrosis in chronic rejection may be due to healing after acute rejection. After all, a strong predictor of chronic rejection is a prior episode of acute rejection; however, chronic rejection can occur in the absence of acute rejection. Other theories include ischemic injury and a chronic DTH (5). In lung transplantation, the development of antidonor HLA antibodies precedes the onset of chronic lung allograft rejection (75). Furthermore, such antibodies have the ability to activate the proliferation of both vascular endothelial cells and lung epithelial cells (70,76). Presumably, such antidonor HLA antibodies may induce the diffuse arteriosclerosis noted in chronically rejected glomeruli and coronary arteries. The mechanism of antibody induction of graft fibrosis remains unclear, however.

Recent studies of chronic rejection championed the importance of indirect alloantigen recognition by $CD4^+$ T cells. In heart, lung, and kidney transplantation, several groups noted recipient $CD4^+$ T cells indirectly recognizing mismatched donor MHC peptides presented by recipient APCs (45,46 and 47). Perhaps cytokine secretion leads to the characteristic graft fibrosis seen on histology. This points to a primary role of $CD4^+$ T cells in this process.

CLINICAL SIGNS OF ALLOGRAFT REJECTION

Kidney Allograft Rejection

Kidney allografts do better than any other solid organ allografts. As indicated in Table 84.1, 1- and 5-year rejection rates for cadaveric allografts are 10.6% and 35.3%, respectively (1). Once procured, the kidney allograft is flushed with University of Wisconsin solution and can be preserved up to 48 hours (3,4). This allows time for HLA matching. As indicated in Table 84.2, a six-HLA antigen match has a 1-year rejection rate of 28.4% compared with 40.7% in completely unmatched grafts (1). Interestingly, a completely unmatched graft from a living unrelated donor does as well as a six-HLA antigen match from a cadaveric donor (1,2).

On the operating table, within minutes of transplantation, hyperacute rejection can occur (3). Preexisting antibodies, typically against ABO blood group incompatibilities, react with donor endothelium and initiate thrombosis and ischemia (9,10,77). Without blood flow, the graft does not become pink, and no urine follows. Grossly, kidney becomes soft, cyanotic, and edematous (30). On pathology, one can detect peritubular infiltrates of polymorphonuclear leukocytes, thrombosis of arteries and glomerular capillaries, and necrosis in the cortex and tubules. Immunoglobulin and complement can be localized to the glomerular capillaries (74,78). Such immunohistochemistry suggest that this phenomenon is mediated by the aforementioned preexisting antibodies. Because of ABO matching and crossmatch test, such rejection is rare (74).

After transplantation, the recipients are closely monitored. Within days to weeks, complaints of fever, pain over the graft, and decreased urine output suggest acute rejection. Transplant physicians may look for fever and graft tenderness; however, physical findings may be subtle (3,4). Of note, similar findings 90 days after transplantation may suggest chronic rejection. Workup includes a serum creatinine measurement. An elevated serum creatinine level documents impaired glomerular infiltration. A nuclear medicine study documents impaired vascular flow to the kidney. The definitive study is a percutaneous biopsy (3,4).

Within days to months after transplantation, acute rejection can occur. Grossly, the kidney may be swollen and edematous (74). There may be focal cortical and medullary hemorrhages. On pathology, interstitial edema with cellular infiltrates of small and large lymphocytes, plasma cells, macrophages, and monocytes may be seen (74). Such cellular infiltrates may be either diffuse throughout the cortex or focal within the periglomerular, peritubular, and perivascular spaces (79). With increasing severity, parenchymal necrosis and interstitial hemorrhage may be seen (Fig. 84.2A). On the other hand, acute rejection may show primarily vascular components. The arteries and arterioles may show subendothelial mononuclear infiltrates or arteritis (74,80). With increasing severity, one may even see fibrinoid thrombus and necrosis. Of note, a severe vascular rejection responds poorly to immunosuppression (30).

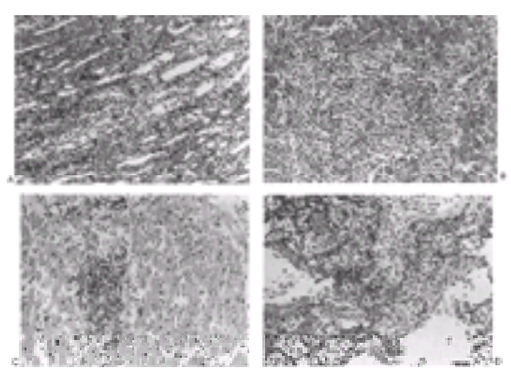

Figure 84.2. Histopathology of acute allograft rejection. A: Kidney: interstitial lymphocytic infiltrate with tubulitis. B: Liver: lymphocytic infiltrate and obliteration of structures in the portal triad. C: Heart: myocytic degeneration with interstitial lymphocytic infiltrate and edema. D: Lung: perivascular lymphocytic infiltrate. (From Kahan BD, Clark JH. Transplantation of solid organs. In: Frank MM, Austen KF, Claman HN, et al., eds. *Samter's immunologic diseases*, 5th ed. New York: Little, Brown and Company, 1995:1502, with permission.)

Sixty to 90 days after transplantation, patients may show subtle signs of chronic rejection. They may have a gradually worsening renal function, with increasing blood pressure, proteinuria, and serum creatinine. Gross pathology can show either normal size or shrunken kidneys, and the cortex may be pale (74). On histology, one primarily notes changes in the arterial circulation. Concentric, subintimal proliferation of myofibroblasts occurs in both small and large arteries. Such proliferation narrows and even occludes the arteries (78). Other common findings include atrophy of tubules, thickening of the basement membranes of tubules, fibrosis in the interstitium, and collapse of the glomeruli. These changes may be attributable to an ischemic injury (30,78,74). Fibrosis, rather than cellular infiltrates, dominates the picture of chronic rejection(Fig. 84.3A).

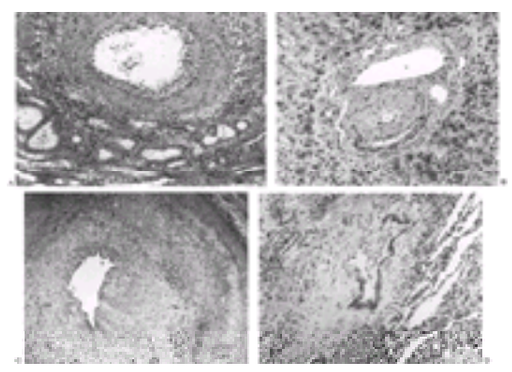

Figure 84.3. Histopathology of chronic allograft rejection. A: Kidney: luminal narrowing resulting from intimal proliferation and foamy macrophages. B: Liver: bile duct degeneration and portal triad fibrosis. C: Heart: coronary artery atherosclerosis with intimal proliferation and concentric narrowing. D: Lung: fibrosis and eccentric scarring of the bronchiole. (From Kahan BD, Clark JH. Transplantation of solid organs. In: Frank MM, Austen KF, Claman HN, et al., eds. *Samter's immunologic*

diseases, 5th ed. New York: Little, Brown and Company, 1995:1503, with permission.)

Liver Allograft Rejection

The liver is unique among the solid organs. Despite ABO blood group incompatibilities, the liver can be successfully transplanted, at least in the short term (3,4). Furthermore, for all liver transplants, the 5-year graft failure rate compares favorably with kidney allografts, the most successful transplants available (1). Why are livers so well tolerated? Some point to the many phagocytic Kupffer cells and donor leukocytes within the allograft. They suggest that chimerism, a “mixing” of donor and recipient leukocytes, can lead to tolerance. Still, many other hypotheses exist, such as the shedding of donor HLA antigen, regeneration potential, and others (4,51).

The liver is special for two other reasons. First, living-related donation may be less of an ethical challenge. After all, one only needs 25% of one's liver to live. Furthermore, the remaining liver can regenerate to the original size. Second, because the liver has two lobes, cadaveric livers have been successfully “split” between two recipients (3,4).

Currently, as a result of ABO blood group matching, hyperacute rejection in liver transplants is rare but has been documented. Some liver allografts fail within hours to days, despite no evidence of antibody induced thrombosis. Such failure, probably not immune mediated, has been termed *primary nonfunction.* Although no single risk factor has been proven in clinical studies, investigators have proposed donor hypotension, donor hepatic steatosis, and preservation injury (4).

Within days to weeks of transplantation, acute rejection can occur. Patients complain of fever, increased ascites, and pain over the graft. On further evaluation, physicians may notice both graft tenderness and a decreased amount of bile output. Initial laboratory tests may note elevated transaminases, bilirubin, and white count (3). As in kidney allografts, biopsy is the gold standard. The pathologist focuses on the portal triads and central veins. Three findings are particularly important. First, there may be a moderate to severe cellular infiltrate of lymphocytes and eosinophils, particularly in the portal triads. Still, a few plasma cells and eosinophils may be found. Second, the inflammatory cells also fill the bile ducts, often with damage to the biliary epithelium. Finally, the portal and central veins show cellular infiltrates beneath the subendothelium (4,81). The presence of all three findings differentiates acute rejection from other processes (Fig. 84.2B). For example, the biliary damage looks similar in primary biliary cirrhosis and chronic GVHD (82).

Sixty days after transplantation, chronic rejection may occur. Overall, one sees signs of portal triad atrophy and fibrosis. Diagnosis of chronic rejection require examination of at least 20 portal triads. Typically, more than 50% of the bile ducts are lost. Some have called this the *vanishing bile duct syndrome* (4). In addition, beneath the intima, foam cells (cholesterol-filled macrophages) and fibroblasts may proliferate and occlude the medium-sized arteries, perhaps adding an ischemic component to the vascular injury (81) (Fig. 84.3B).

Heart Allograft Rejection

Patients with congenital abnormalities, ischemic cardiomyopathy, and other causes of severe heart failure are candidates for heart transplants. As in other transplants, there are far more potential recipients than donors. While waiting, patients can be palliated with mechanical devices, including left ventricular assist devices and the Jarvik totally artificial heart. Patients are so sick and the hearts are so difficult to preserve that HLA matching does not play a significant role; however, the cross match test is performed to rule out prior sensitization to donor HLA antigens (3,4).

After transplantation, signs of heart failure, i.e. worsening shortness of breath, may prompt an evaluation. On lung exam, there may be diffuse crackles. The patient may also have significant jugular venous distension (3). Most rejections, however, can be detected before symptoms develop.

Patients undergo an aggressive surveillance biopsy regimen. Under fluoroscopic guidance, a catheter is threaded through the internal jugular vein into the right ventricle, and a biopsy is obtained from the right ventricular wall. If left ventricular failure is suspected, the femoral artery can be accessed so that a left ventricular biopsy can be obtained (3).

The International Society of Heart and Lung Transplantation standardized a scale for grading (0–4) acute rejection in endocardial biopsies. Grade 0 consists of normal myocardium. Mild inflammation may show a focal lymphocytic infiltrate around blood vessels or in the interstitium, without any evidence of necrosis. With increasing severity, the lymphocytic infiltrate becomes more diffuse with increasing myocyte injury and necrosis. The most severe acute rejection, grade 4, shows a more polymorphous infiltrate, with edema, hemorrhage, vasculitis, and myocyte necrosis (83,84) (Fig. 84.2C).

Chronic rejection presents as accelerated atherosclerosis in the intramuscular coronary arteries. Such rejection is the leading cause of death and graft loss after 1 year (4). Because the donor heart is denervated, the patient does not complain of chest pain. Thus, such patients routinely undergo annual cardiac catheterizations. Unfortunately, this modality is insensitive. Thus, these patients are also monitored by endocardial biopsy (4). On pathology, the subendocardial coronary arteries exhibit concentric subintimal proliferation of myofibroblasts and lipid-filled macrophages, medial sclerosis, interruption of the internal elastic lamina, and thickening of the intimal ground substance. As a result, the subendocardial and intramuscular arteries are severely narrowed (84) (Fig. 84.3C). Epicardial coronary arteries may also be involved. Unlike in nontransplanted patients with coronary artery disease, the transplanted, chronically rejected epicardial arteries are rarely calcified and contain more cells. Given the diffuse nature of the disease, the concentric lesions, and the high incidence in all age groups, such atherosclerosis is probably immune-mediated. Unfortunately, retransplantation is the only option for such patients (4).

Lung Allograft Rejection

In children with cystic fibrosis or pulmonary hypertension, bilateral lung transplantation is a viable option, particularly from living donors. In adults, emphysema is the most common indication. With a 1-year graft failure rate of 23.7%, lung transplantation provides good short-term palliation. With a 5-year graft failure rate of 58%, lung transplantation as a long-term solution suffers from chronic rejection (1).

During acute rejection, recipients may complain of fever, cough, increasing shortness of breath, and decreasing exercise tolerance. Usually, the complaints may be nonspecific. The transplant physician may begin with two noninvasive tests: chest radiograph and pulmonary function tests, both of which are nonspecific. The best test is bronchoscopy because the pulmonologist or surgeon can view the anastomosis and bronchial epithelium directly. Furthermore, transbronchial biopsy or bronchoalveolar lavage can then be done (3,4). As for heart transplantation, pathologic grading has been standardized by the International Society of Heart and Lung Transplantation (83).

Like the other solid organ allografts, ABO blood group matching has made hyperacute rejection a rare entity. Some groups propose that prior pregnancies, blood transfusion, or transplants induce the antibodies against donor MHC molecules. Thus, within minutes to hours of transplantation, the graft may fail. The patient's new lungs become less compliant, and hypoxemia results. On pathology, diffuse thrombosis, inflammation, and necrosis can be seen in the microcirculation (4). To prevent such rejection, most of the lung transplant centers perform assays to detect prior sensitization to HLA antigens. If such sensitization is found, a prospective cross match is performed to select the donors.

Between 4 days to 3 weeks, acute rejection can occur. As mentioned, patients complain of fever, shortness of breath, and decreasing exercise tolerance (3,4). Pathology either from bronchoscopy with biopsy or even from open biopsy is the gold standard for diagnosis. On pathology, one sees many lymphocytes, plasma cells, eosinophils, and neutrophils in the walls of the veins, venules, arterioles, and even medium-sized arteries. There may even be inflammation of the endothelium. Similar changes are seen in the airways, from the bronchi to the respiratory bronchioles. With increasing severity, greater infiltrates, a higher number of involved blood vessels or airways, and even necrosis may be seen. Phagocytic cells, fibrinous exudates, and blood may spill over into the alveoli (4) (Fig. 84.2D). The International Society of Heart and Lung Transplantation standardized a scale (A0–A4) to grade such rejection. Grade A0 shows no such changes. Grades A1 through A4 show minimal, mild, moderate, and severe inflammation, respectively (83).

Chronic rejection deserves special mention. This poorly understood process, bronchiolitis obliterans, involves increasing fibrosis in the airways. With increasing fibrosis, the airways narrow, and the patient has difficulty exhaling. This problem can be documented objectively by the pulmonary function test, the forced expiratory volume in 1 second (FEV_1). With worsening airway obstruction, the FEV_1 diminishes (3). This diagnosis can be verified with biopsy. Because the lesions may be scattered, bronchoscopy with transbronchial biopsy might miss the lesions. On pathology, in early stages, the bronchiolar epithelium and walls become infiltrated with lymphocytes, plasma cells, and histiocytes, followed by concentric, submucosal edema and proliferation of capillaries and fibroblasts. Over time, the infiltrates resolve, and the remaining fibrosis concentrically constricts the bronchiole, resulting in the clinical airway obstruction (Fig. 84.3D). Because of the inability to clear secretions, a pneumonia may result and exacerbate the clinical picture. Similar changes occur within the pulmonary vasculature. The concentric subintimal proliferation of fibroblasts in large elastic and small muscular inflammation results in accelerated pulmonary atherosclerosis. The arteriosclerotic plaques may show foamy histiocytes, cholesterol clefts, and myofibroblasts. Unfortunately, once diagnosed, either by clinical symptoms or by pathology, bronchiolitis obliterans is an irreversible, progressive disease

that responds poorly to aggressive immunosuppression (4). Thus, early prognostic indicators would be helpful.

Although incompletely understood, details of the pathogenesis are emerging. In adult lung transplant recipients, antibodies to mismatched donor HLA molecules precede the onset of bronchiolitis obliterans by about 20 months (75). It has been shown that antidonor HLA antibodies can induce calcium influx and new tyrosine phosphorylation on binding to lung epithelial cells (70,76). Such changes activate the epithelial cells and lead to cytokine secretion. Such cytokines could activate underlying smooth muscle cells and fibroblasts to create the characteristic fibrosis of chronic rejection. A significantly increased precursor frequency of T cells reactive to peptides derived from mismatched donor class I HLA and presented on recipient MHC also has been seen in lung transplant recipients with bronchiolitis obliterans syndrome (BOS) (46). This suggests a role for $CD4^+$ T cells and indirect antigen presentation of donor HLA peptides in the pathogenesis of BOS. Further studies to elucidate the pathogenesis of bronchiolitis obliterans may improve the treatment and prognosis of lung transplants.

IMMUNOSUPPRESSIVE TREATMENT OF ALLOGRAFT REJECTION

As evident from the preceding discussion, many modalities are now available to prevent rejection. By matching ABO blood type and avoiding a positive cross match, recipients are unlikely to have preexisting antibodies that will induce hyperacute rejection. To prevent acute rejection, patients are immunosuppressed. They may be induced, before transplantation, with anti-T-cell antibodies. Afterward, they receive azathioprine/mycophenolate mofetil, cyclosporine/FK506, and corticosteroids. New drugs are continually being developed. For example, sirolimus (85) and intravenous immunoglobulin (86) are showing promise. Details of the mechanism of action for various immunosuppressive drugs, either currently available or being developed, are discussed in Section X: Therapeutic Immunology.

If a patient has acute rejection, he or she is admitted for high-dose steroids and antithymocyte antibodies such as OKT3. For kidney patients, supportive therapy such as dialysis may be needed. Then the patient's maintenance immunosuppression may be changed from cyclosporine and azathioprine to FK506 and mycophenolate mofetil (3).

Chronic rejection is much more difficult to treat. Current immunosuppressive therapy has little effect. Some recipients have lost so much graft function that they may need to be undergo transplantation again.

FUTURE DIRECTIONS

Giving blood transfusions before transplantation decreases the incidence of rejection (3). A natural extension of blood transfusion, that is, bone marrow transplantation, also induces tolerance to donor stem alloantigens. This observation led to studies in which donor bone marrow is administrated before solid organ transplantation to make the recipient tolerant to subsequent allografts (87). Results from these studies are too preliminary at present to advocate this as a general approach to induce organ-specific tolerance.

In oral tolerance models, several studies observed that the oral administration of a given antigen induces a specific Th2 immune response to that antigen. Therefore, when rechallenged with the same antigen given intraperitoneally, orally treated animals do not develop a proinflammatory immune response to the antigens (88). It is likely that these studies may provide a means to induce specific tolerance to an alloantigen. Currently, however, how to use such modalities to induce organ-specific tolerance is not clear.

It is generally accepted that T cells need two signals to become activated. T cells must see antigen (signal 1) and a costimulatory molecule (signal 2). Various studies documented the importance of B7.1, B7.2, and CD40 in eliciting allograft immunity (89,90). Based on these studies, several groups developed methods to block such costimulation resulting in graft-specific unresponsiveness. For example, recent studies with monoclonal antibody specific for CD40 ligand resulted in prolonged allograft survival of kidney (91) and pancreatic islet allografts (92). It needs to be seen whether prolonged acceptance of the graft with this immunosuppressive reagent can cause specific allograft tolerance.

As progress in our understanding of the basic cellular and molecular mechanisms of allograft rejection is made, there is new hope for the development of better immunosuppressive treatment protocols and, eventually, the attainment of organ-specific tolerance. Several techniques for the generation of allospecific immune tolerance in experimental models already have been successful. It is still not possible, however, to accomplish routine clinical organ transplantation without the use of nonspecific immunosuppression. The ultimate goal of transplantation research is to induce organ-specific tolerance to obviate the iatrogenic diseases caused by allograft rejection and the adverse secondary effects of immunosuppressive agents.

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85 IMMUNOLOGY OF XENOTRANSPLANTATION

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The transplantation of organs, tissues, or cells between individuals of different species is referred to as *xenotransplantation*. In recent years, xenotransplantation has been of increasing interest because the use of animals as organ and tissue donors as a source of transplants could overcome the severe and worsening shortage of human organs available for transplantation. This shortage restricts the application of organ transplantation to 5% to 15% of the patients who might benefit in the United States (1), and it is now widely acknowledged to be the major limitation of transplantation. Another reason for interest in xenotransplantation is that the use of animal organs or tissues in lieu of human organs or tissues might, in some cases, avoid the susceptibility to viral infection. Avoiding viral infection was the rationale for several baboon-to-human liver transplants (2) and for the transplantation of baboon bone marrow in a human patient with acquired immunodeficiency syndrome (AIDS). Another reason for interest in xenotransplantation is that, through genetic engineering of the xenotransplant source, the xenograft might provide a vehicle for the delivery of a gene or a diverse set of genes, the expression of which might be difficult to gain enduringly through other means (3). For example, transgenic techniques might be used to express antiviral genes in stem cells.

TISSUE SOURCE AND DONOR FACTORS

The type of organ or tissue transplanted and the phylogenetic distance between the donor and the recipient has profound importance for the immune response to xenotransplantation (Fig. 85.1). The type of organ or tissue grafted determines the nature of the blood supply and, thus, the nature of the contact between the transplant and the immune system of the host (Fig. 85.2), and it determines the relative contribution of donor and host “growth” factors to the survival and function of the graft. The phylogenetic distance determines biologic and immunologic compatibility.

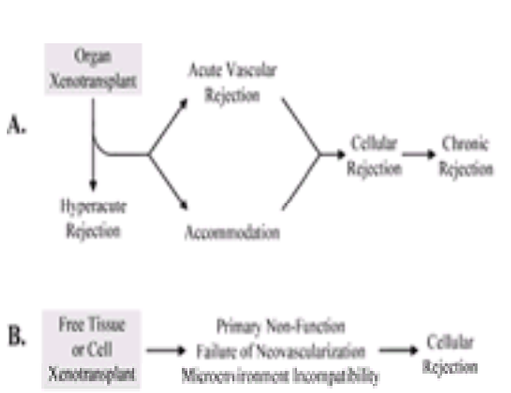

Figure 85.1. The immunologic response to xenotransplantation. The immune response to xenotransplantation can be classified according to whether the graft consists of isolated cells or free tissues, such as islets of Langerhans or of a primarily vascularized organ, such as the kidney or heart. A: Vascularized organ grafts are subject to hyperacute and acute vascular rejection caused by the action of antidonor antibodies on donor endothelium. If hyperacute or acute vascular rejection are averted, the graft may undergo accommodation, a condition in which the graft appears to resist injury despite the return of antidonor antibodies to the circulation and the presence of an intact complement system. A vascularized organ graft also may be subject to cellular rejection and chronic rejection more or less like the corresponding types of rejection observed in allografts. B: Free-tissue grafts are subject to failure caused by primary nonfunction, failure of neovascularization or failure of the microenvironment to support the survival, and function of the foreign tissue. If the free tissue or isolated cells engraft, they are then subject to cellular or humoral rejection. (Reprinted and adapted from *Nature* 1998;392 (Suppl):11–17, Hamilton Magazines, Ltd. with permission.)

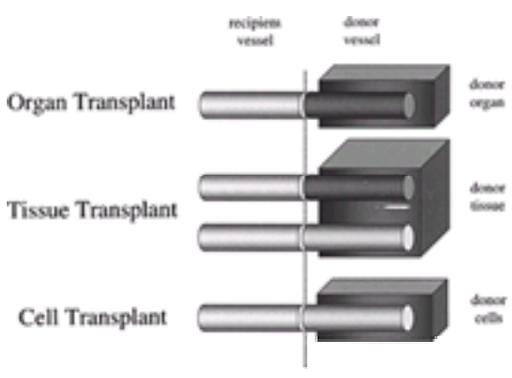

Figure 85.2. Antigen presentation in a free tissue graft: implications for the immunogenicity of xenografts. Free-tissue xenografts are generally vascularized by spontaneous anastomosis of donor and recipient blood vessels and by neovascularization. Spontaneous anastomosis (organ and tissue transplant) allows the presentation of foreign antigen by foreign major histocompatibility complex (MHC) as modeled in the upper left of Figure 85.3. Neovascularization (tissue and cell transplant) results in presentation of foreign antigen by recipient MHC as in the upper right of Figure 85.3. If neovascularization or spontaneous anastomosis were to be impaired, the mechanism of antigen presentation might differ from the mechanism that would predominate in a free tissue allograft.

Xenografts consisting of isolated cells, such as hepatocytes, derive their vascular supply entirely by the ingrowth of blood vessels of the recipient (4) (Table 85.1). Free tissue grafts, such as pancreatic islets or skin, derive their vascular supply in part from the host and in part from the spontaneous anastomosis of donor and recipient blood vessels. The blood vessels of the recipient in cell and tissue grafts pose a barrier between the graft and the immune system of the recipient. This barrier may be sufficient to allow survival of xenografts with no more immunosuppression than allografts (5). In principle, blood vessels of recipient origin might take up and present antigen of the donor. Antigen presented in this way to T-lymphocytes is said to be presented through the “indirect” pathway (6) (Fig. 85.3). The type of rejection typically

seen in cell and tissue xenografts is cellular rejection.

[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
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TABLE 85.1. Classification of Xenografts

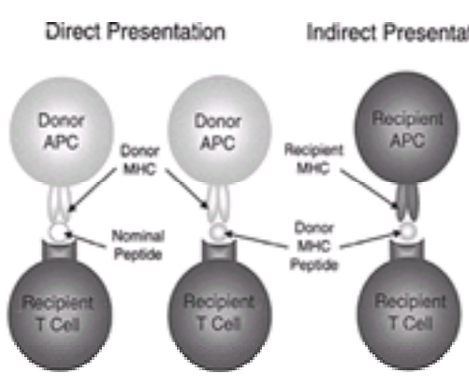

Figure 85.3. Antigen presentation in xenotransplantation. Antigen may be presented by the direct or indirect pathways. When direct antigen presentation occurs, the T cells of the recipient recognize undenatured MHC antigen of the donor expressed by donor antigen-presenting cells (APCs) or other donor cells. The peptide associated with MHC is of donor origin and either dictates specificity or may be nominal with regard to specificity of recognition. For indirect antigen presentation, the T cells of the recipient recognizes donor peptide expressed in association with MHC of the recipient.

In contrast to cellular xenografts, organ xenografts provide their own blood vessels. The interaction of the immune system of the recipient with donor blood vessels gives rise to distinct types of vascular or humoral rejection (Fig. 85.1). Organ xenografts are also subject to cellular rejection. The donor blood vessels of organ xenografts may present antigen to T-lymphocytes through the “direct” pathway (Fig. 85.3). The nature of the blood supply and the microenvironment also determine the biologic viability of the graft (Table 85.1). Cell and tissue grafts may depend on growth factors of recipient origin. If these growth factors are not compatible with the xenogeneic cells, the graft may fail. An important example of such incompatibility is found in the transplantation of xenogeneic bone marrow (7). In contrast, organ xenografts generally provide the factors needed for survival of the xenogeneic cells in the graft.

Genetic differences between the donor and the recipient are another important factor in xenotransplantation (Table 85.2). Phylogenetic distance between the donor and the recipient, of course, determines the number of antigens that might serve as a target of the immune response; however, certain genetically controlled traits have a disproportionate impact on the outcome of a xenograft. For example, the expression of a functional a1,3-galactosyltransferase gene in lower mammals leads to the synthesis of Gala1-3Gal as the terminus of some oligosaccharide chains. This saccharide is recognized by naturally occurring antibodies made by all immunocompetent humans and animals that do not express that sugar (8). This antigen–antibody system constitutes a severe immunologic barrier to xenotransplantation; yet the distribution of that glycosyltransferase in phylogeny is not a function of genetic distance (Table 85.3). Another trait that is presumably under genetic control involves the expression of cell surface molecules that promote activation of the alternative complement pathway of other species, independent of anti-body binding (Table 85.4). For example, the cells of the guinea pig allow unrestrained activation of the rat alternative pathway of complement, and, as a result, organs of guinea pigs transplanted into rats are subject to rapid and vigorous rejection (9,10). Fortunately, the alternative pathway of the human complement system is not activated primarily on porcine cell surfaces (11,12 and 13), and, thus, pigs may serve as a useful source of cells and organs for xenotransplantation into humans. Other genetic differences that may contribute to the outcome of xenografts include the species-specific functioning of cell-associated complement regulatory proteins, such as decay accelerating factor (DAF), CD59 (14), and the potential species-specific functioning of the thrombomodulin vis-a-vis thrombin and protein C (15).

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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[illegible]

TABLE 85.2. Phylogeny and the Susceptibility to Hyperacute Rejection

Species	α1,3-Galactosyl Transferase	Expression of Gala1-3Gal	Natural Anti-Gala1-3Ga Antibodies
Chicken	–	–	+
Mouse	+	+	–
Rat	+	+	–
Pig	+	+	–
Dog	+	+	–
New World monkey	+	+	–
Old World monkey	–	–	+
Baboon	–	–	+
Human	–	–	+

TABLE 85.3. Phylogeny of Gala1-3Gal and Natural Antibodies Specific for that Saccharide

Cells	Serum									
	Cow	Rabbit	Horse	Pigeon	Pig	Human	Dog	Rat	Guinea pig	Sheep
Cow		<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Rabbit	1.5		<0.03	1.4	0.50	1.00	1.7	0.35	0.46	0.41
Horse	0.50	<0.03		0.67	0.41	0.46	2.34	0.24	<0.03	<0.03
Pigeon	1.34	0.48	<0.03		1.4	0.57	1.76	<0.03	<0.03	<0.03
Pig	<0.03	<0.03	<0.03	1.3		<0.03	2.46	0.28	<0.03	<0.03
Human	>4.4	<0.03	<0.03	0.35	0.48		<0.03	<0.03	<0.03	0.13
Dog	<0.03	1.46	1.4	1.65	0.50	4.4		4.1	4.1	>4.4
Rat	0.24	0.28	<0.03	0.57	0.29	0.46	2.1		0.41	0.28
Guinea pig	>4.4	0.46	<0.03	0.65	1.1	0.65	2.46	0.61		>4.4
Sheep	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	0.71	<0.03	<0.03	

From the number of units of complement per milliliter of serum of 12 different species capable of using [illegible] and the [illegible] of the species by alternative pathway.

TABLE 85.4. Activation of Complement on Xenogeneic Cells Via the Alternative Pathway[a]

SELECTION OF A DONOR SPECIES

Immunologic and genetic factors such as those previously described would argue for the selection of species for xenograft sources that are genetically similar to xenograft recipients. For humans, such donors might include the chimpanzee and the baboon. For several reasons, however, pigs rather than nonhuman primates are currently viewed as the most promising sources of xenografts. First, pigs are available in large numbers and in appropriate sizes; in contrast, nonhuman primates are relatively scarce and generally are much smaller than adult human men. Second, the use of pigs does not raise such severe concerns regarding zoonosis as the use of a species more closely related to humans. Third, there is the possibility of genetically engineering pigs using technologies that, for practical reasons, could not be applied to nonhuman primates.

One major hurdle to using the pig as a source of organs for clinical transplantation is that the organs of the pig are subject to severe and irreversible rejection reactions that occur when porcine organs are transplanted into primates (16). Until recently, these rejection reactions seemed to pose an insurmountable hurdle to clinical application of xenotransplantation. Studies during the past few years, however, revealed much about the molecular basis of xenotransplant rejection, particularly humoral rejection, and thus have encouraged the view that these reactions might be addressed by novel and specific therapies.

This chapter summarizes some of the recent progress regarding the humoral immune reactions that pose a hurdle to transplanting porcine organs into humans. The reader is referred to recent reviews for more detailed consideration of this subject (16,17,18 and 19).

BIOLOGIC RESPONSES TO XENOTRANSPLANTATION

Figure 85.1 shows a model for the biological responses to xenotransplantation. These responses, which manifest predominantly as rejection reactions, are also seen in allotransplants, albeit with less severity and frequency. The sections that follow summarize the manifestations of rejection of organ xenografts and the immune mechanisms that underlie these manifestations. A later section of the chapter is devoted to cell and tissue xenografts that are susceptible mainly to cellular rejection.

Whole-organ xenografts are subject to various types of rejection (Fig. 85.1). For the most part, these types of rejection are manifested as vascular disease arising in response to the interaction of the immune system of the recipient with donor blood vessels. Figuring predominantly in these types of rejection is intravascular coagulation caused by the conversion of blood vessels from anticoagulant to procoagulant and ischemia caused by the constriction or occlusion of blood vessels in the graft.

Free-tissue xenografts are not subject to hyperacute rejection but generally undergo rejection over a period of days, a tempo often more rapid than that observed for free tissue allografts. Rejecting free tissue xenografts may be infiltrated by host T cells and macrophages (20,21 and 22). Free tissue and cellular xenotransplants also may fail because of “primary nonfunction” (23), which may result from immune or nonimmune factors. The extent to which free-tissue xenografts are subject to humoral rejection following vascularization has not been established; however, studies involving the transfer of immune serum demonstrate that antidonor antibodies can mediate rejection of free-tissue grafts and, thus, suggest that humoral responses could be an important determinant of graft outcome (24,25 and 26).

Recent work in our laboratory and the laboratories of others elucidated much about the molecular mechanisms underlying xenograft rejection. These new insights are the subjects of this review. This review discusses those aspects of cellular and chronic rejection that might differ from the corresponding types of rejection seen in allografts.

HYPERACUTE REJECTION

Organs transplanted between certain species are subject to hyperacute xenograft rejection (27,28) (Table 85.2, Fig. 85.4). Combinations of donor and recipient species in which hyperacute rejection regularly occurs in unmodified recipients are called *discordant*; combinations of donor and recipient species in which hyperacute rejection rarely occurs are called *concordant* (29). In most, but not all, cases, susceptibility to hyperacute rejection and, thus, discordance is found to be a function of phylogenetic distance between donor and recipient (Table 85.2). Hyperacute xenograft rejection is characterized histologically by interstitial hemorrhage, edema, platelet thrombi, and severe injury to endothelial cells (16,30,31,32,33 and 34). In some cases, prominent infiltration of neutrophils is observed. Evidence of injury to graft endothelial cells—the presumed target of the hyperacute rejection reaction—includes swelling, vesiculation, alteration in cellular junctions, detachment, and, in some cases, lysis.

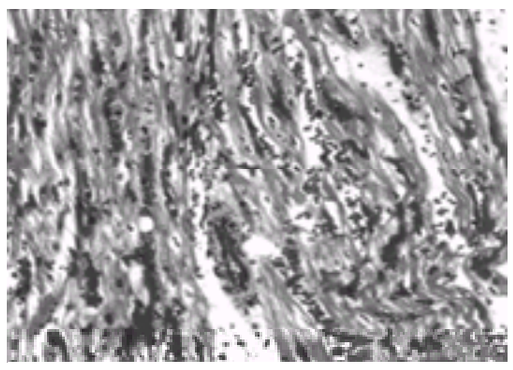

Figure 85.4. The histology of hyperacute rejection. Porcine cardiac tissue 30 minutes after heterotopic transplantation into a baboon. Hyperacute rejection is characterized by interstitial hemorrhage, edema, and thrombosis.

The pathogenesis of hyperacute rejection depends absolutely on the activation of complement, which, as discussed later in the text, may occur as a result of (a) the binding to the graft of complement-fixing xenoreactive antibodies, (b) the direct activation of complement via the alternative complement pathway on the foreign cell surfaces, and/or (c) the failure of complement regulation in the foreign organ (35). Regardless of how complement is activated, most evidence suggests that endothelial cells are the primary target of complement in this type of rejection (12,14). In addition to the structural damage to endothelium, the immunopathology of hyperacute rejection invariably reveals complement components and often immunoglobulin of the recipient along endothelial surfaces of graft blood vessels (10,12,30,32,36,37). The earliest structural change is the aggregation of platelets in small blood vessels (38). Platelet aggregation may be caused by various factors, including the interaction of platelets with matrix exposed by the action of complement on endothelium and the direct effects of complement on platelets (39). Our concept of the pathophysiology of hyperacute rejection is that it involves the loss of barrier and anticoagulant functions of endothelial cells (18). The loss of these functions allows the escape of vascular contents from small blood vessels and the attachment and aggregation of platelets leading to formation of platelet thrombi. The sections that follow discuss the components of the immune system that contribute to the development of hyperacute rejection and the mechanism through which these components might bring about hyperacute rejection.

Xenoreactive Natural Antibodies

Xenoreactive natural antibodies are thought to initiate hyperacute rejection of organs from lower mammals transplanted into humans and nonhuman primates. Xenoreactive natural antibodies are among the naturally occurring antibodies that are synthesized without a known history of sensitization (40,41). Some natural antibodies are “polyreactive” because they bind to multiple target antigens; other natural antibodies recognize blood groups (40,42,43) and bind to the surface of xenogeneic cells (44). Xenoreactive antibodies appear to be related to some anti–blood group antibodies, appear to be distinct from polyreactive antibodies (8,45), and have been implicated in the pathogenesis of xenograft rejection, particularly hyperacute xenograft rejection (27,46).

The concept that natural antibodies initiate xenograft rejection has dominated the field of xenotransplantation for 30 years (27,47). Perper and Najarian suggested that a given individual is particularly likely to have cytotoxic natural antibodies specific for the cells of disparate species and these antibodies initiate hyperacute xenograft rejection (27). Several observations point to the importance of natural antibodies in the immunopathogenesis of hyperacute xenograft rejection. First, naturally occurring antibodies directed against donor cells can be found in the serum of all mammalian species combinations (40,41). Second, xenoreactive antibodies are rapidly deposited in xenografted organs (12,28). Third, depletion of xenoreactive antibodies prevents hyperacute rejection of a subsequently engrafted organ (12,27,30). Fourth, hyperacute rejection does not occur in some species combinations in which the newborn lacks circulating xenoreactive antibodies (48). Fifth, hyperacute rejection can be induced by the administration of xenoreactive antibodies to a xenograft recipient (36).

A major advance in the field of xenotransplantation came with determination of the specificity of xenoreactive natural antibodies. Although it might be expected that the repertoire of xenoreactive natural antibodies serum would be broad, human xenoreactive antibodies predominantly recognize one epitope, Gala1-3Gal (49,50,51 and 52). Gala1-3Gal was studied extensively by Galili and colleagues (53,54), who showed that the sugar is expressed on the cells of New World monkeys and lower mammals but not on the cells of humans, apes, or baboons (Table 85.3) (51). Humans, apes, and baboons, which do not express the sugar, have specific natural antibodies for it (55). Most individuals have 5 to 40 µg of immunoglobulin M (IgM) per milliliter of plasma directed against Gala1-3Gal (56). The amount of IgG specific for Gala1-3Gal varies from 0 to 20 µg per milliliter of plasma (45). Parker and colleagues showed that the functional properties and concentration in serum of xenoreactive natural antibodies specific for Gala1-3Gal are similar to those of antibodies against blood group A and B antigens and, on this basis, proposed that the xenoreactive natural antibodies and the anti–blood group A and B antibodies may be members of a common family of natural antibodies (8,45,56).

Evidence that Gala1-3Gal might be important in xenotransplantation was first suggested by Good et al., who found that the binding of antibodies in human serum to porcine cells could be blocked by purified Gala1-3Gal but not by unrelated structures (49). Neethling et al. (52) found that antibodies directed against Gala1-3Gal can be eluted from porcine organs perfused by human plasma. Sandrin et al. demonstrated that transfection of COS cells with the murine a1,3-galactosyltransferase gene, which catalyzes the addition of aGal residues to Galb1-4GlcNAc-R to yield Gala1-3Galb1-4GlcNAc-R, induces binding of human natural antibodies to the transfected cells (50). Collins et al. demonstrated that enzymatic removal of a-galactose residues from porcine cells abrogates binding of xenoreactive antibodies to the treated cells (51). Collins et al. also showed that expression of Gala1-3Gal provides a sufficient basis for the development of hyperacute xenograft rejection when the heart of a New World monkey is transplanted into a baboon, which has antibodies specific for Gala1-3Gal (57). Sachs and Sablinski (58) and Kozlowski et al. (59) showed that depletion of Gala1-3Gal from the blood of baboons prevents the hyperacute rejection of porcine organ xenografts (58,59).

Of natural anti-Gala1-3Gal antibodies, it is IgM rather than IgG that appears to be of greatest significance in initiating the rejection of porcine organs by primate recipients. Although both human natural IgM and IgG anti-Gala1-3Gal bind to porcine cells (44), it is the binding of IgM and not of IgG that predominately initiates complement activation (13,56,60). In fact, xenoreactive natural IgG may consist largely of IgG2, which inhibits complement activation by blocking the binding of IgM (61). Consistent with this concept is the observation that administration of human IgG to a xenograft recipient does not promote the rejection of a xenograft; in fact, by diverting reactive complement proteins away from the graft endothelium, it may prevent hyperacute rejection (62). Kearns-Jonker et al. showed that antibodies may be restricted to the IGHV 3-11 and IGHV 3-74 germline progenitors in humans exposed to bioartificial livers containing porcine hepatocytes (63).

Although the epitope recognized by xenoreactive antibodies is a simple sugar, the conditions that allow these antibodies to bind to that epitope are complex. Parker et al. (56), Platt and Holzknecht (64), Cotterell and colleagues (65) showed that expression of the Gala1-3Gal epitope by itself may not be sufficient to result in significant binding of complement fixing xenoreactive antibodies. Rather, natural antibodies seem to bind preferentially to certain glycoproteins bearing that epitope. This finding emerged from analysis of the mechanisms determining the avidity of natural IgM antibodies for the Gala1-3Gal on cell surfaces (56,64). One important factor in antibody binding appears to be the clustering of epitopes, which allows multivalent interactions between antibody molecules and the cell surface. Multivalent interactions are necessary because the dissociation constant (K_d) for monomeric interactions is as low as 10^{-4} *M*, whereas the effective avidity for intact IgM is characterized by a K_d of approximately 10^{-10} *M*. The optimal clustering of epitopes is not random, however, because the binding of xenoreactive antibodies to purified proteins containing similar numbers of Gala1-3Gal substitutions varies by more than approximately 1,000-fold (64).

Further evidence that the manner in which Gala1-3Gal is expressed rather than the total number of epitopes dictates the extent of antibody binding emerged from studies on variations in antigen expression by the population of potential donors (66). Analysis of the level of binding of xenoreactive antibodies to cells from populations of pigs suggests that there is a range of antigen expression of up to tenfold (66,67). The variation appears to have a genetic basis, although it is not a simple Mendelian trait. The implications of this observation were tested by Cotterell et al. (65), who found that porcine organs with low levels of antigen expression absorb significantly fewer xenoreactive natural antibodies during perfusion with baboon blood than organs with a high level of antigen expression. Cotterell et al. also found that although binding of human IgM to porcine cells varies over a range of nearly tenfold, that range is independent of the total expression of Gala1-3Gal (65), suggesting that the way in which sugar is expressed, rather than its absolute level of expression, determines antibody binding.

The core structures bearing the Gala1-3Gal modifications recognized by xenoreactive natural antibodies include proteins of the integrin family and von Willebrand factor (64,68). The attachment of antibodies to endothelial cell integrins could disrupt endothelial integrity by hindering the ability of the integrins to contribute to cell–cell interactions (69). Antibody binding to endothelial cell integrins also may deliver signals to the cells (70,71), potentially contributing to endothelial cell activation, which, as discussed in a later section of this chapter, is thought to be important in acute vascular xenograft rejection. The binding of antibodies to endothelial cell–associated von Willebrand factor potentially might alter the interactions between endothelial cells and platelets (72,73 and 74).

Based on current understanding of the specificity of xenoreactive antibodies, it is possible to devise specific strategies for depletion of xenoreactive antibodies from the circulation of xenograft recipients. Affinity columns have been used previously for depleting isohemagglutinins, allowing transplantation of organs across ABO barriers (75), and it is reasonable to think this approach also could be used for depleting xenoreactive antibodies. One hope is that temporary depletion of xenoreactive antibodies would allow the development of accommodation for xenografts as it does for allografts. Another way to prevent graft injury initiated by xenoreactive natural antibodies is to inhibit their binding using soluble ligands. This approach has been used to prevent rejection of ABO-incompatible kidney transplants (76,77). Unfortunately, because the binding of xenoreactive antibodies to cell surfaces is extremely avid, high concentrations of a monomeric inhibitor would be needed. Nevertheless, such approaches are being developed (78,79).

Yet another approach to preventing the interaction of xenoreactive antibodies with a xenograft is to seek out or develop pigs that have low levels of antigen expression. With the identification and cloning of the gene for the glycosyltransferase responsible for the synthesis of Gala1-3Gal (80,81), the possibility of genetically engineering donor animals with decreased expression has been proposed. The recent cloning of pigs by nuclear transfer (82) and the demonstration that these approaches might allow gene targeting (83) raise the possibility that a1,3galactosyltransferase might be “knocked out.” An alternative strategy proposed by Sandrin et al. (84) and by Sharma et al. (85) involves the introduction of another glycosyltransferase, a1,2-fucosyltransferase, which would compete with a1,3-galactosyltransferase for adding the terminal saccharide onto oligosaccharide chains. Transgenic mice and pigs expressing the H transferase have been made and found to have decreased expression of Gala1-3Gal (Fig. 85.5). Yet another strategy might involve the expression of agalactosidase in transgenic animals (86).

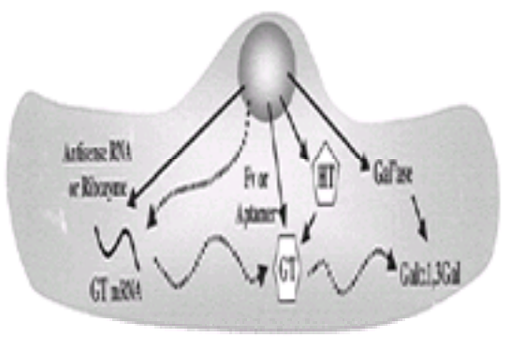

Figure 85.5. Possible ways in which Gala1,3Gal expression could be decreased by genetic engineering. Synthesis of Gala1,3Gal is catalyzed by a1,3-galactosyltransferase (GT), whose biosynthetic pathway is shown by the dashed line. Four approaches to minimizing the synthesis of the Gala1,3Gal epitope are shown here. First, the expression of antisense RNA or a ribozyme might disrupt the structure or function of the GT mRNA. Second, the introduction of a gene whose

product, such as an appropriate Fv (an antibody-like molecule), inhibits GT function. Third, overexpression of another glycosyl transferase, such as H transferase (HT), which adds terminal fucose residues, might compete for the subterminal residues that GT recognizes. Fourth, expression of a glycosidase, such as a-galactosidase, might lead to the cleavage of antigenic saccharide chains. (Reprinted and adapted from *Nature* 1998;392 (Suppl):11–17, Macmillan Magazines, Ltd. with permission.)

As another approach to obtaining donors with low levels of antigen, there is the possibility of exploiting the natural variation in antigen expression, as previously mentioned. Geller et al. (66) and Cotterell et al. (65) found that expression of lower levels of antigen by some pigs has a genetic basis. Perfusion of organs from low antigen-expressing animals with the blood of baboons leads to the deposition of little IgM and C4, in contrast to similar experiments in which organs from normal pigs are perfused.

Complement

The activation of complement is an essential step in the development of hyperacute rejection. The importance of complement in xenograft rejection is suggested by three observations. First, upon perfusion of a discordant xenograft with blood of the recipient, serum complement levels decrease precipitously because of consumption of complement in the graft (32,87). Second, complement accumulates rapidly in rejecting xenografts (12). Third, the survival of a discordant xenograft is always prolonged if complement is inhibited (88,89), if the recipient is depleted of complement by agents such as cobra venom factor (90,91), or if the graft is placed in a recipient that is congenitally deficient in complement component C6 (92,93). The role of complement in xenotransplantation was recently reviewed (16,19,94,95).

Given the importance of complement as a mediator of xenograft rejection, a critical question is how the complement system of the recipient becomes activated in the xenograft. One mechanism that may lead to complement activation is activation by the binding of complement-fixing xenoreactive antibodies to the graft. Such a mechanism of graft injury originally was proposed by Gewurz et al., who showed that, following repercussion of a xenogeneic organ graft, both xenoreactive antibodies and complement are depleted from the recipient's serum (87). Perper and Najarian suggested that a relationship exists between the phylogenetic distance between the donor and recipient of a xenograft and the importance of cytotoxic antidonor antibodies (27). Although the importance of xenoreactive antibodies in the activation of complement in xenografts has been questioned (29,96), several lines of evidence support the importance of this mechanism in clinically relevant pig-to-primate xenograft models. First, immunopathologic analysis of rejecting xenografts demonstrates diffuse deposition of the classical complement component C4 colocalized with IgM but only focal deposition of alternative pathway components factors P and B (12). Second, depletion of IgM or C2, but not factor B, from a human serum prevents activation of complement when that serum is applied to porcine endothelial cells (11,13,56,60,64,68), suggesting that complement activation requires antibody binding and an intact classical complement pathway. Third, activation of complement on porcine cells during exposure to a human serum is inhibited by C1 inhibitor (97). Fourth, depletion of xenoreactive antibodies under conditions in which the complement system remains intact prevents hyperacute rejection of pig-to-primate xenograft (12). Fifth, pig hearts transplanted into newborn baboons, which have only low levels of xenoreactive antibodies, do not undergo hyperacute rejection (48).

On the other hand, in some experimental models, hyperacute rejection can occur in the apparent absence of antidonor antibodies (98), suggesting that components of natural immunity other than xenoreactive natural antibodies may mediate immune recognition (Table 85.3). The possibility that complement activation in a xenograft might arise independent of xenoreactive antibodies was envisioned by Calne (29). Such mechanisms might include activation of the alternative pathway or foreign cell surfaces and attachment to cells or proteins other than natural antibodies capable of activating complement. In fact, the alternative complement pathway is activated on xenogeneic cell surfaces in many combinations of donor and recipient transplant models (99). Miyagawa et al. showed that activation of the alternative pathway in a guinea pig-to-rat xenograft model could trigger hyperacute rejection (9). Activation of the alternative complement pathway probably also initiates the rejection of porcine organs transplanted into dogs, a point of historic interest, as much of the early work in xenotransplantation involved analysis of pig-to-dog renal xenografts (27,28). Evidence indicating the alternative complement pathway in these models includes (a) that the recipient of a xenograft that is rejected hyperacutely can be shown to have low or undetectable levels of antidonor antibodies (9,96); (b) that further depletion of those antibodies does not prevent hyperacute rejection (10); and (c) that the alternative, but not classical, pathway activity in the recipient's plasma decreases after the graft is reperfused (100). Hyperacute rejection initiated by activation of the alternative complement pathway is particularly fulminant, perhaps because the formation of C3 convertase complexes proceeds rapidly and diffusely and does not depend on the kinetics of antibody–antigen interaction. The possibility also remains that activation of the alternative complement pathway by xenoreactive antibodies will be found to be important in some experimental models.

Complement Regulation

Xenografts may be especially susceptible to complement-mediated injury because of the regulation of complement activation. Under physiologic conditions, the activation of complement on autologous cells is controlled in part by cell-associated glycoproteins such as DAF (CD55) and membrane cofactor protein (MCP, CD46), which inhibit complement activation at the C3 convertase step, and CD59, which prevents formation of the membrane attack complex (101). These proteins may have a limited ability to control activation of heterologous complement (102), and as a result a xenograft might be especially susceptible to complement-mediated injury (9,14,103). The potential impact of a defect in complement regulation on endothelial cells surfaces is suggested by the work of Matsuo et al., who found that inhibition of decay accelerating factor by blocking antibodies in a rat model causes development of prominent vascular changes (104). It is possible that the species specificity of complement regulatory proteins is conditional on the system in which specificity is tested. For example, Miyagawa et al. found that the function of complement regulation depends on other components of the cell surface of the target cell (105). This point is not uncontroversial because recent studies by Van den Berg and Morgan suggested that CD59, and perhaps other complement regulatory proteins, can function normally between species (106). In any case, work from my laboratory showed that hearts from transgenic pigs expressing even low levels of human DAF and human CD59 transplanted into baboons, in many cases, resisted the development of hyperacute rejection (107,108). This finding suggested that control of complement is an important element of the definition of discordance and, thus, the immune barrier to xenotransplantation (19).

In addition to the control of complement by cell membrane–associated proteins, there is the potential role of immunoglobulin as a regulator of complement. Basta et al. showed that immunoglobulin can serve as an alternative acceptor for C3 and C4 (109), diverting the activated components away from target cell surfaces. Magee et al. recently tested this concept in a xenograft model (89). Administration of gamma globulin, which contains xenoreactive IgG, to primate recipients of porcine cardiac xenografts, prevented the development of hyperacute rejection in most cases. The immunopathology of the xenografts in the treated recipients revealed host immunoglobulins and C1q along endothelial surfaces, but little or no evidence of other complement components was found.

Components of Complement Involved in the Pathogenesis of Hyperacute Rejection

Another important issue is which components of complement actually mediate tissue injury in rejecting xenografts (110). Two lines of evidence suggest that the occurrence of hyperacute rejection depends on the assembly of terminal complement complexes. First, hyperacute xenograft rejection does not occur in recipients inherently deficient of C6 (92,93,111). Second, inhibition of complement using anti-C5 antibodies prevents some features of hyperacute rejection (112). Although terminal complement complexes are necessary for the development of hyperacute rejection, the formation of the membrane attack complex may not be essential. We recently found that organs from transgenic pigs expressing human CD59 significantly resist assembly of the membrane attack complex and some aspects of tissue injury; however, the development of hyperacute rejection is not averted (113). How terminal complement components might cause hyperacute rejection without assembly of the membrane attack complex may be explained by the ability of C5b67 complexes to disrupt the integrity of endothelial monolayers (114), as discussed later. On the other hand, the formation of terminal complexes, including the membrane attack complex, may not be sufficient to cause hyperacute rejection because hyperacute rejection is not seen in many ABO blood group incompatible allografts, even though the membrane attack complex can be shown to be present (115).

If assembly of terminal complexes is absolutely necessary for the development of hyperacute rejection to occur, that does not exclude the likelihood that other active components of complement may influence the well-being and function of an organ during the hours following transplantation. For example, formation of C3bi on graft endothelium provides a ligand for neutrophils bearing complement receptors (116) and other cells that might amplify the impact of ischemia reperfusion. Formation of C5a may contribute to this process by activating phagocytic cells. It also may mediate the release of heparan sulfate from endothelial cells (117), a process that may deprive endothelial cells of a number of physiologic functions (11), as discussed later.

Natural Killer Cells

Some recent studies suggested the possibility that natural killer (NK) cells might accumulate in and specifically damage a vascularized xenograft. One or more of several mechanisms might underlie the recognition of a xenograft by NK cells. First, the accumulation of NK cells in the graft and activation of those cells might be enhanced by xenoreactive antibodies deposited in the graft (118). Second, host NK cells that ordinarily are controlled by receptors specific for major histocompatibility complex (MHC) class I molecules may fail to recognize the disparate MHC class I molecules expressed in a xenograft (119). Although NK cells have not been detected in large numbers in grafts undergoing hyperacute xenograft rejection (10, 91,120), these cells have been seen in acute vascular rejection (121), and it would seem reasonable to think they may play a role in the evolution of this lesion.

Pathogenesis of Hyperacute Rejection

Hyperacute rejection is perhaps best understood as a condition in which the critical functions of endothelial cells are lost (18). It is as though the blood vessels had, in a sense, lost endothelium. The loss of barrier functions allows the rapid formation of interstitial hemorrhage; the interaction between platelets and the extravascular compartment promotes thrombosis. Loss of the protective property of endothelium allows injury by oxidants, complement, and perhaps other inflammatory agents. The sections that follow consider how the activation of complement in a xenogeneic organ graft might lead to the dramatic picture of hyperacute rejection. For more detailed consideration of this subject, the reader is referred to a recent review (122).

Complement-mediated Cytotoxicity

One mechanism that might account for loss of endothelial function would involve complement-mediated lysis of endothelial cells. Lysis or loss of endothelial cells would allow the egress of blood cells and fluid from blood vessels and would expose the underlying matrix that contains components capable of initiating platelet aggregation and thrombosis. Endothelial destruction occasionally is seen in emerging hyperacute rejection lesions, particularly in the more aggressive models such as guinea pig-to-rat. Under optimal conditions, however, endothelial cell death is not a major finding early in the course of hyperacute rejection in pig-to-primate xenografts (12,16,34). Thus, complement-mediated changes probably involve noncytotoxic processes.

Release of Heparan Sulfate

One noncytotoxic event that may contribute to the pathogenesis of hyperacute rejection is the loss of heparan sulfate from endothelium. Heparan sulfate is an acidic polysaccharide that is involved in many of the physiologic functions of blood vessels. Heparan sulfate contributes to the endothelial barrier function, which excludes blood cells and plasma proteins, and enhanced anticoagulation and protection against complement and oxidants (123,124). Thus, changes in the metabolism of heparan sulfate might account for some of the events associated with xenograft rejection (11). Consistent with this possibility, exposure of endothelial cells to human serum was found to cause the release of up to 50% of endothelial cell–associated heparan sulfate within 30 to 60 minutes (11). Release of heparan sulfate from endothelial cells depended on the binding of xenoreactive antibodies to the cells and the generation of C5a (117) and involves activation of endothelial cell–associated proteases (125). Stevens et al. showed that the same process occurs *in vivo*; about 50% of biosynthetically labeled heparan sulfate is lost within 5 minutes of the reperfusion of vascularized xenografts, and manipulations that inhibit loss of heparan sulfate also prolong the survival of xenografts (126). Other inflammatory events may cause the release of heparan sulfate from endothelial cells, perhaps accounting for other inflammatory or immune-mediated changes in blood vessels. For example, Key et al. showed that elastase released from stimulated neutrophils causes the release of endothelial cell heparan sulfate (127), and Magee et al. showed that this process may be amplified in the presence of oxidants (128).

Complement-mediated Changes in Endothelial Cell Shape and the Formation of Intercellular Gaps

Another noncytotoxic mechanism that might bring about the loss of endothelial cell function is a change in endothelial cell shape with corresponding disruption of cell–cell attachments. Saadi et al. showed that activation of complement on endothelial cells causes a dramatic change in endothelial cell shape, leading to the formation of intercellular “gaps” (114). The formation of gaps could be mediated by C5b67 (or C5b6), although the membrane attack complex accelerates the process. The formation of gaps was associated with increased production of second-messenger metabolites. For example, like other changes in the barrier function and morphology of endothelial cells, complement-mediated alteration in cell shape is associated with changes in the level of cAMP (129,130). Adding dibutyryl-cyclic adenosine monophosphate (cAMP), forskolin, an activator of adenylate cyclase, or methylisobutyl xanthine, an inhibitor of cyclic nucleotide phosphodiesterase, to the medium bathing endothelial cells prevents gap formation in response to antibody binding and complement activation. Consistent with the potential importance of this mechanism, the ultrastructure of endothelial cells in xenograft tissues obtained 5 minutes after reperfusion with recipient blood reveals the interposition of platelets between adjacent endothelial cells (Fig. 85.6).

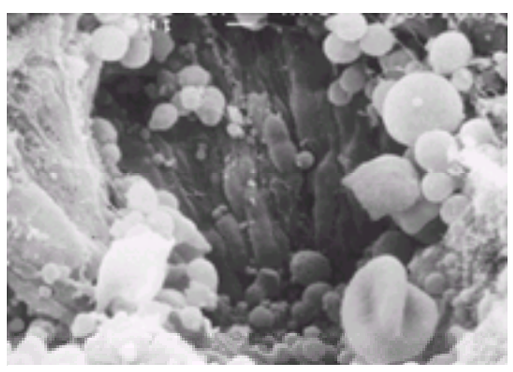

Figure 85.6. Platelet attachment during hyperacute rejection. Electron microscopy reveals platelet attachment to the endothelial lining of a porcine blood vessel undergoing hyperacute rejection after reperfusion with baboon blood.

Other Complement-mediated Changes

Activation of complement on endothelial cell surfaces causes a variety of other changes that may contribute to the manifestations of hyperacute or other types of xenograft rejection. For example, formation of the membrane attack complex on endothelial cell surfaces triggers the secretion of von Willebrand factor (131,132), which might trigger platelet aggregation and the formation and release of cell membrane vesicles, which might promote coagulation. In addition to recruiting platelets, the secretion of von Willebrand factor may contribute to the overall humoral reaction in xenografts because it can serve as a target of complement-fixing human natural antibodies (64). The membrane attack complex also changes the alteration to the surface properties of endothelial cells so as to promote assembly of prothrombinase complexes (133). Stimulation of endothelial cells and platelets also causes expression of P-selectin, which can serve as a ligand for neutrophils and platelets (134,135). These events and perhaps the generation of small amounts of thrombin may account for the observation that platelet aggregation is the earliest morphologic change observed in hyperacute rejection. In addition to causing the formation of thrombi and obstruction to blood flow, the aggregation and activation of platelets may have a direct impact on the functions of endothelial cells (38).

Tissue damage in hyperacute xenograft rejection may be amplified by reperfusion injury. Reperfusion injury is thought to involve local activation complement and to lead to endothelial cell dysfunction through various mechanisms, including the generation of toxic oxygen species, such as superoxide anion and the release of proteolytic enzymes. These mechanisms alter endothelial cell structure and function, potentially rendering the xenograft more susceptible to injury by natural antibody and complement. Consistent with this idea, we demonstrated that oxidant stress increases the susceptibility of porcine endothelial cells to the noncytotoxic effects of human natural antibody and complement (128) and that providing the second messenger, cAMP, which inhibits some manifestations of preservation injury (136), also mitigates some of the effects of natural antibody and complement on endothelium (137). These studies provide preliminary evidence supporting the idea that reperfusion injury, even at a level too low to yield dysfunction of an isograft or allograft, may contribute to tissue injury in a xenograft.

ACUTE VASCULAR REJECTION

If hyperacute rejection is prevented, a discordant xenograft is subject to another type of rejection, which we have called *acute vascular xenograft rejection* (10). Acute vascular rejection is the primary type of rejection seen in concordant xenografts and in some allografts (138,139 and 140). It is characterized pathologically by endothelial injury and swelling, ischemia, and diffuse thrombosis. An infiltrate consisting of mononuclear leukocytes and neutrophils is often observed. Acute vascular rejection is sometimes called *delayed xenograft rejection*; however, the latter term may be misleading because it suggests incorrectly that this type of rejection is a delayed type of hyperacute rejection.

That acute vascular rejection is not, indeed, a delayed form of hyperacute rejection is suggested by several lines of evidence. First, acute vascular rejection typically occurs when the complement system of a xenograft recipient is inhibited, a condition that always prevents the occurrence of hyperacute rejection (10,16). Second, acute vascular rejection occurs in complement-deficient animals, which are unable to mount an hyperacute rejection response (93). Third, treatments such as the administration of antileukocyte antibodies, which suppress acute vascular rejection by inhibiting inflammatory cells, have no impact on hyperacute rejection (141). On the other hand, administration of gamma globulin, which diverts complement away from the graft but increases antibody binding to the graft (89), prevents hyperacute rejection but not acute rejection and may even make the latter worse. Fourth, the most dramatic pathologic manifestations of acute vascular rejection, especially endothelial swelling, focal ischemic injury, and diffuse fibrin thrombi are not typically seen in hyperacute rejection (10,18).

Role of Xenoreactive Antibodies in the Pathogenesis of Acute Vascular Rejection

Several lines of evidence suggest that acute vascular xenograft rejection is caused by the continuing interaction of xenoreactive antibodies with the graft (19). First, during the days following the extracorporeal circulation of blood of patients with fulminant hepatic failure through porcine livers, the levels of antiswine antibodies in the blood of the patients increase, and this increase is observed at a time that corresponds to the period during which a xenograft is subject to acute vascular rejection (62). Second, the removal of antibodies from the circulation of xenograft recipients or inhibition of antibody synthesis by treatment with cytotoxic agents delays or averts acute vascular rejection (58,(142,143,144 and 145). Third, a type of rejection similar or identical to acute vascular rejection of xenografts is observed in allografts (146,147) and xenografts (148,149) in association with *de novo* appearance of antidonor antibodies. Fourth, acute vascular rejection of allografts and xenografts can be induced by the infusion of antidonor antibodies (36).

Role of Complement

At present, it is uncertain whether complement contributes to the pathogenesis of acute vascular xenograft rejection and how complement might do so. Clearly, the development of tissue lesions characteristic of acute vascular rejection is easily envisioned as a reflection of acute complement-mediated changes in endothelial cell structure and function, as previously discussed. Such a role for complement in acute vascular rejection is difficult to reconcile with the fact that acute vascular xenograft rejection typically is observed in xenograft recipients that had been depleted of complement (10). This difficulty may be resolved by three considerations: (a) even the most potent inhibitors of complement are incompletely effective; (b) under conditions of inflammation, endothelial cells synthesize complement components; and (c) some endothelial changes, such as the alteration in heparan sulfate metabolism, can be induced by low levels of complement. For instance, less than 1% of the complement activity in normal serum is sufficient to mediate the release of heparan sulfate from porcine endothelial cells (11,125), and less than 10% of normal complement activity is sufficient to stimulate procoagulant and proinflammatory changes in endothelial cells (150). If complement contributes to the pathogenesis of acute vascular rejection, the membrane attack complex may not be essential. Acute vascular rejection develops in transplant recipients deficient in C6 (92,93).

Endothelial Cell Activation

Based on the histopathology of acute vascular rejection, which includes infiltration of the organ by neutrophils, the thickening of endothelial cells, and pronounced thrombotic changes (10), the author proposed that the pathogenesis of acute vascular xenograft rejection might involve the activation of endothelial cells and thus the acquisition of new endothelial functions (18). This idea was supported by the observation that endothelial cells in grafts undergoing acute vascular rejection display phenotypic changes characteristic of activation (121). One new function presumably acquired by endothelium in acute vascular rejection is the ability to promote coagulation. Unlike quiescent endothelium, which prevents coagulation by serving as a barrier between tissue factor activity in the underlying matrix and plasma coagulation proteins, the endothelium in rejecting xenografts allows coagulation proteins to come in contact with tissue factor in the underlying matrix. In addition, the formation of the membrane attack complex on endothelial cell surfaces stimulates the formation of IL1a, which in turn stimulates the *de novo* synthesis of tissue factor (150) and proinflammtory products (18). Other changes, such as loss of thrombomodulin, increased expression of E selectin (151), chemokines (152), prostaglandins (153), synthesis of plasminogen activator inhibitor type 1, and changes as previously discussed, such as the alteration of cell surface promoting the formation of prothrombinase complexes, also may contribute to the procoagulant posture. In addition, the production of small amounts of thrombin, platelet activating factor, or thomboxane A2 may stimulate platelets in the vicinity of the endothelium.

Role of Platelets

Whereas the thrombi in acute vascular rejection consist mainly of fibrin, the fibrin is invariably admixed with platelets. Bustos and Platt tested the idea that platelets activated by small amounts of thrombin might activate endothelial cells (38). Indeed, human platelets so activated were found to express interleukin-1 (IL-1)-a on the surface, which together with secreted cytokines, could contribute to activation of endothelium. The relative importance of platelets and complement in triggering endothelial cell activation, and thus acute vascular rejection, remains uncertain.

Role of Inflammatory Cells

Blakely et al. (121) suggested that monocytes, which are known to express tissue factor, a co-factor for the formation of prothrombinase complexes, might cause the fibrin deposition and thus the tissue manifestations of acute vascular rejection. This view was supported by the observation that acute vascular rejection in rodents is associated with the influx of macrophages expressing tissue factor. On the other hand, the lesions early in the course of acute vascular rejection do not reveal significant numbers of macrophages (89,154), and the earliest expression of tissue factor is on graft endothelium (155). Nor is the onset or the pathology of acute vascular rejection more than modestly inhibited by the administration of agents that inhibit the interaction of inflammatory cells with xenogeneic endothelium (141), in contrast to the results achieved by antibody depletion, which significantly prolongs the survival of the transplants (154). Nor, conversely, do allotransplants with cellular rejection, which often have large numbers of invading macrophages, necessarily exhibit lesions typical of acute vascular rejection (156). Thus, although a role for macrophages cannot be excluded at this juncture, it seems more likely that these cells are a marker for tissue injury rather than the cause in acute vascular rejection of xenotransplants.

Yet another mechanism that might contribute to the pathogenesis of acute vascular rejection is the action of lymphocytes or NK cells on graft endothelium. The potential involvement of NK cells has been of special interest because the cells might be expected to be activated in xenograft recipients through stimulation carbohydrate and Fc receptors and failure of stimulation of MHC class I receptors (118,157). Consistent with this concept, human NK cells exert cytotoxic and noncytotoxic effects, such as induction of procoagulant activity on porcine endothelial cells *in vitro* (158,159). Although NK cells and other lymphocytes have been found in some rodent xenografts (160), this finding is not always observed (10) because they are not major components of pig-to-primate xenografts (89,154). Therefore, whereas lymphoid cells may well contribute to tissue injury in acute vascular rejection, they may not be essential for the manifestation of tissue lesions.

Accommodation

When antidonor antibodies are depleted from a xenograft recipient, acute vascular xenograft rejection may not occur, even after the antibodies return to the circulation. This condition, in which the graft seems to resist acute vascular rejection despite presence in the circulation of all inciting factors, is called *accommodation* (14). We first observed accommodation in ABO-incompatible kidney allografts (76,161) and postulated that a similar phenomenon might occur in vascularized xenografts (12,14,162). Accommodation also may be observed in graft recipients with circulating antidonor human leukocyte antigen (HLA) antibodies (163,164).

We have postulated that accommodation may arise through one or more of three mechanisms (12,14). First, there is the possibility that the antidonor antibodies may change in their functional properties or specificity or both. Supporting this possibility were studies in ABO-incompatible allografts in which antidonor antibodies were detected in the circulation but were generally not observed in biopsies of the transplanted organ (76,161). Another potential change in the antidonor antibodies is toward predominance of IgG2, which activates complement poorly and might compete with complement fixing antibodies for binding to target cells (61). Second, there might occur a change in the antigen. This concept is supported by the finding that carbohydrate synthesis in the kidney changes following transplantation (165). Third, accommodation might involve a change in the endothelium so that the graft becomes inured to antibody binding and complement activation. The possibility that an allograft or xenograft might acquire resistance to tissue injury and rejection is supported by recent studies showing that accommodation in a rodent model is associated with expression of “protective” genes by endothelial cells (166,167) and by observations that, with continued stimulation of endothelial cells by antibodies or complement, the sensitivity of those cells to injury decreases (168,169 and 170). As an example of one potential scenario, continued stimulation of endothelial cells with endotoxin or with IL-1 causes the cells to develop resistance to restimulation (171). Not only may the sensitivity to restimulation decrease, but the sensitivity to injury also may decrease. For example, stimulation of endothelial cells may cause an increased synthesis of DAF (172), which inhibits complement activation at the level of C3 convertase.

Regardless of the biologic basis for accommodation, further understanding of how this process is induced could be extremely useful, if not critical, to the clinical application to xenotransplantation. Not only does the occurrence of accommodation eliminate the need for continuing depletion of antidonor antibodies from a graft recipient, it also provides clues to manipulations of the donor or recipient that might prevent more chronic forms of graft rejection. The study of accommodation also could yield insights into how blood vessels might be manipulated to alter sensitivity to other injurious processes.

Therapeutic Approaches to Acute Vascular Rejection

Three general strategies might be envisioned for preventing acute vascular rejection. The first strategy involves the induction of accommodation. As discussed, accommodation might be induced by temporary depletion of xenoreactive antibodies. Studies in our laboratory have indicated that accommodation of pig organs transplanted into nonhuman primates can be achieved by depletion of all Ig (154) or of anti-Gala1-3Gal antibodies (59,173).

The second approach to preventing acute vascular rejection might involve induction of immunologic tolerance, lending to decreased production of antixenograft antibodies. Humoral tolerance has been achieved in a1,3galactosyltransferase knock-out mice (these mice make anti-Gala1-3Gal antibodies like humans) (174) by the

transplantation of normal murine bone marrow to yield mixed chimerism and autologous cells transduced with a1,3galactosyltransferase (175). These approaches seem capable of preventing rejection of Gala1-3Gal murine hearts expressing epitopes by a1,3galactosyltransferase knock-out mice. Whether the strategy would be effective between disparate species is still unknown because of the difficulties of transplanting bone marrow between species. It is hoped that treatment with porcine cytokines might allow the enduring engraftment of porcine bone marrow (7).

A third strategy for preventing acute vascular rejection involves decreasing expression of Gala1-3Gal in the transplant. To the extent that Gala1-3Gal is the major target of the xenoreactive antibodies that cause acute vascular rejection, as our work suggests (51), this strategy would have the theoretical advantage of limiting the immunosuppressive treatments that would have to be applied to the recipient. Various genetic strategies for decreasing the expression of Gala1-3Gal are summarized in Fig. 85.5. Of these, the expression of H transferase, which catalyzes production of H antigen, to which humans are tolerant, in lieu of Gala1-3Gal, has been applied with some success in pigs (84). Studies by Parker et al., however, suggest that, to effectively prevent acute vascular rejection, Gala1-3Gal expression would have to be decreased by greater than 95% (176). The recent cloning of pigs by transfer of nuclei from cultured somatic cells to enucleated zygotes (82) raised the possibility the a1,3galactosyltransferase could be "knocked out." It is also possible that the elimination of Gala1-3Gal might subject the recipient to increased risk of infection by porcine viruses, such as the porcine endogenous retrovirus (PERV), which otherwise would be neutralized by anti-Gala1-3Gal antibodies (177). Which of the preceding or other strategies will be most effective in preventing acute vascular rejection is uncertain. This question is a critical one because acute vascular rejection would appear to be the most difficult immune hurdle to xenotransplantation of organs.

XENOGENEIC CELLULAR IMMUNE RESPONSES

The initial type of rejection observed in cellular or free tissue xenografts is acute cellular rejection. If hyperacute and acute vascular rejection are averted, an organ xenograft would next be subject to rejection by cell-mediated immunity of the host against the donor (20,58,178). The central questions regarding cellular rejection of xenografts are the extent to which the cellular immune response to a xenograft might differ from the cellular immune response to an allograft and whether this response would be more severe and less subject to immune modulation than the corresponding allogeneic response. Preliminary answers to these questions have not been forthcoming because, until recently, it has been difficult to bring about survival of organ xenografts for more than a few days without the administration of massive doses of immunosuppressive agents. For example, the studies performed by Alexandre et al. involving the transplantation of porcine kidneys into baboons (142) provided some evidence for the importance of T cells inasmuch as decreases in graft function were reversed by administration of immunosuppressive agents. Also preliminary, but nonetheless intriguing, are studies by Leventhal and co-workers demonstrating that rejection of guinea pig-to-rat cardiac xenografts could be hastened by transfer of lymphocytes from presensitized animals (179). Unfortunately, these studies did not clarify the role of the transferred cells in rejection.

Studies of the cellular immune response to xenografts of free tissues such as the islets of Langerhans or the skin have provided some insight into the similarities and potential differences between the cellular immune responses to xenografts and allografts. Similar to allografts, islet and skin xenografts are subject to cellular rejection because they rapidly fail if the recipient is a normal mouse but survive if the recipient is a nude or a severe compromised immunodeficiency disease (SCID) mouse. In some cases, however, the failure of a free tissue xenograft occurs notably earlier than the failure of a free tissue allograft. This difference may reflect a greater susceptibility of the xenograft to "primary nonfunction." What is not yet clear is whether the early failure of the xenografts was caused by the immune response of the recipient as in primary nonfunction of allografts (23) or whether, at least to some extent, the failure was caused by incompatibility of the graft with the foreign microenvironment. As one example of the latter, there is the possibility that neovascularization of a free tissue xenograft may be impaired in comparison to neovascularization of a free tissue allograft. Such impairment could result in the xenograft being deprived of nutrition and the ready removal of waste products. Impaired neovascularization also could influence the way foreign antigens are presented in a xenograft (Fig. 85.2). On the other hand, free-tissue xenografts may survive for extended periods of time in recipients treated with immunosuppression that is not more intense than the immunosuppression used for allografts (180,181). Moreover, anti-CD4, which has limited effectiveness in allotransplantation, may be especially effective in preventing xenograft rejection (22).

Some recent *in vitro* observations have shed light on the potential differences between xenogeneic and allogeneic cellular immune responses. Proliferative responses to xenogeneic cells in mixed leukocyte cultures are often less exuberant than allogeneic responses or even undetectable (182). This decreased proliferative response to xenogeneic stimulation was thought to reflect the smaller repertoire of T cells that might recognize xenogeneic cells directly, a reflection in turn of positive selection leading to more cross-reactivity with allogeneic than with xenogeneic MHC. Indeed, the dampened *in vitro* response led to the suggestion that cellular immunity to a xenograft might be less intense than cellular immunity to an allograft (20,182). This idea was further supported by studies suggesting that recognition of xenogeneic cells occurs predominantly through the indirect pathway, the T cells of the host being specific for peptides of xenogeneic origin complexed with MHC on host antigen-presenting cells. Because indirect recognition would lead to activation of a smaller fraction of the T-cell repertoire, the response arising in this way would be less intense and less rapid than the response to allostimulation arising through the direct pathway. The dependence of the xenogeneic response on the indirect pathway also reflects defects in the ability of T cells to respond to xenogeneic antigen-presenting cells brought about by "incompatibility" of CD8 or CD4 with xenogeneic MHC leading to decreased coreceptor function (183,184) and by incompatibility of costimulatory pathways and cytokines (185). Recent studies have shown, however, that mouse and human T cells can recognize xenogeneic cells, such as porcine endothelial cells, directly and that under the proper conditions xenoimmune responses arising *in vitro* may be as vigorous as alloimmune responses (186,187,188 and 189), although there is some evidence of a limited frequency of T-cell precursors with the ability to recognize directly highly disparate MHC (184).

There are reasons to think, however, that the cellular response to a xenograft might be as strong or stronger than the cellular response to an allograft. First, virtually all foreign proteins contain amino acid sequences that differentiate one species from another. Thus, a xenotransplant, in contrast to an allotransplant, might give rise to a vast array of foreign peptides that could stimulate a strong response, even though the response is restricted to the indirect pathway. This concept is supported by the work of Murray et al. (186), which demonstrated that human T cells could respond to primary stimulation by porcine peptides, whereas primary responses to allogeneic peptides cannot be detected (190). These results suggest the existence of a much higher frequency of human T cells committed to respond to porcine peptides than to human peptides. Second, to the extent that the direct recognition contributes to immunoregulatory responses, defects in the direct pathway, such as those previously described, might lead *in vivo* to impaired immunoregulation and thus to stronger cellular immune response. Third, the humoral response to xenotransplantation, as it occurs *in vivo*, may give rise to increases in expression of cell adhesion molecules or release of proinflammatory mediators, which could in turn amplify cellular immune responses (150). As one example, heparan sulfate released from endothelial cells by antiendothelial cell antibodies and complement (11,124,125) may activate antigen-presenting cells leading to increased ability to stimulate proliferative (191,192 and 193) and cytolytic T-cell responses (194). In addition to depriving endothelium of the function of heparan sulfate, the release of that molecule may have other consequences. Wrenshall et al. found that heparan sulfate in soluble form activates antigen-presenting cells, leading to amplified proliferative (192) and cytolytic (194) T-cell responses. These changes are caused by the direct action of heparan sulfate on antigen-presenting cells, leading to stimulation of several immunomodulatory pathways (191,195).

Another aspect of the cellular immune response to xenogeneic cells that remains to be elucidated is the extent to which the response will be directed against MHC versus other proteins. There is evidence that human cellular immune responses generated against porcine cells *in vitro* can be directed against porcine MHC (189). Whether the porcine MHC would be the major target of *in vivo* responses remains to be determined. Also uncertain is whether the response will be subject to the kinds of regulation that impact on alloimmune responses.

Another question of potential import is the nature of the humoral immune response that would be elicited by a xenograft and how such a response might impact elicited cellular immunity. Already discussed is the observation that, when a xenograft recipient is treated with immunosuppressive therapy, the antibodies in the serum of the recipient following xenotransplantation still may recognize Gala1-3Gal as the predominant target (62). On the other hand, it seems likely that antibodies against other donor antigens, particularly polypeptides, are likely to arise and pose an additional hurdle, perhaps in the form of acute or chronic vascular rejection. This question is clearly an important one for future investigation.

Whether cellular immune responses to xenotransplants can be controlled by the same therapeutic approaches used to control cellular immune responses to allotransplants is a question of obvious practical import. Specific advice on this matter cannot be offered at present, except to consider the apparently increased efficacy of anti-CD4 antibodies in preventing rejection of cellular xenotransplants (vide supra). Some suggested that the cellular immune response to xenotransplants would be so intense that induction of tolerance would be necessary to prevent rejection (58). If tolerance is required to prevent cellular rejection of xenotransplants, it is uncertain which approach to induction and tolerance would be most effective. Studies in rodents have shown that bone marrow transplantation lending to mixed chimerism (196,197) and thymus transplantation in conjunction with bone marrow transplantation (198,199) might be effective.

ELICITED HUMORAL IMMUNE RESPONSES

Little is known about the barrier posed by elicited humoral immune responses to xenotransplantation. The potential contribution of such responses to acute vascular xenograft rejection was previously discussed. There is also the possibility that elicited antidonor antibodies will contribute to more indolent or chronic types of rejection (200). Although the importance of these responses for xenografts of all types might seem intuitive, there is some evidence that humoral responses will not cause the demise of free tissue or cellular grafts. Whether in the case of free tissue grafts the limited impact of humoral responses will prove to reflect lesser immunogenicity of the graft or the relative protection of the foreign tissue by recipient blood vessels remains to be determined.

OTHER HURDLES TO XENOTRANSPLANTATION

The preeminent hurdle to the transplantation of organs and tissues between the same species is the immune response directed against MHC antigens. Although

immune responses against MHC antigens undoubtedly pose a hurdle to xenotransplantation, such immune responses are far from the only hurdle. Discussed above is the substantial hurdle to xenotransplantation posed by natural immunity—natural antibodies, complement, and NK cells—and the likely contribution of elicited immune responses against antigens other than those encoded in the MHC complex.

If the intense and diverse responses to xenotransplantation were not enough, two other hurdles warrant brief comment. One other hurdle is the physiology of the graft: the possibility that a graft might fail or fail to function because of incompatibility with the recipient. At present, the question of physiology would appear to be more theoretical than a real hurdle as recent studies have shown that the heart, kidney, and lungs can function in highly disparate recipients. In the end, the greatest physiologic hurdle may prove to be impairment in graft function caused by xenoimmune responses.

One other hurdle worthy of comment is that of infection. There is a possibility of transferring infections from the graft to the xenogeneic recipient (i.e., zoonosis). The main agent of concern at present is the porcine endogenous retrovirus (PERV), which is a type C retrovirus that can infect human lymphoid cells in culture (201,202). Thus far, infection of humans has not been observed (203,204). Of more immediate concern is the possibility that immunosuppression or tolerance regimens might render the recipient relatively immunoincompetent and thus severely subject to infection. Still another concern is that viral infections of xenotransplants might not be adequately controlled by cell-mediated immune responses because viral peptides would be presented by xenogeneic MHC and, thus, relatively unrecognized by the T-cell repertoire of the recipient.

Despite all the hurdles discussed herein, there is increasing enthusiasm about the aspects for clinical xenotransplantation. In part, this enthusiasm springs from the urgent need for donor organs and a sense of willingness to consider xenotransplantation as a rational approach to that problem. In part, this enthusiasm also arises with the recent success in the genetic engineering of pigs as potential xenotransplant donor and from the long-term survival of experimental xenografts performed using organs from these pigs (144). Indeed, some of these results would seem to suggest that there are no fundamental incompatibilities that would stand in the way of enduring survival of a xenograft. Whether the enthusiasm for xenotransplantation proves to be well founded, progress in the field of transplantation provides dramatic evidence of how the approach to medical problems is changing. As molecular hurdles to xenotransplantation are discovered, those hurdles are being addressed through the rational design of drugs and the genetic engineering of donor animals. Thus, if it is not possible to predict that xenotransplantation will enter the clinical arena in a few years, it does seem certain that knowledge in this area will continue to advance rapidly and that knowledge will increasingly benefit the broader fields of immunology and clinical medicine.

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86 CALCINEURIN AS A THERAPEUTIC TARGET

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Calcineurin, a serine/threonine phosphatase involved in multiple biologic functions, is the molecular target of both immunosuppressants cyclosporine A (CsA) and tacrolimus (originally termed FK506). The discovery of a single enzymatic target changed our understanding of drug action and our approach to drug development, but it also initiated focused study of calcineurin-dependent signaling pathways. Comparison of the biologic outcomes of CsA and tacrolimus administration, both *in vitro* and *in vivo*, allowed the attribution of action to a single, identifiable enzymatic activity; from this understanding, both the upstream regulators and downstream targets could be investigated. Furthermore, differences between the action of CsA and tacrolimus action now are understood in terms of drug metabolites or in terms of the different endogenous receptors, termed immunophilins, for these drugs. Immunophilins are a large family of widely expressed and well-conserved proteins that differ in their core hydrophobic pockets that are able to bind drug. Cyclophilins bind CsA, whereas tacrolimus (FK506)-binding proteins (FKBPs) bind tacrolimus and a structurally related, although biologically distinct, immunosuppressive agent, sirolimus (rapamycin). FKBPs, however, do not bind CsA, and cyclophilins are unable to bind either tacrolimus or sirolimus. The complexes formed by CsA binding to cyclophilins and by tacrolimus binding to FKBPs target and inhibit the serine/threonine phosphatase calcineurin. This chapter reviews the central role of calcineurin in lymphocyte and other cellular actions; in addition, the therapeutic and side-effect profiles of CsA and tacrolimus are discussed.

IDENTIFICATION OF THE IMMUNOSUPPRESSIVE DRUGS: CYCLOSPORINE, TACROLIMUS, AND SIROLIMUS

The immunosuppressant cyclosporine (also termed cyclosporine A, CsA) was first identified in 1970 by screening soil samples containing new strains of fungi for immunosuppressive properties (1,2) (Table 86.1). Isolated and later purified from the fungus *to lypocladium inflatum*, cyclosporine inhibited the proliferation of lymphocytes cultured in a mixed lymphocyte reaction, demonstrating its ability to suppress an allospecific response, a property that was later confirmed in *in vivo* studies (3,4). Its clinical utility is discussed in detail in the following section.

Property	Cyclosporine (Cs)	Tacrolimus	Sirolimus
Alternative names	CsA	FK506	Rapamycin
Mr (Da)	1203	822	914
Structure	Cyclic undecapeptide	Macrolide	Macrolide
Intracellular immunophilin receptor	Cyclophilins	FKBPs	FKBPs
Target of drug: receptor complex	Calcineurin	Calcineurin	mTOR
Function	Inhibition of cytokine and gene transcription	Inhibition of cytokine and gene transcription	Inhibition of growth-factor sign p70 S6 kinase activity, protein translation machine growth and proliferation
Major side effects			
Nephrotoxicity	++	++	-
Neurotoxicity	++	++	-
Hypertension	++	++	-
Hyperlipidemia	-	-	++
Leukopenia	-	-	+
Thrombocytopenia	-	-	+
Susceptibility to infection	++	++	+

FK BPs, tacrolimus (FKBP, FKBP-binding protein); mTOR, mammalian Target of Rapamycin; MW, molecular weight

TABLE 86.1. Immunosuppressant Agents

Tacrolimus also was identified by its ability to inhibit allospecific responses, and the compound was purified from soil samples. In 1975, its chemical structure was determined and shown to be a macrolide, most similar to a product isolated from *Streptomyces hygroscopicus*, termed *sirolimus* (originally rapamycin) (5) (Fig. 86.1). Whereas tacrolimus and sirolimus share structural similarity, their biologic action and their molecular targets differ (discussed later). On the other hand, whereas tacrolimus is not apparently structurally related to cyclosporine, a cyclic undecapeptide (Fig. 86.1), both agents share many of the same biologic characteristics, both are immunosuppressive *in vitro* and *in vivo*, and both target calcineurin.

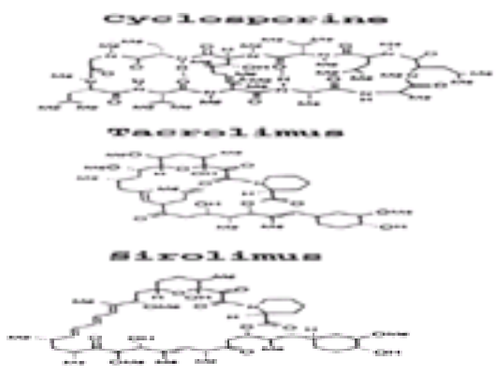

Figure 86.1. Structure of immunophilin binding agents: CsA, tacrolimus, sirolimus. Chemical structure of cyclosporine A (CsA), a cyclic undecapeptide, M_r 1,203 Da; tacrolimus (FK506, Prograf), a macrolide antibiotic, M_r 822 Da (**B**); and sirolimus (rapamycin), another macrolide antibiotic, M_r 914 Da (**C**).

Cyclosporine binds to a family of cyclophilins, whereas tacrolimus (and sirolimus) bind the family of proteins, FKBPs. The cyclophilins and FKBPs, collectively termed *immunophilins*, share the capacity to catalyze the *cis*-trans isomerization of proline-containing proteins. Within the cell, immunophilins appear to be involved in protein folding and transport (6,7 and 8). The isomerase activity of cyclophilins is inhibited by cyclosporine and that of FKBPs is inhibited by the specific ligands tacrolimus and sirolimus.[1]

Inhibition of immune function results not from inhibition of isomerase activity (9,10) but from the ability of the complexes formed by cyclosporine binding to cyclophilins and tacrolimus binding to FKBPs to bind to and inhibit the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin (11,12). Cyclosporine and tacrolimus themselves are considered prodrugs; only upon binding to their intracellular immunophilin do they acquire the ability to bind to calcineurin, leading to inhibition of calcineurin phosphatase activity.

IMMUNOPHILIN RECEPTORS FOR CYCLOSPORINE, TACROLIMUS, AND SIROLIMUS

Although the families of immunophilins differ in drug binding, they share a number of important characteristics, including species conservation and function: the ability to catalyze the cis-trans isomerization of peptidyl-prolyl bonds. Both cyclophilins and FKBPs are abundant proteins and are well represented throughout the animal and plant kingdoms, and of keen biologic interest. A complete summary of each member of the two families is beyond the scope of this chapter, but certain principles are discussed. It is important to appreciate that the effects of drug binding to these different immunophilins, leading to inhibition of endogenous immunophilin function, may be responsible for the side-effect profile or toxicities of drug administration. Only with significant understanding of the biologic properties of these immunophilins, and of their cell-type specific expression and function, will their contribution to drug effects be appreciated.

Cyclophilins

The cyclophilins all share a cyclosporine-binding domain of approximately 165 amino acids, a domain that cannot bind tacrolimus or sirolimus. More than a dozen mammalian cyclophilins have now been identified. An 18-kd cyclophilin termed *cyclophilin A* (CyPA) has been shown to mediate cyclosporine inhibition (13) and to function, in the absence of drug, in protein folding (14,15 and 16). CyPB has been shown to be localized to the endoplasmic reticulum (17) and may be secreted in inflammatory states (18). CyPB interacts with a protein termed *calcium-signal modulating cyclophilin B ligand* (CAML), localized to the endoplasmic reticulum, which appears to play a role in calcium signaling and calcium storage (19). Both CyPA and CyPB bind the third hypervariable loop of the human immunodeficiency virus gp120 envelope glycoprotein (20,21). CyPC, a 23-kd protein bound to membrane attack complex (MAC)-2 binding protein, is localized to cytosolic vesicles; its function is unknown (22). In the presence of cyclosporine, the interaction of CyPC with MAC-2 binding protein is inhibited, and CyPC may be secreted (12). Localized to mitochondria, CyPD appears to regulate mitochondrial permeability in a cyclosporine-sensitive fashion (23). Cyclophilin proteins have been detected on the surface of natural killer (NK) cells (e.g., NK-TR150, (24)) and T cells (e.g., CyPB) (25), in the nucleus (e.g., CyP-60) (26), and the cytoplasm (e.g., CyP-40) (27). CyP-40 binds to heat shock protein (hsp) 90 through its carboxyterminal tetratricopeptide repeat (TPR) domain, a 34-amino-acid degenerate motif involved in protein–protein binding (28). Whereas a complex composed of CyP-40 and hsp90, among others, has been shown to be a component of the glucocorticoid receptor complex, CyP-40/hsp90 binding is not sensitive to cyclosporine treatment (29). Therefore, the endogenous role of the cyclophilins remains an active area of current study.

FK506 Binding Proteins

The spectrum of FKBP family members is as diverse as the cyclophilin family (30,31). An FKBP of 12 kd, termed *FKBP12*, is the principal cytosolic FKBP responsible for both tacrolimus and sirolimus action IQ(Fig. 86.2) (13,32). It is an abundant protein, expressed in essentially all eukaryotic cells, and it binds both tacrolimus and sirolimus with high affinity; drug binding inhibits its endogenous isomerase activity. FKBP12.6 is a related FKBP that is robustly expressed in muscle and appears to be responsible for tacrolimus action in specialized tissue. FKBP13 is localized to the endoplasmic reticulum and has been shown to bind to erythrocyte protein 4.1 (33). FKBP23 also localizes to the endoplasmic reticulum but appears to bind calcium (34). FKBP25 is localized to the nucleus, where it has been shown to bind nucleolin. FKBP51 and FKBP52 are homologous to one another; like CyP-40, FKBP52 is a component of the steroid receptor complex (35). Of note, neither CyP-40 nor FKBP52 appears required to activate hormone binding by the glucocorticoid receptor (36).

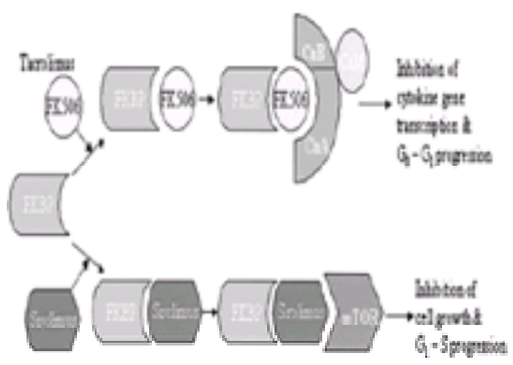

Figure 86.2. FKBP binding partners. Schematic illustration of the different binding partners of one FKBP immunophilin, FKBP12. FKBP12 binds to tacrolimus (FK506) and the complex of FKBP12 †tacrolimus, like cyclosporine †cyclophilins, binds to and inhibits the activity of the calcium/calmodulin-dependent phosphatase calcineurin, thus inhibiting cytokine gene transcription and G_0 to G_1 progression. FKBP12 binds sirolimus (rapamycin) and the complex of FKBP12/sirolimus binds to and inhibits the activity of mammalian Target of Rapamycin (mTOR), leading to inhibition of cytokine signaling, protein expression, and the G_1-S transition required for cell growth.

In the absence of tacrolimus or sirolimus, FKBP proteins, specifically FKBP12 and FKBP12.6, play important roles in ion channel function (37,38). FKBP12 binds the type 1 tumor growth factor-b (TGF-b) receptor (39) and negatively regulates internalization of the TGF-b receptor (40). FKBP12 and FKBP12.6 bind the ryanodine receptor (RyR1 in skeletal muscle and RyR2 in cardiac muscle, respectively, and both proteins bind the inositol-3-phosphate receptor (IP_3R). The IP_3R and Ryanodine receptor (RyR) channels regulate calcium-mediated signaling in diverse cell types, including muscle, heart, and immune cells. The association of FKBP12 (and of FKBP12.6) with the IP_3R and the RyR stabilizes the conformation of the receptor, modulates calcium conductance and channel gating (37,38). Furthermore, protein kinase A-dependent phosphorylation of RyR2 mediates the dissociation of RyR2 from FKBP12.6 (37) while the presence of an FKBP may provide a docking site for the phosphatase calcineurin (see below). Kinases and phosphatases, in turn, regulate receptor phosphorylation and thereby calcium channel gating (41,42). Another 12-kd FKBP, termed *inositol phosphate-binding protein* (IPBP12) is a 12-kd tacrolimus- and sirolimus-binding FKBP that binds inositol phosphates as well; it may regulate calcium flux as well (43). The importance of the FKBP/ion channel in clinical settings such as heart failure is currently being explored (42,44,45).

CALCINEURIN, A CENTRAL MOLECULAR TARGET

Because the specific molecular target of the immunosuppressant/immunophilin complexes, CsA/cyclophilin and tacrolimus/FKBP, is calcineurin (11,12), an understanding of the biologic role and function of calcineurin and of its regulation leads to an appreciation of the action of both immunosuppressant compounds. Indeed, since the discovery that calcineurin is the specific target of both CsA and tacrolimus, these agents have been used to reveal the fundamental role and importance of calcineurin in biologic systems.

Calcineurin Structure

Calcineurin is a calcium- and calmodulin-dependent serine/threonine phosphatase. A heterodimer, calcineurin, is composed of a 59- to 61-kd catalytic calcineurin A chain (CnA) and a 19-kd regulator calcineurin B (CnB) chain (Fig. 86.3); CnB is required for both proper protein folding and enzymatic activity (46). The calcineurin subunits exist in the cytoplasm of the cell as a holoenzyme. Three isoforms of the CnA subunit have been identified, and they are approximately 75% homologous to one another. Whereas CnAa and CnAb are expressed in all tissues, CnAb is expressed preferentially in cardiac tissues, and expression of the CnBg subunit is limited to the testis (47). Two isoforms of CnB have been identified: CnBa is expressed ubiquitously, and expression of CnBb is testis-specific. Furthermore, alternatively spliced forms of both CnA and CnB subunits have been identified. Little is currently known about isoform-specific expression and their biologic function. Differences in isoform-specific expression may correlate with or be important for developmental maturation or, alternatively, may help to explain tissue-specific differences in the role of calcineurin.

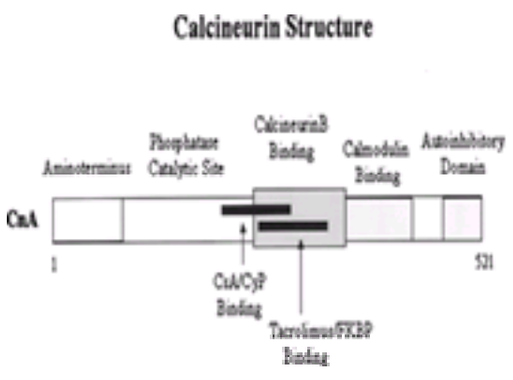

Figure 86.3. Calcineurin structure. Calcineurin is a heterodimer composed of a 61-kd catalytic subunit (CnA) and a 19-kd regulatory subunit (CnB). CnA has a phosphatase active domain, a CnB binding domain, a calmodulin-binding domain, and an autoinhibitory (AI) domain. The AI domain appears to bind at or near the phosphatase active site in the inactive form. Tacrolimus/FKBP and cyclosporine/cyclophilins bind near the CnB binding domain and prevent substrate docking.

The CnA subunit consists of the catalytically active domain, a domain for CnB binding, a domain for calmodulin binding and a carboxyterminal portion (Fig. 86.3). Both calmodulin, which associates with the carboxyterminus of CnA, and CnB independently bind calcium; calcium binding is required for enzymatic activity. The carboxyterminal domain of CnA contains a putative autoinhibitory domain as limited proteolysis or genetically engineering CnA to be deleted of the carboxyterminus has been shown to render the enzyme constitutively active (48). The carboxyterminal 97 amino acid fragment functions as an intrasteric, competitive inhibitor of calcineurin phosphatase activity (49). The solution of the structure of calcineurin, complexed with drug/immunophilin, confirmed that the carboxyterminus bound the phosphatase active site and that this catalytic site was in close proximity to, but not overlapping with, the substrate-binding site (50,51).

Calcineurin Activity

Calcineurin activity is stimulated by engagement of a number of extracellular receptors and, as predicted from its structure, regulated by calcium influx and calmodulin binding (52) and by reactive oxygen species (53). Calcineurin phosphatase activity is critical to the function of many intracellular processes and biologic functions (Fig. 86.4). These include nuclear translocation of certain transcription factors, such as nuclear factor of activated T cells (NFAT), ion channel regulation such as the gating of the RyR, protein transport, and enzymatic activity. The dependency of many of these processes on calcineurin has been revealed by the ability of cyclosporine and tacrolimus (but not sirolimus) to inhibit them.

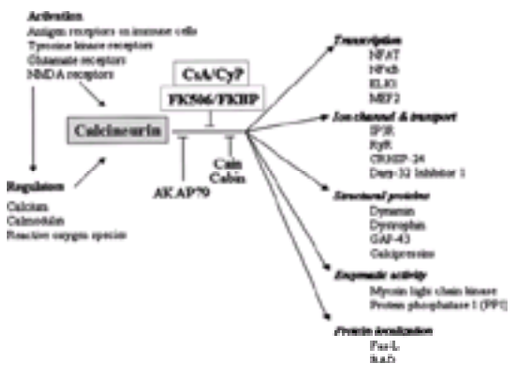

Figure 86.4. The central role of calcineurin. The calcium/calmodulin-dependent serine/threonine phosphatase calcineurin serves a number of roles, including regulation of transcriptional activity, ion channel and transport, protein interactions and localization. Calcineurin is activated by a number of receptors, as shown. Its activity is regulated by calcium, calmodulin, and reactive oxygen species, among others.

NFAT: A Target of Calcineurin

Early research focused on the role of the immunosuppressive agents in T-cell inhibition, and therefore the role of calcineurin in T-cell activation was the subject of study. Engagement of the T-cell receptor (TCR) initiates a complex series of signaling events (summarized in Fig. 86.5) that involves activation of protein kinase C (PKC) coupled with influx of extracellular calcium to the intracellular compartment. Calcium influx, together with calmodulin, leads to calcineurin activation; calcineurin-mediated dephosphorylation is required for the nuclear translocation of the transcription factor NFAT (54,55,56 and 57) as well as a number of other transcription factors (see later discussion). NFAT is a family of related transcription factors, many of which are important for transcription of the cytokine genes, including interleukin-2 (IL-2), IL-3, IL-4, tumor necrosis factor-a (TNF-a), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-g (IFNg) among others. Once in the nucleus, NFAT binds to and transactivates the DNA recognition sequence (2,58,59,60,61,62,63 and 64). Drug/immunophilin-mediated inhibition of calcineurin activity results in inhibition of nuclear translocation of NFAT and of expression of all the NFAT-dependent genes, notably the cytokine genes (64,65,66,67 and 68).

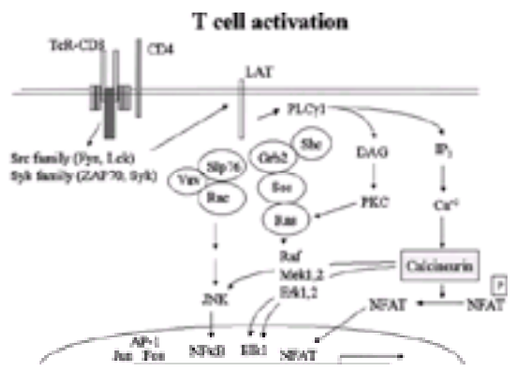

Figure 86.5. Effects of immunophilin/binding agent complex on T-cell signaling. Stimulation of T-lymphocytes via the T-cell receptor (TCR) mediates intracellular signaling events that include the activation of protein kinase C as well as an increase in intracellular calcium levels. Calcium and calmodulin are recruited to activate calcineurin, a phosphatase important for activation of a number of transcription factors. The calcium/calmodulin-dependent activation of serine/threonine phosphatase calcineurin is inhibited by both CsA/CyPA and FK506/FKBP12 complexes and *in vivo* by Cain/Cabin I.

It is important to note, however, that NFAT is not synonymous with calcineurin dependency; other mechanisms of calcineurin dependency exist. Maximal IFN-g transcription, for instance, depends on a calcineurin-dependent composite site made up of NFAT and kB elements (69). The NFAT protein is related to Rel/NFkB proteins (64,70,71) and, although different signaling pathways optimally regulate NFAT and NFkB, both proteins may bind common elements, as has been shown for the IL-4 promoter (72). This paradigm further allows for the possibility that the same element may regulate both calcineurin-dependent and calcineurin-independent transcription, depending in part on the inductive stimulus for transcription.

Transcription of a number of genes is activated by the binding of cooperating elements to composite sites on the enhancer or promoter; calcineurin dependence may relate to the sensitivity of one or more of the cooperating proteins. IL-3 and GM-CSF, for instance, are related genes that lie10 kb from one another (73) but are differentially regulated. Whereas GM-CSF is induced in T cells and in endothelial cells, cyclosporine-sensitive IL-3 transcription is induced only in T cells and NK cells. DNase-hypersensitivity of an enhancer element is eliminated after T-cell induction suggesting transcription factor binding; this site has been shown to bind Oct and NFAT elements cooperatively (74) and resembles those found in IL-2 and IL-4.

The cell type and differentiation state of the cell appear to be critically important for directing transcription and, therefore, may also be potentially crucial for calcineurin dependence. $CD4^+$ T-helper (Th) cells can be induced to differentiate to Th1-specific cells that secrete IFN-g and IL-2 and promote cell-mediated immunity or to Th2-specific cells that secrete IL-4, IL-5, and IL-10 preferentially and promote humoral immunity, immunity to intracellular parasites, and IgE secretion (for review, 75). In this context, the same transcription factor, NFAT1, is able to bind a 3' enhancer element of the IL-4 gene in activated Th2 cells but only to the IFN-g promoter in activated Th1 cells (76). Transcription of both IL-4 and IFN-g is cyclosporine sensitive, but the specific cytokine genes transcribed are controlled by access to the chromosomal site *in vivo.*

Calcineurin: NFAT Interaction

The site of NFAT interaction on calcineurin has been mapped extensively by mutagenesis and other molecular biologic techniques (for review, see 77,78). Shared among all NFAT family members is the DNA binding domain at the N-terminus; calcineurin binds to a region just aminoterminal to the DNA binding domain, a region that is not conserved in NFAT5. Furthermore, a short peptide was developed that inhibits NFAT-dependent activation of cytokine response genes but does not inhibit calcineurin phosphatase activity (78,79). This PxIxIT motif is thought to inhibit docking of NFAT to calcineurin and thus specifically inhibit this calcineurin substrate or others that rely on this binding motif (unidentified to date). If small molecules that mimic this PxIxIT-based peptide can be developed, the opportunity for selective T-cell and cytokine inhibition may be afforded. These NFAT-specific pharmacologic inhibitors may not affect other calcineurin substrates that potentially could be responsible for drug toxicity.

In addition to a specific docking site on calcineurin, NFAT contains a nuclear localization sequence that is masked, in the resting state, by serine phosphorylation on the regulatory domain (56). Neither the serine kinase responsible for NFAT phosphorylation nor the amino acids phosphorylated have been identified to date, and it remains possible that different kinases are active and responsible for the phosphorylation of different targeted residues within the NFAT family members. Calcineurin activity is required not only for NFAT nuclear translocation (56,80) but also for optimal DNA binding and transcriptional activity (81,82). Identification of calcineurin substrates coupled with molecular appreciation of the determinants of the interaction may allow for development of novel immunosuppressive agents with greater specificity and more limited toxicity.

Other Calcineurin-dependent Transcription Factors

Transcription factors other than NFAT have been identified that are regulated by calcineurin, factors often revealed by inhibition by cyclosporine or tacrolimus (83,84 and 85). Calcineurin has been shown to cooperate with PKC to activate the IkB kinase (IKK) complex. When dephosphorylated, this complex sequesters NFkB in the cytoplasm; NFkB is thus regulated by cyclosporine and tacrolimus (86,87 and 88). Furthermore, calcineurin can cooperate with another PKC isoform, PKCt, to induce activation of the MAP kinase family member Jun kinase (JNK) (89), which is responsible for c-Jun and activating transcription factor-2 (ATF-2) activation (90,91). Downstream substrates dependent on c-Jun or ATF-2 activity will therefore be inhibited by cyclosporine and tacrolimus.

Elk-1 appears to be a direct substrate for calcineurin, at least *in vitro* (92). Mitogen-activated protein (MAP) kinases induce serine phosphorylation of Elk-1, which is required for its activity. Calcineurin dephosphorylates the serine amino acid and thus represses Elk-1-dependent transcription (92) including c-Fos. Depending on the cell type, calcineurin has been shown to regulate the cyclic adenosine monophosphate (cAMP)- and calcium-responsive transcription factor cAMP-responsive element (CRE)-binding protein (CREB) (93,94,95,96,97 and 98). The molecular characteristics of this inhibition, whether direct or indirect, are yet to be explored.

Calcineurin-dependent Cytokine Effects Other than Transcription Factor Regulation

Calcineurin and JNK activity have both been shown to stabilize mRNA transcripts of a number of cytokine genes, including IL-3 and IL-2, containing *cis*-acting AUUUA-rich element in their 3' untranslated region (99,100). Cyclosporine and tacrolimus prevent this mRNA stabilization and promote degradation. Whether prevention of mRNA stabilization contributes to immunosuppressive action is not as yet clear. Other cytokines also can be affected by cyclosporine: cyclosporine increased hepatocyte growth factor expression by mechanisms that are as yet unclear.

Cyclosporine has been shown to upregulate expression of the immunosuppressive cytokine transforming growth factor-b (TGF-b) apparently through an effect on calcineurin (101,102 and 103). The effects of TGF-b expression on various immune and nonimmune cell types are complex but include induction of the cell-cycle inhibitor p21 (104), leading to inhibition of cell proliferation. Cyclosporine-dependent TGF-b promoted cancer progression and anchorage-independent growth in nude mice (105), an effect that some investigators relate to the increased incidence of secondary malignancies in patients receiving chronic immunosuppression.

Other Calcineurin Targets

Calcineurin has a role in a number of functions in addition to regulation of transcription factor localization and activity. The phosphatase has a major role in subcellular location of proteins generally, in addition to the transcription factors discussed already. A few examples will serve to illustrate the paradigm.

The proapoptotic protein Bcl 2 proapoptotic family member (BAD), a member of the Bcl-2 family, is bound by 14-3-3 proteins in the cytoplasm. Calcineurin-dependent dephosphorylation of BAD provokes the dissociation of BAD from 14-3-3, allowing binding to Bcl-X_L (106). BAD binding to Bcl-X_L prevents the latter from fulfilling its antiapoptotic function, resulting in induction of apoptosis, or cell death. It should be noted that regulation of BAD phosphorylation is not the only mechanism by which calcineurin affects the choice between survival and cell death: calcineurin activity is required for FasL (CD95L) expression and appears to be dependent on NFAT activity (107).

CLINICAL USE OF CYCLOSPORINE AND TACROLIMUS

The U.S. Food and Drug Administration has approved both cyclosporine and tacrolimus for prophylaxis and treatment of solid organ rejection, specifically in liver and kidney transplantation. In addition, these agents have been used extensively in other forms of solid organ transplantation, hematopoietic stem cell transplantation, hematopoietic stem cell disorders such as aplastic anemia, psoriasis, and a variety of autoimmune disorders such as rheumatoid arthritis and multiple sclerosis. A complete compendium of the clinical experience is beyond the scope of this chapter. Consultation with specialists familiar not only with the specific condition but, importantly, with the clinical use of these potent immunosuppressive agents should be encouraged.

It is important to appreciate that organ graft and patient survival is equivalent in controlled, randomized, multicenter treatment trials comparing tacrolimus and cyclosporine, each in combination with other agents (see, for instance, 108,109)). The utility of these earlier studies is limited by the appreciation that the optimal range for our current therapeutic blood level for tacrolimus is lower than that used in earlier efforts: the lower blood level may decrease toxicity without adversely affecting efficacy (108,109). Importantly, long-term follow-up comparing cyclosporine and tacrolimus, including late graft rejection and late toxicities (e.g., secondary malignancies, infectious complications) have not yet been reported. Cost differences will be important when the patent window expires. Finally, the importance of evaluating newer approaches to designing immunosuppressive regimens, including using cyclosporine and tacrolimus in combination with other agents, may well supersede direct comparisons of these two agents in clinical trials.

Toxicity and Side-Effect Profile of Cyclosporine and Tacrolimus

The clinical use of cyclosporine and tacrolimus is limited by extensive toxicity, particularly when used chronically. The most common side effects are nephrotoxicity, neurotoxicity, hypertension, and hyperbilirubinemia; an anticipated side effect of prolonged immunosuppression is susceptibility to infections (reviewed in 110,111 and 112). Acute nephrotoxicity with decreased glomerular filtration rate appears to be due to vasoconstriction and local ischemia. The incidence and severity of neurotoxicity, including headaches, seizures, and reversible posterior leukoencephalopathies, appear to be aggravated by coincident hypertension, hypocalcemia, and hypomagnesemia. These complications often respond to decreasing the dose of or discontinuing drug, but permanent sequelae may result. Because both cyclosporine and tacrolimus are extensively metabolized by the hepatic cytochrome P450 system, the development of hyperbilirubinemia challenges the ability of physicians to dose the medications appropriately; vigilant monitoring of blood therapeutic levels is indicated. Drug levels also should be assessed whenever medication changes are introduced, particularly those that affect the hepatic cytochrome P450 system. Therapeutic levels should be measured in the trough state (unless the patient is receiving drug by continuous infusion) and preferably performed by the same laboratory. There is a general, but not strict, correlation between levels, efficacy, and toxicity for both cyclosporine and tacrolimus; clinical judgment and experience must guide choice of agent, administration, and dosing.

Both cyclosporine and tacrolimus are lipophilic agents that are available in both oral and intravenous formulations. The diluent used for intravenous administration for both agents is a cremaphor (in olive oil) that occasionally is associated with burning of the hands and feet, a side effect often attenuated by decreasing the rate of infusion or changing to oral administration if possible. The absorption of both cyclosporine and tacrolimus is highly variable and dependent on a number of factors, including the presence of bile salts for cyclosporine. A new oral, microemulsion formulation of cyclosporine is available that is more readily absorbed and that has less variable pharmacokinetic parameters. The improved bioavailability is particularly helpful in patients with gastrointestinal difficulties or malabsorption (e.g., graft-versus-host disease, liver transplant recipients) and may translate into less erratic pharmacokinetics and decreased toxicity.

It has been difficult to segregate the side-effect profiles of cyclosporine from those of tacrolimus. The common side-effect profiles suggest that inhibition of calcineurin activity, which is common with these two drugs, may be responsible (113). Indeed, differences between different patients or patient populations may relate not to the mechanism of toxicity but rather to the specific underlying disease or organ(s) affected. It is possible but unlikely that the side effects and toxicities may be related to drug metabolites or the specific role of the immunophilin receptors. Of course, these possibilities are not mutually exclusive. A few side effects, such as hirsutism and gingival hyperplasia, have been observed only in cyclosporine-treated patients to date; these discordant toxicities are likely secondary to differences in immunophilin

expression (resulting in localized calcineurin inhibition by one but not the other agent), the biologic consequences of inhibition of specific immunophilins, or individual drug metabolites. The molecular causes of toxicity must await more careful analysis of animal models or the development of specific agents that target calcineurin substrate selectivity. It is hoped that small molecule mimetics and synthetic, bioavailable peptides or products will be developed that are specific for the biologic targets of calcineurin; these novel agents will have greater selectivity and, potentially, diminished toxicity.

CONCLUSIONS

Introduced into clinical practice only a quarter century ago, cyclosporine has forever changed the outlook for transplantation medicine. Current understanding of the intracellular receptors for cyclosporine and tacrolimus, and of the molecular target(s) of cyclosporine/cyclophilin and tacrolimus/FKBP is again modifying approaches to transplantation biology. This understanding, coupled with the development of new agents that have a different molecular mechanism and spectrum of side effects, will enlighten the design of equipotent, and less toxic, therapeutic regimens. Novel immunotherapies targeting specific, tissue-restricted proteins may limit toxicities to other organs and will further identification of such unique targets. We are approaching a molecular understanding of the biology of immunologic tolerance with the ultimate aim of inducing tolerance to allogeneic (and xenogeneic) antigens and reinstating tolerance to autoantigens in disorders of autoimmunity. At that time, targeted immunosuppressive therapy will be tailored to the molecular signaling pathway, to the affected organ system, and to the patient.

[1]It is important to consider the possibility that specific drug toxicities may be related to the effects of inhibiting immunophilin isomerase activity: If this were the case, one would predict that some toxicities of tacrolimus and sirolimus might overlap (inhibition of FKBPs), whereas that of CsA might differ.

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87 T-Cell Antigen and Costimulatory Receptors as Therapeutic Targets

Maria-Luisa Alegre, Ph.D., and Jeffrey A. Bluestone, Ph.D.

OVERVIEW

The prevalence of immune-mediated diseases in America and, indeed, around the world is a significant health care problem that requires an aggressive and innovative approach toward the development of new treatment solutions. Immune-mediated diseases include a broad spectrum of autoimmune illnesses such as rheumatoid arthritis, diabetes mellitus, lupus and multiple sclerosis, solid tumors and hematologic malignancies, infectious diseases, asthma and various allergic conditions. These diseases affect tens of millions of Americans and result in annual medical and other indirect costs of more than 100 billion dollars. Furthermore, immune-mediated graft rejection impacts more than 20,000 Americans suffering from nonimmunologic diseases who receive organ transplants each year.

Historical Context

Over the past 40 years, improved results in the treatment of immune-mediated diseases have been achieved primarily through the development of increasingly potent nonspecific immunosuppressive drugs to inhibit immune responses. Immunotherapies, such as steroids, cyclosporine A (CsA) and pan-reactive monoclonal antibodies (mAbs) have met with a degree of clinical success in treating conditions such as acute immune rejection of organ transplants and severe autoimmune diseases. Such therapies, however, require life-long use that nonspecifically suppress the entire immune system, exposing patients to considerably higher risks of infection and cancer. In addition, the chronic use of these nonspecific therapies has proven to have a negative impact on long-term graft survival (1). The calcineurin inhibitors and steroids in particular are nephrotoxic and diabetogenic. For instance, in nondiabetic kidney allograft recipients, the combination of calcineurin inhibitors and prednisone are associated with the development of an insulin-dependent diabetic state in up to 25% of the patients (2). Furthermore, the use of these drugs increases financial costs and side effects (3) and is of limited usefulness in the autoimmune, allergy, and asthma settings. Finally, the calcineurin inhibitors CSA and tacrolimus appear to inhibit the development of tolerance in multiple animal models as described in Chapter 86 and subsequently herein

Immune Tolerance: The Holy Grail for Immune Therapy of Transplantation and Autoimmune Diseases

A major goal for the treatment of immune disorders is the induction of immune tolerance - selective short-term immunotherapies targeted toward eliminating only the pathogenic immune response while preserving normal, beneficial immune function. The advances in the development of tolerogenic strategies in clinical transplantation have been fueled by multiple anecdotal cases in which transplant recipients have discontinued all immunosuppression and have not rejected their transplants (4). Whereas in most cases discontinuation of immunosuppression results in either fulminate acute or chronic rejection, these successful “experiments of nature” indicate that tolerance is achievable in clinical transplantation and suggest that the establishment of allograft tolerance using intentionally designed therapies is indeed an attainable goal (5,6). Such therapies are now a real possibility because molecular techniques have begun to help unravel the fundamental processes responsible for immune regulation. In fact, unparalleled progress made over the past few years in the conceptual understanding of the mechanisms operative in the induction of tolerance and restoration of self-tolerance has led to the development of unique and selective immunomodulatory strategies.

The normal processes generally thought to regulate tolerance are based on negative selection of autoreactive T cells in the thymus (*central tolerance*) and on five peripheral mechanisms: ignorance of antigen, clonal inactivation, clonal deletion, suppression, and cytokine-dependent immune deviation (Fig. 87.1). The relative contributions of the individual processes may vary depending on the nature of the antigen and the location in which “tolerization” occurs. Recently, numerous new approaches are making their way into clinical trials based on the demonstration of long-term kidney and islet allograft survival in nonhuman primates combined with strong preclinical data in autoimmunity. These therapies include the use of anti-CD154 antibodies (6,7) and cytotoxic T-lymphocyte (CTL)A-4 (CTLA-4)Ig (8) to block T-cell costimulation, a new generation of anti-T-cell mAbs that selectively inhibit the inflammatory subset of primed autoreactive effector T cells (9,10), and a number of therapies that alter the balance of the pathogenic and nonpathogenic T-cell responses. These accomplishments signal a quantum leap in our approaches to circumvent autoimmune destruction and graft rejection and now provide realistic opportunities for major advances in immunotherapy.

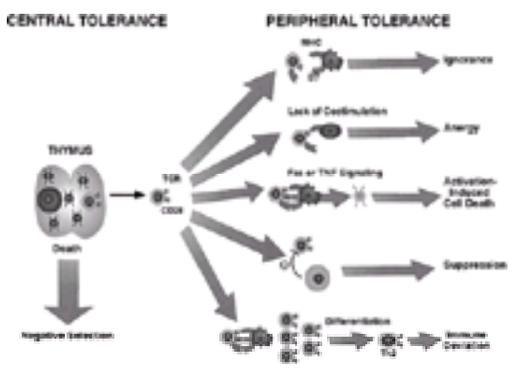

Figure 87.1. Mechanisms of T-cell tolerance. Lack of reactivity against self-antigens is thought to rely on a central mechanism of negative selection of highly self-reactive thymocytes. In addition, autoreactive peripheral T cells that may have escaped negative selection can be prevented from inducing autoimmunity by several known mechanisms: lack of attraction to the potential site of autoimmunity, killing or inactivation of reactive T cells, or induction of differentiation into cells with reduced cytotoxic potential.

Specific Immune Enhancement: a Goal for Cancer and Infectious Disease Therapies

In contrast to transplantation and autoimmune settings, infectious disease and tumor settings would benefit from specifically activating relevant T cells without stimulating bystander T cells that ultimately may give rise to lymphocyte malignancies or autoimmune disorders. Recent advances in gene therapy as well as in vaccination protocols to activate selectively tumor-specific T cells indicate that T-cell receptor (TCR) and costimulatory receptor signals are valid targets for T-cell activation as well. These approaches have combined our knowledge of the basic biologic processes that control immunization with the identification of novel or overexpressed normal proteins on infectious agents and tumor cells. Thus, the field of immune potentiation and therapeutic vaccinations provides a new means to combat these deadly diseases.

T-CELL RECEPTOR AND COSTIMULATORY MOLECULES: STRUCTURE AND FUNCTION

$CD4^+$ T cells play a major role in adaptive immune responses by recognizing specific antigens and subsequently providing help to different cell types eventually to eliminate the infectious agent or foreign antigen. A typical immune response begins when an antigen enters the body through the skin and migrates via lymphatic vessels to draining lymph nodes or penetrates through the gastrointestinal tract and migrates to the tonsils and Peyer patches. In some instances, the antigen enters the blood, where it is carried in the bloodstream to the spleen. The antigen is processed by specialized antigen-presenting cells (APCs) and presented on the cell surface in the context of major histocompatibility complex (MHC) class II molecules. The immune response is initiated when a small proportion of naive T cells, specific for a single antigenic peptide (as few as one in 10^5 or 10^6), expand in response to the antigenic peptide presented typically on macrophages or dendritic cells as APCs. The activated $CD4^+$ T cells interact with naive B cells that have soluble antigens bound by surface immunoglobulins, constituting the B-cell receptor (BCR). Signals delivered through the BCR induce changes on the surface of B cells that promote B-cell differentiation. Cell surface molecules, such as the ligands for costimulatory receptors on T cells and the antigenic peptide–MHC complexes, are upregulated on the activated B cells. This promotes the reactivation and expansion of the antigen-specific T cells. As a consequence of this activation, T cells secrete cytokines that promote B-cell proliferation, differentiation, and immunoglobulin isotype switching. Thus, the most potent immune responses combine optimal specific presentation of antigen and maximal expression of costimulatory ligands and receptors, further promoting B- and T-cell activation and differentiation into effector lymphocytes. As importantly, T-helper (Th) cells support the differentiation of cytotoxic T cells and natural killer (NK) cells.

Naive $CD4^+$ (often referred to as helper T cells, or Th cells) and $CD8^+$ T cells (also called *cytotoxic* T cells, or Tc cells) differentiate following activation into phenotypically different subsets. Th1 and Tc1 cells produce interleukin-1 (IL-2), tumor necrosis factor-a (TNF-a), interferon-g (IFN-g) but no IL-4, whereas Th2 and Tc2 cells secrete IL-4, IL-5, IL-6, IL-10, but no IFN-g. Each subset affects immune responses differently by potentiating either proinflammatory (T1 subsets) or humoral/allergic (T2 subsets) responses. For instance, Th1-type cells facilitate B-cell differentiation and immunoglobulin isotype switching toward complement-fixing immunoglobulin isotypes that promote inflammation and cytotoxicity. In contrast, the production of IL-4 and IL-5 by Th2-type cells is critical for the development of allergic and asthmatic diseases through the recruitment of histamine-producing mast cells. Therefore, T-cell differentiation plays a critical role in the pathogenesis of autoimmune and allergic diseases. Thus, therapies aimed at interfering with differentiation could prove beneficial for the induction of tolerance and for immune enhancement.

T-Cell Receptor: Structure and Function

T-Cell Receptor

The TCR is constitutively expressed on the surface of T-lymphocytes and comprises two disulfide-linked chains, termed a and b, that specifically recognize antigenic peptides bound to MHC molecules on the surface of APCs. The TCR is noncovalently associated with a complex of transmembrane small-molecular-weight polypeptides collectively called *CD3 proteins* and designated CD3g, d, e, h, and z. The intracytoplasmic domains of the CD3 molecules are longer (about 40 amino acids) than those of the a and b chains (5 amino acids) and contain phosphorylation and immunoreceptor tyrosine-based activation (ITAM) motifs for the binding of intracellular signaling molecules. On TCR engagement, these motifs become phosphorylated by intracellular kinases such as *lck* and *fyn,* inducing the recruitment of the z-associated protein (ZAP-70). These events result in activation of the phospholipase Cg1 and lead to intracellular accumulation of Ca^{2+}, an event that triggers the activation of a number of Ca^{2+}-dependent enzymes. This series of biochemical steps is followed, in turn, by the activation and nuclear translocation of transcription factors, resulting in the production of the multiple proteins expressed by activated cells. Depending on the subset of T cells as well as the stage of differentiation, these activation markers can be adhesion molecules and homing receptors necessary for trafficking, cytokines important for autocrine or paracrine functions, cytolytic enzymes that destroy cells expressing antigens recognized as foreign, and so on. Although an individual organism maintains a large diversity of TCRs, TCR molecules are in general of one species with specificity for a single ligand in each individual T cell (11).

STIMULATION BY ALTERED PEPTIDE LIGANDS

T-cell receptors recognize peptides 8 to 15 amino acids long bound to MHC class I or class II molecules. T cells can respond to variants of the same antigenic peptide by generating different effector functions, ranging from the secretion of cytokines in the absence of proliferation to altered differentiation. Recent data indicate that the different strengths of signal delivered by individual peptides can affect T-cell differentiation toward Th1 versus Th2 T cells (12,13). Under some circumstances, modifications of a peptide resulting in suboptimal interactions of the TCR with the peptide–MHC complex can lead to partial T-cell signaling, resulting in T-cell inactivation. These variant peptides, which are capable of interacting with the same TCR but lead to different phenotypic T-cell outcomes, have been termed *altered peptide ligands* (14). Biochemically, this is reflected by modified activation cascades compared with agonistic antibodies, such as partial phosphorylation of the CD3z chain, diminished Ca^{2+} flux, and reduced activation of certain kinase pathways.

Costimulatory Molecules

Ligation of the TCR, along with either the CD4 or CD8 coreceptors, by MHC molecules containing antigenic peptide is the first essential step for T-cell activation. TCR engagement is not sufficient, however, to mount a full immune response. An additional signal provided by a costimulatory receptor is also required. In fact, *in vitro* studies using human and murine T-cell clones showed that ligation of the TCR in the absence of costimulation can lead to a long-lasting state of T-cell unresponsiveness termed *clonal anergy* (15) (Fig. 87.2). On restimulation, anergic T cells are unable to secrete IL-2, the principal T-cell growth factor, even when TCR restimulation is performed in the presence of costimulatory molecules.

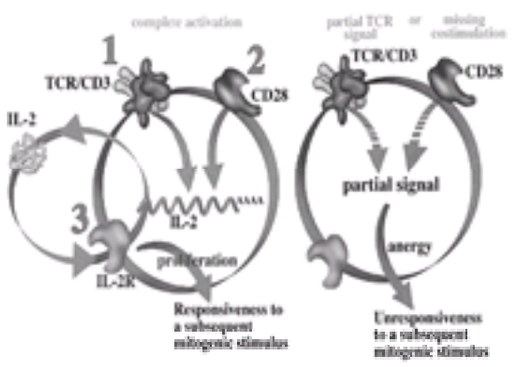

Figure 87.2. T-cell activation signals. T cells require at least two signals for complete activation. Signals delivered through the TCR (signal 1) and through a costimulatory receptor such as CD28 (signal 2) result in interleukin-2 (IL-2) production, upregulation of the IL-2R allowing IL-2 to act as an autocrine growth factor, and proliferation. In addition, T cells activated in this manner are susceptible to subsequent restimulation. In contrast, T cells receiving a partial signal through their TCR in the presence of costimulation or a complete TCR signal in the absence of costimulation become unresponsive to subsequent restimulation, a state that has been termed anergy in T-cell clones.

CD28/CTLA-4/B7 Pathway

A well-characterized costimulatory receptor on T cells is CD28 (16). CD28 is a homodimeric 44-kd glycoprotein constitutively expressed on virtually all murine T cells, all $CD4^+$ human T cells, 50% of $CD8^+$ human T cells, developing thymocytes, and plasmocytes. Signaling through CD28 in the presence of suboptimal TCR stimulation enhances T-lymphocyte proliferation as well as the production of multiple cytokines, including IL-2, TNF-a, granulocyte macrophage–colony-stimulating factor (GM-CSF), and IFN-g, as a consequence of both increased transcription and stabilization of cytokine mRNA. In addition, costimulation through the CD28 molecule also serves to improve T-cell survival, through the increased secretion of IL-2 and the upregulation of Bcl-x_L, a member of the Bcl-2 family of antiapoptotic genes. CD28 signaling appears to also favor differentiation of T cells into an IL-4-producing phenotype (17,18). T cells from mice made genetically deficient in CD28 by homologous recombination have reduced cytokine production and proliferation *in vitro* in response to various stimulators. Moreover, these mice have a significant defect in developing T-cell-dependent antibody responses. Some *in vivo* responses are well preserved in these mice, however, including the successful elimination of certain viruses and the ability to reject skin allografts (19,20) and heart allografts (21). These results suggest that either certain cells can respond in the absence of effective costimulation (specifically certain $CD8^+$ T cells) (22) or additional costimulatory molecules can compensate the lack of CD28.

CD28 has been shown to bind two ligands, B7-1 and B7-2 (also called CD80 and CD86, respectively), on APCs. CD80 is expressed on activated B cells and

monocytes as well as on dendritic cells and Langerhans cells. CD80 is upregulated on B cells on MHC class II engagement, and expression is enhanced further by the addition of exogenous IL-2 and IL-4. CD86 is 25% homologous with CD80 and is expressed on resting monocytes, dendritic cells, T cells, and activated NK cells, B cells, and other APCs. Both CD80 and CD86 are expressed on activated T cells, but the role of these molecules on T cells remains elusive. Mice deficient in B7-1, B7-2, or both have been generated. Mice lacking both B7 family members fail to form germinal centers and have immunoglobulin G1 (IgG1) and IgG2a responses, but humoral responses are more severely affected by lack of B7-2 than by lack of B7-1 (23).

Cytotoxic T-lymphocyte A4 is a 30- to 40-kd glycosylated protein that shares about 50% homology with CD28 and also binds CD80 and CD86 on APCs, although with higher affinity than CD28 (16). Unlike CD28, however, CTLA-4 is expressed on T cells only following T-cell activation and may serve to downregulate T-cell responses. Indeed, antibodies that block the binding of CTLA-4 to its ligands induce an increase in T-cell proliferation and cytokine production*in vitro*. *In vivo*, blocking mAbs increase antitumor immune responses, accelerate the onset of autoimmune diseases such as diabetes mellitus or experimental allergic encephalomyelitis, augment responses to pathogens, and accelerate cardiac allograft rejection in experimental murine models (21,24,25,26 and 27). Conversely, cross-linking of CTLA-4 reduces cytokine production and arrests T cells in the G1 phase of the cell cycle (28,29). CTLA-4-deficient mice develop a lymphoproliferative disorder and die a few weeks after birth, supporting a role of CTLA-4 in maintaining T-cell homeostasis (30,31). In addition, lack of CTLA-4 appears to promote Th2 differentiation in a CD28-dependent manner (32,33), and CTLA-4 cross-linking favors Th1 differentiation (34).

Therefore, T-cell activation depends on a subtle balance between costimulation through the constitutively expressed CD28 receptor and downregulation of the response by the inducibly expressed CTLA-4 glycoprotein. Thus, CTLA-4 may serve as a negative feedback mechanism to prevent perpetuation of T-cell activation once a productive immune response has occurred.

INDUCIBLE CO-STIMULATOR (ICOS) PROTEIN

ICOS is a novel costimulatory molecule that is expressed on T cells as a 55- to 60-kd homodimer about 20% that is homologous to CD28. Unlike CD28, ICOS is not present on naive T cells but is upregulated following T-cell activation and is retained in memory T cells. Cross-linking of ICOS results in enhanced T-cell proliferation, expression of adhesion molecules, and B-cell help. Ligation of ICOS, however, appears to augment production of IL-10 rather than IL-2. The ligand for ICOS was identified and termed *LICOS* (35). A ligand for the mouse homolog of the human ICOS also was found and termed **BB7RP-1** or *B7h*. B7RP-1 is expressed on B cells and macrophages (36). Its expression is induced by TNF-a, perhaps as a mechanism to increase antigen recognition during inflammation (37). Mice previously sensitized to the antigen oxazolone and treated with an agonist fusion molecule B7RP-1-Fc during antigen challenge display enhanced contact hyperreactivity compared with controls (36). Because of the recent discovery of the ICOS molecule, no data are yet available on the effects of ICOS blockade or cross-linking in experimental models of disease. Because of its potent effects *in vitro*, ICOS also may prove a useful target for immunomodulation *in vivo*.

Another distant member of the B7 family of molecules was discovered. B7-H1 is only 20% homologous to CD80 and CD86 and does not bind CD28, CTLA-4, or ICOS. Ligation of B7-H1 to a receptor on T cells results in increased T-cell proliferation and preferential induction of IL-10 production (38).

THE TNF/TNF RECEPTOR (TNFR) FAMILY OF COSTIMULATORY MOLECULES

The TNF/TNFR-related proteins are a growing family of molecules that include a number of costimulatory molecules, CD40L, CD30, 4-1BB, OX40, LIGHT, and TNF-related activation-induced cytokine (TRANCE). These are all expressed on activated T cells and, in some cases, have dual roles because they can enhance TCR-dependent proliferation but also trigger T-cell death under some circumstances. The ability to induce apoptosis or proliferation depends on the cell type, the stage of differentiation or activation, the transformation status, and the presence of other stimuli. Because T cells express both TNF-like and TNFR-like molecules, T-lymphocytes potentially can undergo autocrine costimulation or fratricidal programmed cell death. CD30 and CD40 lack the death domains present in the cytoplasmic tails of TNFR1 and Fas that are necessary for the latter molecules to promote apoptosis. The mechanism by which CD30 and CD40 mediate cell death is not clear but may depend in some cases on the production of TNF and subsequent signaling through the TNFR1 receptor (39).

CD40/CD40 Ligand (CD40L) Pathway

CD40, a 50-kd glycoprotein, is a member of the TNFR superfamily. It is expressed on APCs such as B cells, macrophages, and dendritic cells. CD40L (gp39, CD154), a member of the TNF superfamily, is expressed on activated T cells, mast cells, eosinophils, dendritic cells, activated platelets, and endothelial cells (40). CD40 is an important costimulatory molecule for B cells, the ligation of which promotes antibody production and proliferation. Importantly, cross-linking of CD40 also induces B7 on dendritic cells, macrophages, and B cells, therefore also increasing costimulation of T cells via CD28 binding. Recent data, however, indicate that CD40L cross-linking on human T cells can costimulate T cells directly in a CD28-independent manner and results in increased secretion of TNF-a, IFN-g, and IL-10, but not of IL-2, but also increased T cell death (41). Signaling through CD40 using agonistic antibodies has been shown to activate APCs, rendering them capable of directly costimulating $CD8^+$ cytolytic cells, bypassing the requirement for help by $CD4^+$ cells (42,43 and 44). Signals delivered through CD40 lead to activation of the transcription factor NF-kB, the accumulation of antiapoptotic molecules such as Bcl-x_L, and the production of IL-12 by dendritic cells. Mice deficient in CD40 or CD40L lack IgG, IgA, and IgE antibody responses and germinal center formation, underlining the importance of CD40L costimulation on T cells for the generation of B-cell help (45). CD40L on T cells is also necessary for priming of mice with antigen *in vivo* (46). In addition, B cells deprived of CD40 signaling appear to induce T-cell tolerance rather than priming (47). In humans, lack of CD40L on T cells results in a hyper-IgM syndrome, a human immunodeficiency associated with severely impaired humoral responses.

CD30/CD30L

CD30 was initially identified as a surface marker for neoplastic cells of Hodgkin lymphoma. Later, CD30 was found to be expressed on activated $CD4^+$ and $CD8^+$ T cells, and levels of expression were maximum if TCR stimulation was delivered in association with CD28 and IL-4 signals (48), resulting in preferential expression of CD30 on Th2-type cells (49). CD30 cross-linking induces activation of TNF receptor-associated factors (TRAF) TRAF1, TRAF2, and TRAF5 and results in activation of the transcription factor NF-kB (50). CD30 has both positive and negative roles on T-cell responses. Cross-linking of CD30 during TCR stimulation induces upregulation of IL-5, suggesting that CD30 may play a role in the pattern of cytokines produced in an immune response (49). In contrast, CD30-deficient mice display impaired negative selection (51), and CD30 overexpression enhances programmed cell death in the thymus (52), indicating that the CD30 receptor influences cell death signaling. Similarly, blockade of CD30 appears to reduce apoptosis of peripheral $CD8^+$ T cells induced by withdrawal of a TCR stimulus (53). CD30L (mCD153) is expressed on naive and activated B cells, on eosinophils, and on transiently on activated T cells. CD30L stimulates B-cell proliferation and antigen-specific antibody production in a CD40-independent manner (55).

4-1BB (CD137)/4-1BBL

4-1BB is a 39-kd glycoprotein induced on the surface of $CD4^+$ and $CD8^+$ T cells following TCR stimulation. 4-1BB is also found constitutively on monocytes, whereas 4-1BBL is expressed on activated APCs. Cross-linking of 4-1BB augments TCR-driven T-cell proliferation and cytokine secretion (56), and it increases production of IL-8 and TNF-a, but it inhibits IL-10 expression by monocytes (57). Despite being expressed on all activated T cells, 4-1BB signaling may preferentially affect $CD8^+$ T cells inasmuch as 4-1BB-deficient mice exhibit reduced antiviral $CD8^+$ T-cell responses to lymphocytic choriomeningitis virus (LCMV); $CD4^+$ T cells appear minimally affected (58). In addition, cross-linking 4-1BB *in vivo* prevents deletion of a higher proportion of $CD8^+$ than of $CD4^+$ T cells following staphylococcal enterotoxin A injection, presumably by promoting survival of $CD8^+$ T cells (59). Experiments performed using CD28-deficient mice indicate a role for 4-1BB in potentiating suboptimal CTL responses *in vivo* and *in vitro* in a CD28-independent manner (60). Finally, 4-1BB appears preferentially expressed on Tc2 rather than Tc1 cells following differentiation *in vitro*, and cross-linking antibodies on $CD8^+$ T cells result in upregulation of IL-4 by Tc2 but not IL-2 or IFN-g by Tc1 (61). Data indicate, however, that *in vivo* blockade of 4-1BB results in $CD4^+$ T-cell anergy, leading to abrogation of T-dependent humoral responses and that this event occurs in the absence of $CD8^+$ T cells (62). Like other TNFR family members, 4-1BB activates TRAF2 and results in activation of NF-kB. In addition, some costimulatory properties of 4-1BB, such as enhancement of IL-2 production by T cells, appear to be absolutely dependent on TRAF-2 activation because they do not occur in T cells from TRAF-2-deficient mice or mice transgenic for a dominant negative form of TRAF-2 (63).

Other TNF/TNFR Family Members

Other members from the TNF/TNFR family of molecules also exert costimulatory functions in T cells.

OX40 (CD134), a TNFR family member, is expressed on activated T cells, whereas OX40L is induced on B cells stimulated through CD40 and the BCR and on CD40-activated dendritic cells. T cells from OX40-L-deficient mice display reduced recall responses postantigenic priming and impaired contact hypersensitivity responses (64,65). In OX40-deficient mice, $CD8^+$ T-cell responses appear normal, whereas $CD4^+$ T cells exhibit reduced proliferation and IFN-g production, suggesting that OX40 plays a predominant role in costimulation of $CD4^+$ T cells (66). In addition, OX40 ligation on $CD4^+$ T cells may favor, directly or indirectly, upregulation of CXCR5 expression and migration of T cells into B-cell zones to support germinal center formation (67).

CD27, a member of the TNFR superfamily, is expressed on most T cells, B cells, and NK cells, whereas its ligand CD70 is expressed on activated T and B cells. *In*

vitro, anti-CD27 mAb or CD70-transfected cells can augment T-cell proliferation and cytokine production induced by PMA, anti-CD3 mAbs, or anti-CD3 plus anti-CD28 mAbs (68,69).

Expressed on activated $CD4^+$ T cells, TRANCE interacts with receptor activator of NF-kB (RANK) apparently restricted to mature dendritic cells. TRANCE shares sequence homology with CD40L and seems to share some of the functions of CD40L. In fact, blockade of TRANCE in CD40-deficient mice led to severely impaired $CD4^+$ T-cell responses to LCMV following infection *in vivo*, but it had no effect in wild-type mice, suggesting that TRANCE may be an alternative pathway bypassing CD40L for T-cell activation (70). $TRANCE^{-/-}$ mice exhibit osteopetrosis because TRANCE also plays a role in osteoclast differentiation. The absence of TRANCE also results in partial blockade in T- and B-cell development, however, underlining a crucial role of this molecule in lymphopoiesis (71).

A member of the TNF superfamily, LIGHT was recently cloned in human and mouse mononuclear cells. LIGHT appears to have a strong, CD28-independent costimulatory effect on T cells, leading to increased proliferation and secretion of IFN-g and GM-CSF. Gene transfer of LIGHT into tumor cells has resulted in rejection of an otherwise progressively growing tumor in mice, whereas blockade of LIGHT promoted reduced graft-versus-host disease (GVHD). These results suggest that LIGHT could be an important therapeutic target in clinical settings (72).

RECEPTORS ON T-CELLS FOR SOLUBLE COSTIMULATORY FACTORS

Soluble cytokines can exert costimulatory functions via receptors expressed on T cells. These cytokines can be divided into two categories based on whether they regulate expansion or differentiation of T-lymphocytes, although some may have a dual function. As stated, following activation, T cells differentiate into specific phenotypes. Most prominent, especially in the mouse, are the type-1 cells that produce IL-2, TNF-a, and IFN-g and the type 2 cells that secrete IL-4, IL-5, IL-6, IL-10, and IL-13. Cytokines can directly regulate T-cell growth and expansion. IL-2 is the prominent growth factor for type-1 cells, and IL-4 is responsible for both T-cell expansion and differentiation of the type 2 phenotype. Other cytokines act indirectly (sometimes via the APCs) to influence T-cell differentiation. For instance, IL-12 and IL-18 strongly promote type 1 differentiation. Finally, cytokines can influence other cytokines to effect differentiation. IFN-g and IL-10 can cross-regulate each other to promote Th1 and Th2 development, respectively. Therefore, cytokine administration can greatly influence the differentiation and expansion state of individual T cells bearing the same TCR, thus, potentially influencing the outcome of the immune response in the clinical setting. For the purpose of this chapter, only those cytokines/cytokine receptors that have been targeted clinically for therapeutical purposes will be discussed.

IL-2/IL-2R

Interleukin-2 is the prototype of a cytokine playing a role in cell expansion. IL-2 is produced by naive T cells following TCR stimulation as well as by T cells differentiated along the type 1 pathway. IL-2 is used in an autocrine and paracrine manner to promote T-cell proliferation, but it is also a growth factor for B cells, NK cells, monocytes, macrophages, and oligodendrocytes. It binds the IL-2 receptor (IL-2R), expressed transiently on activation of T cells, B cells, monocytes, and macrophages. The IL-2R is a complex of three polypeptide chains. The a-chain (p55, CD25) and b-chain (p75, CD122) have a low affinity for IL-2. The third subunit (g chain, p64) does not bind IL-2 but allows the formation of heterotrimeric abg complexes with high affinity for IL-2. The g-chain is also a component of IL-4R, IL-7R, IL-9R, and IL-15R and therefore is often referred to as the *common* g-chain. Mutations in the g-chain are responsible for X-linked severe combined immunodeficiency (X-SCID) because the g-chain is located on the X chromosome. X-SCID is a lethal disease characterized by reduced numbers of T cells and greatly impaired immune responses. Binding of IL-2 to its receptor induces tyrosine phosphorylation of residues within the receptor that serve as docking sites for multimolecular signaling complexes. This initiates three major pathways: the Janus kinase (Jak-STAT) pathway controlling gene transcription, the Ras-MAPK pathway leading to cell proliferation and gene transcription, and the PI3-kinase pathway involved in survival and organization of the cytoskeleton (73). In addition, IL-2 also may increase the susceptibility of cycling T cells to cell death by upregulating proapoptotic genes (74). In fact, IL-2-deficient and IL-2Ra-deficient mice develop a lymphoproliferative disease, resulting in early lethality (75,76).

IL-4/IL-4R

Interleukin-4 is involved in promoting both T-cell expansion and T-cell differentiation toward an IL-4-producing type 2 phenotype. It is produced by T cells, $NK1.1^+$ T cells, and mast cells and exerts multiple effects on T cells, B cells, monocytes, endothelial cells, fibroblasts, neuroblasts, myocytes, and others. IL-4 also induces isotype switching in B cells, leading to production of IgG1 and IgE by mouse B cells and IgG4 and IgE by human B cells. IL-4R is composed of a high-affinity a-chain (p140, CD124), which determines the biochemical nature of the induced signals and of the common g-chain, which is required for the transduction of such signals. A soluble form of the receptor is produced by alternative splicing and acts as an IL-4 antagonist. IL-4-induced growth depends on tyrosine phosphorylation events on the a-chain of the receptor. IL-4-driven differentiation depends on a region of the a-chain of the IL-4R comprising STAT6-binding sites. Indeed, IL-4 signaling activates the transcription factor STAT6, and IL-4-deficient and STAT6-deficient mice have impaired generation of type 2 responses (77,78), but STAT6-deficient T cells retain the ability to proliferate to exogenous IL-4 (79).

IL-12/IL-12R

Interleukin-12 is a heterodimeric cytokine that is composed of two chains, p35 and p40. It is produced by dendritic cells, macrophages, and B cells, and it has been shown to induce IFN-g secretion by T cells and NK cells, to enhance T-cell proliferation, and to induce differentiation into type 1 T cells. IL-12 induces direct activation of the transcription factor STAT4 (78). The receptor for IL-12 found on NK cells and activated T cells comprises a b1 and a b2 subunit, and the latter appears to play a critical role in regulating IL-12 responsiveness. Recent evidence suggests that the IL-12R may be expressed on B cells (80). Costimulation via the CD28/B7 pathway promotes IL-2 and IL-12 production by T cells and APCs, respectively, which, in turn, induce expression of IL-12Rb2. In addition, IL-12 may directly or indirectly induce B7 expression by APCs (81). Consistent with the known functions of IL-12, T cells from IL-12-deficient mice have reduced IFN-g production and defective differentiation into a type 1 pathway (82).

IFN-a

Type I interferons, IFN-a and -b, constitute a family of inducible secreted cytokines that are known to promote differentiation of human T cells toward a type 1 phenotype. This does not occur in mouse T cells, however, because IFN-g signaling indirectly activates STAT4 in human but not mouse $CD4^+$ T cells (83). IFN-g signaling in human T cells induces upregulation of IL-12 receptor, increased IL-12 responsiveness, and subsequent secretion of IFN-g. In addition, IFN-a also induces amplification of $CD8^+$ T-cell-driven responses, resulting in more potent antiviral responses (84). IFN-a is produced by lymphocytes, monocytes, and macrophages, whereas its receptor is present on most cell types. The ubiquitous expression of the receptor and the numerous effects of type I IFNs on non-T cells make it difficult to determine whether the therapeutic consequences of IFN administration to animals or humans depend on its direct effects on T-cell differentiation or on the innate immune system.

IMPORTANCE OF TCR AND COSTIMULATORY SIGNALS *IN VIVO*

It is clear that TCR and costimulatory signals are important for the generation of immune responses *in vivo*. Despite activation of a common set of enzymes and transcription factors, each costimulatory receptor appears to have unique properties in experimental settings *in vivo*. A window into the role of the respective molecules is provided by the study of immune responses in specific gene knockout mice, as discussed previously. For example, CTLA-4-deficient mice develop a lymphoproliferative disorder; mice deficient in CD28, CD40, CD40L, and B7 all lack germinal center formation; CD30-deficient mice have impaired negative selection; and so on. The role of costimulatory receptors as therapeutic targets in different models of disease has been given particular attention in recent years.

Transplantation

Strong experimental evidence indicates that TCR and costimulatory signals are essential for transplant rejection. T cells are required for acute allograft rejection to occur because animals devoid of T cells retain grafts long over the long term. In transplantation settings, interactions between receptors on T cells and their ligands are complicated by the fact that TCRs may be stimulated either directly by MHC molecules on the graft or indirectly by alloantigenic peptides presented by the MHC expressed by the host APCs (Fig. 87.3). In addition, costimulation has the potential of being delivered either by the donor or host cells and either by the same APC that presents antigen or in trans by other donor or host APCs. Although these scenarios multiply the possible combinations of events that can lead to T-cell activation, it is largely assumed that $CD4^+$ T-cells receive both the TCR and costimulatory signals from the same APCs because professional APCs of the host and donor express both MHC class II and costimulatory ligands. Effector $CD8^+$ cells, in certain cases, may receive costimulation from a different source than that expressing antigen because many target cells that express MHC class I are devoid of specific ligands for costimulatory receptors on T cells. Furthermore, different donor tissues contain different sorts of APCs and may be more or less competent at providing T-cell costimulation. As pointed out already, $CD8^+$ T cells may not always require costimulation allowing for direct activation of this subset on costimulation-deficient APCs. Thus, the use of costimulatory blockade may have different outcomes in transplanted tissues, such as skin, that have an abundant number of Langerhans cells that express high levels of costimulatory ligands versus heart allografts that contain few, if any, APCs. Therefore, each organ may elicit T-cell responses in a different manner and require different a immunotherapy for tolerance induction. The availability of B7-1- and B7-2-deficient mice as well as of B7-double knockout mice enabled investigators to address the role of donor versus host costimulation for allogeneic T-cell responses in certain models. It appears that rejection of cardiac allografts depends on costimulation by host rather than donor B7 (85), whereas lack of B7 on host cells

or host and donor cells is not sufficient to prevent rejection of skin allografts (86). This suggests that costimulation of other receptors on T cells is important for skin rejection or that skin rejection is costimulation independent. In contrast, CD28-deficient mice are competent at rejecting cardiac allografts, albeit with slower kinetics that wild-type mice (21), suggesting that CD28-mediated co-stimulatory signals are dispensable under some circumstances in this graft setting.

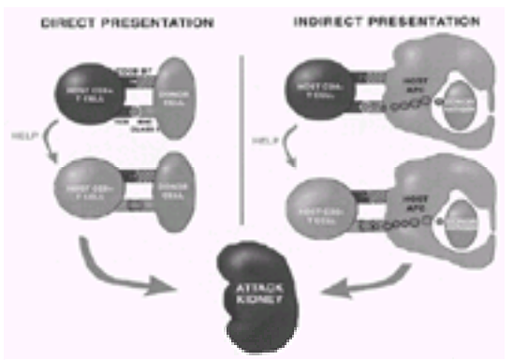

Figure 87.3. Antigen presentation in the context of allogeneic transplantation. Transplantation of allogeneic tissue introduces cells expressing foreign major histocompatibility complex (MHC) molecules to transplant hosts. Therefore, alloantigens are different from regular antigens in that they can be presented to $CD4^+$ and $CD8^+$ T cells either directly by the allogeneic MHC molecules present on graft cells or indirectly through representation of non-self peptides in the context of MHC molecules on host cells. Once activated, $CD4^+$ T cells can provide help to $CD8^+$ T cells and either subset can then directly or indirectly (via activation of other cell-types) mediate episodes of acute allograft rejection.

Autoimmune Diseases

New developments in understanding mechanisms of autoimmune diseases suggested novel immunotherapeutic approaches, such as the targeting of lymphocyte activation pathways and induction of lymphocyte anergy. New strategies are likely to affect immune regulatory pathways so that lasting tolerance rather than global immunosuppression can be achieved. Several lines of evidence implicate T cells in the pathogenesis of many autoimmune diseases. There is a striking correlation between disease progression and accumulation of $CD4^+$ T-helper cells and inflammatory cytokines in diseased tissues. Furthermore, genetic studies implicated the MHC class II complex. Multiple studies have shown that HLA-DRB*0401, *0404, *0101, and related subtypes are linked to certain autoimmune diseases, such as rheumatoid arthritis (RA) and type 1 diabetes. This association strengthened the concept that interactions between human leukocyte antigen (HLA)-DR molecules on APCs and TCRs regulate the disease process.

There is increasing evidence that costimulation plays a critical role in the development and progression of autoimmunity. A few studies showed increased expression of CD28 and CD80/CD86 in synovial fluid and tissue from patients with RA. Studies in animal models of arthritis, diabetes, multiple sclerosis (MS), and other autoimmune disorders provide strong evidence for the role of CD28 and CD40L in autoimmunity. For instance, experimental acute encephalomyelitis (EAE) is a rodent model for MS, where animals display brain infiltrates and limb paralysis following immunization with myelin-derived proteins. The CD28/B7 pathway appears to be important both for the induction and the effector phase of the disease. Mice deficient in CD28 or B7 molecules exhibit reduced disease severity than wild-type controls, and adoptive transfer of encephalitogenic T cells results in milder disease in B7-deficient than wild-type hosts (87). Similarly, MRL/lpr mice constitute a spontaneous model of lupus, in which mice develop lymphoproliferation, circulating auto-antibodies, and glomerulonephritis. The disease is also less severe in MRL/lpr mice deficient in CD28 molecules (88). Unexpectedly, deficiency in CD28 seems to accelerate and exacerbate spontaneous autoimmune diabetes in nonobese diabetic (NOD) mice (89). Why this particular autoimmune disease is accelerated whereas others are attenuated in CD28-deficient mice remains unclear but may reflect changes in the Th1/Th2 balance or development of regulatory T cells. In any case, these unexpected findings underscore the importance and complexity of the CD28/CTLA-4/B7 pathway in autoimmunity. Similar observations have been made for other costimulatory pathways. Mice transgenic for a TCR-specific for myelin basic protein (MBP) failed to develop EAE following immunization if their T cells were genetically deficient in CD40L. CD40L-deficient T cells could be induced to secrete cytokines following MBP challenge if stimulated with B7-expressing APCs, suggesting that reduced disease may result, at least in part, from the absence of CD40 signals on APCs, leading to subsequent defect in B7 upregulation (90).

A line of evidence in support of a critical role of costimulation in autoimmunity is the genetic association between certain costimulatory alleles and autoimmunity. In the NOD mouse, a diabetes susceptibility locus has been found on chromosome 1 within a region encompassing ICUS, CTLA-4 and CD28 (IDDM5), and Colucci et al. found that there is defective expression of CD28 and CTLA-4 in these mice (91). An association between polymorphisms in the CTLA-4 gene and a number of autoimmune diseases has been observed in humans (92,93,94,95 and 96). The role of CTLA-4 in controlling T-cell responsiveness makes this locus an interesting candidate gene for disease susceptibility.

Cancer and Infectious Diseases

T cells are essential for initiating or mediating tumor rejection, as most tumor cell-lines can form tumors when injected into mice that lack T cells genetically, or that have been T cell-depleted before the injection of tumor cells. In addition, chronic immunosuppression, such as that experienced by human immunodeficiency virus (HIV) patients or by patients treated long-term with immunosuppressive drugs, leads to an increased incidence of tumors, usually of viral origin. Numerous different tumor antigens have been identified that can be recognized by T cells. These antigens can be divided predominantly into three categories. The first group is composed of antigens that are not expressed in normal tissues and are therefore truly tumor specific. This is the case for the family of mitogen-activated protein (MAGE) antigens, for example, expressed in melanoma cells but not in normal cells with the exception of testis, where it is likely to be expressed exclusively in germline cells that are devoid of MHC class I and cannot present the neoantigen. The second group consists of antigens that are normally expressed in embryonic or differentiating tissues but are downregulated following maturation. An example of this is the carcinoembryonic antigen (CEA) protein produced by colon cancers. The third group comprises antigens that are expressed in normal cells but that are mutated (p53) or overexpressed (tyrosinase) in cancer cells. T cells have been shown to recognize and be activated by peptides belonging to each of these subclasses of tumor antigens. Even though T cells can react with these tumors, cancers still arise in many instances in otherwise immunocompetent patients. This may occur because of deletion or inactivation of tumor-specific T cells or because some tumor antigens do not get effectively presented to T cells. At least one example exists in mice, where the presence of an antigen expressed by tumor cells induces unresponsiveness of specific T cells, leading to cancer growth (97). Lack of costimulatory molecules on tumor cells may be a factor promoting a form of anergy of tumor-specific T cells. Indeed, costimulation appears to be important for tumor rejection. Using mouse models in which injected tumor cells can form tumors only in a subset of the animals, rejection, when it naturally occurs, depends on B7 molecules expressed by host APCs (98,99). This result confirms the importance of the CD28 pathway in normal tumor rejection.

Infectious Diseases

Antibody and CTL responses have critical roles in eliminating many pathogen infections. The important role of costimulatory signals in this setting has been increasingly realized. The absence of optimal CD28/B7, CD40L/CD40, and even 4-1BB interactions profoundly reduce generation of virus and other pathogen-specific immunity (100,101 and 102). Together, these results suggest therapeutic opportunities wherein tumor or infectious pathogen immunity might be enhanced in patients by promoting more effective T-cell costimulation in the context of immunogenic peptides. In fact, the immunologic benefit of coexpression of costimulatory molecules such as B7 or CD40 with target antigen has been exploited in a variety of vaccination protocols. In some systems, retroviruses have been used to transfect costimulatory molecules into antigen-bearing cells. In others, both the antigen and costimulatory molecules have been admixed to achieve high levels of cell surface expression. These approaches led to the increased efficacy of vaccination protocols in animal models. At present, several recombinant poxvirus, retrovirus and adenovirus vectors are currently being evaluated to induce antigen-specific immunity to a variety of infectious disease agents. This new threshold of T-cell activation has broad implications in vaccine design and development.

THERAPEUTIC APPROACHES TARGETING THE TCR

Immunotherapies that block TCR or costimulatory signals or that eliminate T cells usually result in immunosuppression and are used in settings of autoimmune diseases and transplantation where inactivation of T cells is warranted. Other treatments result in potentiation of TCR or costimulatory signals and are sought after for treatment of various cancers or infectious diseases in which activation of antigen-specific T cells is desired. Strategies that target TCR and costimulatory molecules for immunosuppression or immune enhancement are reviewed in the following.

Immunosuppressive Therapies

Antigen/MHC complex binding to the TCR/CD3 complex initiates a cascade of signaling events, beginning with the activation of several cytoplasmic protein tyrosine kinases. Recruitment of the CD4 (or CD8) coreceptor and its associated tyrosine kinase, lck, into the vicinity of the TCR complex is believed to induce phosphorylation

of CD3 proteins, ultimately leading to downstream signal progression. *In vitro* studies suggest that interruption of this signaling pathway at a number of points may lead to tolerance. Thus, mAbs directed at the TCR, altered TCR ligands, and MHC-derived peptides present novel approaches to tolerance induction that are ready to be applied clinically.

ANTI-CD3 AND ANTI-TCR MONOCLONAL ANTIBODIES

Antibodies directed at CD3 have been used since the early 1980s in human transplantation and have proven to be extremely potent immunosuppressive agents. The original mouse IgG2a antihuman CD3 mAb, OKT3, is still used to revert corticosteroid-resistant rejection episodes in the clinic, but it suffers from a high rate of "rebound" rejection when treatment is stopped (103). Several mechanisms lead to immunosuppression following anti-CD3 mAb injection. Antibody treatment induces rapid internalization of the TCR, thereby preventing subsequent recognition of antigen. In addition, administration of the mAb is rapidly followed by depletion of most T cells from the bloodstream and the peripheral lymphoid organs, such as lymph nodes and spleen. This absence of detectable T cells from the usual lymphoid regions is secondary in part to cell death following complement activation and activation-induced cell death but also to margination of T cells onto vascular endothelial walls and redistribution of T cells to nonlymphoid organs, such as the lung. Finally, anti-CD3 mAb also induces a downregulation of the function of nondepleted T cells, as defined by lack of IL-2 production and a great reduction in the production of multiple cytokines, perhaps with the exception of IL-4 and IL-10. Several problems have limited the usefulness of this mAb in transplantation and prevented the widening of its use to the treatment of autoimmune diseases. First, whereas OKT3 therapy promotes long-term graft survival, there is no evidence of clinical tolerance induction. In fact, patients often suffer from high rate of "rebound" rejection when treatment is stopped. Second, before inducing immunosuppression, anti-CD3 mAb administration results in the production of multiple cytokines, including GM-CSF, TNF-a, IL-2, IL-3, IL-4, IL-6, IL-10, IFN-g, resulting in side effects designated as first-dose reactions. Symptoms range from moderate to extremely serious and consist of high fever, headaches, nausea, vomiting, diarrhea, pulmonary edema, seizures, and coma and are due to the direct toxicity of these cytokines. Usually, only the first administration of anti-CD3 mAb induces these side effects because the TCR becomes internalized after the initial dose and is not susceptible subsequently to stimulation by the mAb. Experiments in mice using a model anti-CD3 mAb, 145-2C11, revealed that high doses of corticosteroids prevent the initial release of cytokines and reduce first-dose reactions considerably (104,105). Administration of corticosteroids prior to the injection of OKT3 is now a standard procedure in the clinic and has improved tolerance to the drug (106). Fc receptor (FcR) binding by the Fc portion of anti-CD3 mAb appears to be the cause for the initial activating properties of this agent (107). Finally, the third drawback to repeated use of the mAb is the occurrence of immunization against the mouse determinants of the antibody, which can neutralize OKT3 and prevent its immunosuppressive efficacy. Recently, genetically altered anti-CD3 mAbs were developed that are humanized to minimize the occurrence of anti-mAb antibody responses and mutated to prevent binding to FcRs (108). An analog of these humanized anti-CD3 mAbs, the chimeric antimouse CD3 mAb 2C11-IgG3, was generated by fusing the variable region of a hamster antimouse CD3 with the constant region of mouse IgG3 Fc that has intrinsic low affinity for FcRs. This new generation of anti-CD3 mAb induces selective immunomodulation in the absence of toxicity associated with conventional anti-CD3 mAb therapy (109). FcR nonbinding anti-CD3 mAbs have short-lived effects on naive T cells but deliver a partial signal in activated T cells, resulting in clonal inactivation of Th1 cells and proliferation/cytokine production by Th2 cells (110). These selective effects make these new agents particularly useful for targeting the imbalance of autoreactive Th1 cells and immunoregulatory T cells that favors certain autoimmune diseases and allograft rejection. 2C11-IgG3 also was evaluated in transplantation tolerance induction models (107,109); a short course of the mAb not only results in long-term graft survival but allows a second genetically identical graft, placed at a later date, to survive without additional therapy. Interestingly, CsA inhibits the ability of a second genetically identical graft to survive without rejection (J. Bluestone, unpublished observation), supporting the hypothesis that, although calcineurin inhibitors may have an additive immunosuppressive effect in combination with nonactivating anti-CD3 therapy, they can inhibit tolerance induction by the low FcR affinity anti-CD3 mAb. Further studies in the autoimmune disease setting have shown that short-term treatment with anti-CD3 mAb restores peripheral self-tolerance to b-cell-associated autoantigens in overtly diabetic NOD mice when applied within 7 days of the onset of diabetes (10). Moreover, unlike untreated diabetic NOD mice, these animals do not destroy syngeneic islet grafts. In an initial clinical trial, the humanized FcR nonbinding anti-CD3 mAb reversed acute renal allograft rejection in the absence of first-dose cytokine release syndrome (111). In addition, initial results from two other clinical trials are showing absence of side effects in new-onset type 1 diabetes patients (K. Herold and J. Bluestone, unpublished observations) as well as in patients with psoriatic arthritis (M. Clark and J. Bluestone, unpublished observations). Clinical efficacy of the drug in these autoimmune settings is being evaluated. Other nonmitogenic anti-CD3 mAbs also were generated. BC3 is a mouse antihuman CD3 IgG2b that has been used to treat acute GVHD in 14 patients, resulting in improvement of GVHD symptoms in most patients with minimal acute toxicity (112). Monovalent anti-CD3 mAb has been used in renal transplant patients as another means of preventing TCR cross-linking and cytokine release (113). Finally, a humanized rat antihuman CD3 in which the cytoplasmic tail was mutated to prevent glycosylation, thereby also preventing FcR binding, has shown promising results in the treatment of acute renal allograft rejection, leading to few side effects while retaining immunosuppressive properties (114).

In addition to anti-CD3 mAbs, anti-TCR mAbs have also been used in clinical trials to induce global immunosuppression in solid organ and bone marrow transplantation settings. These include T10B9, a mouse IgM, and BMA031, a mouse IgG2b (Fig. 87.4). Both these mAbs are less mitogenic than OKT3, resulting in somewhat reduced side effects, while still inducing potent lymphopenia and immunosuppression. Clinical efficacy in preventing allograft rejection was similar or slightly reduced compared with OKT3, and the incidence of immunosuppression-related infections was also comparable (115,116,117 and 118).

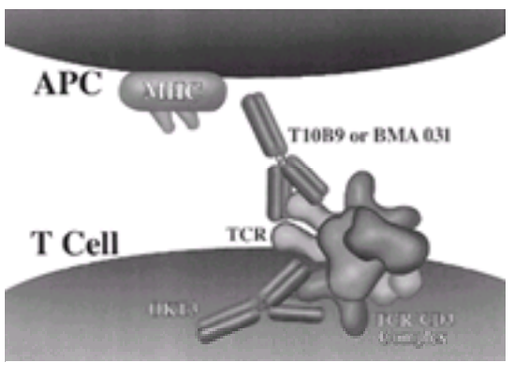

Figure 87.4. Antibodies directed to the T-cell receptor (TCR)/CD3 complex. Several monoclonal antibodies (mAbs) have been generated that recognize either the TCR or the CD3 complex of T cells. Binding of the mAbs to the TCR/CD3 complex results in TCR internalization, complement-mediated deletion of a subset of T cells, and, in some cases, inactivation of residual T cells, possibly because of the lack of simultaneous costimulation. Because all T cells constitutively express TCR and CD3 molecules, these mAbs lead to global immunosuppression.

ANTIGEN-BASED THERAPIES

Research that involves experimental therapies aimed at inducing tolerance is shifting from a global T-cell immunosuppression approach to more limited targeting of antigen-specific T cells. Therefore, attempts are being made to administer whole proteins or peptides recognized by defined TCRs in a manner that results in specific T-cell tolerance. For example, in transplantation settings, peptides derived from the donor MHC can activate T cells via the "indirect" pathway. In multiple sclerosis in humans and EAE in rodents, peptides derived from MBP and proteolipid protein can induce disease when administered in adjuvant. Different modes of administration of antigens or peptides have been used over the past years to induce tolerance rather than full activation of T cells. These include intrathymic injection of antigen or antigen-containing cells, systemic injection of soluble antigen, administration of altered peptide ligands, and oral or nasal administration of antigen. Moreover, these techniques can be combined to induce tolerance because, for example, altered peptide ligands can be administered orally. In each case, the mechanism of tolerance may be different and is described subsequently.

Intrathymic Injection of Antigen

Negative selection is a central mechanism of tolerance whereby thymocytes encountering high affinity antigen in the thymus are deleted, thus avoiding autoimmunity. Therefore, intra-thymic injection of antigen as a means of inducing deletion of antigen-specific T cells has been attempted in multiple experimental models of transplantation and autoimmune diseases. For example, intra-thymic injection of pancreatic islets in combination with depletion of $CD4^+$ T cells in mice has been shown to induce donor-specific tolerance to alloantigen by inducing clonal deletion of thymocytes directly recognizing alloantigen (119). Similarly, intrathymic injection of peptides derived from allogeneic MHC class I in combination with T-cell depletion of the recipient rats with antilymphocyte serum led to permanent acceptance of allogeneic islets. In contrast, animals treated with antilymphocyte serum, alone or in combination with intravenous injection of the peptide, did not become tolerant to the transplant (120). Tolerance following intrathymic administration of antigens appears to result both from depletion of antigen-specific thymocytes as well as from reduced IL-2 production by the remaining mature antigen-specific T cells (121). In addition, induction but not maintenance of tolerance may depend on CTLA-4 signaling because injection of anti-CTLA-4 mAb shortly after priming prevented induction of tolerance by intrathymic injection of MBP in a mouse EAE model (122). These data suggest that intrathymic injection of known antigens may have clinical applications for tolerance induction in transplantation and autoimmune settings. Thymus involutes rapidly in adulthood in humans, and whether enough tissue remains to allow for maturation of new thymocytes in the presence of the injected antigen remains a matter of debate.

Systemic Administration of Antigen

The milieu in which TCR encounters antigen appears to affect subsequent immune responses to that antigen. Thus, the route and context of antigen exposure (i.e., soluble antigen versus antigen emulsified with adjuvant, subcutaneous versus intravenous injection, or peptide fragments versus whole protein) can greatly alter the immune response. Indeed, the route of antigen administration affects the type of cells that will bind the peptide or process the antigen for presentation to T cells as well as the environment where this happens. In addition, the presence of adjuvant may induce the production of IL-12 by APCs, upregulation of costimulatory molecules or other inflammatory events, resulting in full activation rather than tolerization of T cells. As an example, skin from transgenic mice expressing galactosamine as a transgene is rejected rapidly by syngeneic recipients presensitized with galactosamine in an adjuvant solution. In contrast, recipients became tolerant to the skin transplant when galactosamine was injected intravenously in a soluble form (123). Peripheral tolerance achieved following intravenous injection of antigen depends on depletion of antigen-specific T cells and anergy because remaining T cells have reduced cytokine production on restimulation. Tolerance induced by the injection of peptides is short-lived, however, even if the emigration of new T cells is precluded by thymectomizing the tolerant mice. Reversal of tolerance probably occurs because of the limited half-life of soluble peptides, but it can be maintained with repeated peptide injections (124). Similar results have been obtained in humans. Intravenous but not subcutaneous or intrathecal injection of a synthetic immunodominant MBP peptide to patients with MS resulted in tolerance to MBP as assessed by undetectable levels of MBP autoantibodies in the serum of patients. Although a single injection of peptide reduced MBP autoantibodies for 3 months, effects lasted longer than 1 year in most patients who received two injections (125). Similarly, intravenous injection of MP4, a fusion molecule between human MBP and the extracellular domain of proteolipid protein (PLP) has been shown to prevent or ablate established EAE in mouse and primate models (126). Thus, the use of intravenous peptide administration has emerged as a potentially important therapeutic opportunity, with multiple clinical trials initiated at this time.

Administration of Altered Peptide Ligands

As stated above, ligation of a TCR by different variants of a single peptide can result in distinct T cell responses ranging from clonal anergy to Th1/Th2 skewing, to full activation. Peptides derived from human papilloma virus and from *Bacillus subtilis* contain a motif also found in a major antigenic peptide of MBP and function as altered peptide ligands on T cells specific for MBP. Recently, it has been shown that injection of these peptides prevented onset of EAE by MBP immunization in rodents and induced IL-4-producing suppressor T cells capable of preventing EAE induction (127). Similarly, altered peptide ligands can be used to tolerize encephalitogenic T-cell clones *in vivo*, thus preventing T-cell infiltration in the CNS and paralysis of the mice. This effect is dependent on IL-4 production. (128).

An example of administration of a peptide in humans that can downregulate autoimmunity is that of Copolymer 1 (Copaxone) for the treatment of MS. Copaxone is a synthetic copolymer that mimics MBP, but instead of being encephalitogenic, it causes suppression of MBP-induced EAE. Copaxone binds MHC class II HLA-DR molecules and can serve as an antagonist of the 82-100 epitope of MBP. It is thought to function as an altered peptide ligand, resulting in differentiation of MBP-reactive T cells into a less aggressive phenotype, or inducing regulatory T cells that suppress MBP-dependent inflammatory responses. In animals, Copaxone induces suppressor T cells that can transfer tolerance into naive hosts protecting against induction of EAE. Copaxone-specific T cells secrete IL-4, but no IFN-g in response to MBP, whereas they can secrete IFN-g in response to Copaxone itself. Copaxone is routinely administered subcutaneously to patients with MS with few side effects. Patients receiving Copaxone were more likely than controls to be neurologically improved and have fewer relapses (129), suggesting that altered peptide ligands may be useful in clinical settings.

Oral or Nasal Tolerance

A well-recognized approach to the induction of antigen-specific tolerance is the mucosal (oral or nasal) administration of antigens or peptides. Oral tolerance can be achieved following feeding of either low or high doses of antigen, through an effect on gut-associated lymphoid tissue such as Peyer patches. Low-dose oral tolerance appears to result from the induction of regulatory T cells that secrete antiinflammatory cytokines such as transforming growth factor-b (TGF-b), IL-4, and IL-10. High-dose oral tolerance is thought to depend on the deletion or anergy of antigen-reactive T cells.

Multiple antigens have been used successfully for the induction of oral tolerance in autoimmune diseases in small animals. The effects of these peptide/proteins in humans has had mixed results, however. The use of Copaxone for MS is an example of this therapy resulting in amelioration of a disease. Copaxone-specific T cells generated after oral administration of the polymer appear to suppress T cells reactive against MBP and also T cells responding to other myelin antigens, presumably via the secretion of inhibitory cytokines. Oral administration of Copaxone was associated with elevation of serum IL-10, TGF-a, and IL-4 as well as with the reduction of the proinflammatory cytokine TNF-a, suggesting a shift from a Th1 to a Th2-type responses in humans. Oral Copaxone-1 has been approved for the treatment of relapsing MS and has been shown to stabilize disease in 90% of patients as well as to reduce the annual rate of disease relapse. Whether the oral route of administration provides any added benefit beyond that noted due to its altered peptide ligand activity remains to be seen.

“Immunogenic” peptides and proteins have been tested orally in MS patients. These tests include oral administration of bovine myelin (130) or a combination of MBP and proteolipid protein (131). Initial phase 1 and 2 studies noted fewer relapses in treated groups compared with control groups. In addition, oral treatment with antigen promoted generation of antigen-specific TGF-b-producing T cells, suggesting a possible mechanism for suppression of inflammation of the target organs.

Type II collagen has been administered to patients with RA (132). Some efficacy was observed at the lowest dose tested, consistent with the findings in animal models that oral administration of low dose antigens induces generation of disease-suppressing regulatory T cells.

Patients with autoimmune thyroid disease have received oral animal thyroglobulin. Treatment did not produce changes humoral immunity parameters but resulted in reduced proliferation *in vitro* to certain thyroid peptides (133).

Autoimmune uveitis has been treated with oral administration of an HLA class I peptide that mimics a retinal autoantigen. Treatment resulted in reduced intraocular inflammation and unchanged or increased visual acuity (134).

Taken together, these results indicate that oral administration of antigen does have systemic effects on T cells and may be useful for the treatment of autoimmune diseases. Some larger trials, however, failed to allow definitive conclusions about the efficacy of this approach, and worsening of autoimmune diseases after mucosal administration of antigen was anecdotally reported in a animal models (135). Therefore, the use of oral administration of these immunogens awaits further examination in larger clinical trials.

ADMINISTRATION OF PEPTIDES DERIVED FROM THE TCR

The V-region hypothesis, proposed in the late 1980s, suggested that, in some instances, autoimmune diseases were mediated by a limited number of T-cell clones, each associated with a specific use of a TCR V-region. This led several investigators to hypothesize that immunization with a peptide derived from the dominant TCR might alter the pathogenicity of the T cells and ameliorate disease. This TCR peptide immunization protocol was originally developed in EAE because it was shown that T cells that recognized MBP in rats preferentially utilized Vb8.2, a specific b-chain of the TCR. Immunization of rats with synthetic peptides derived from that TCR region was effective in both preventing and, more significantly, treating EAE (136,137 and 138). Similar results were found in mice with EAE. The mechanism of action of this approach is unclear. TCR peptide immunization has been shown to activate peptide-specific T cells, stimulate production of anti-TCR mAbs, and perhaps tolerize disease-causing antigen-specific T cells. In patients with MS, vaccination with a peptide from Vb5.2 sequence, expressed on MBP-reactive T cells, reduced MBP responses and stabilized disease in the few responding patients (139). Peptide-specific Th2 cells inhibited MBP-specific Th1 cells *in vitro* via secretion of IL-10. Further clinical studies in this indication are planned.

Specific Vb subsets also are overexpressed in synovial fluid and tissues from patients with RA. A recent clinical trial evaluated immunization of RA patients with peptides derived from Vb5, Vb14, and Vb17 in incomplete Freund adjuvant (140). This therapy was well tolerated and showed a tendency toward improvement of clinical outcome; however, the true efficacy of TCR peptide immunization in these patients has yet to be determined.

Strategies for Immune Enhancement

ANTI-CD3 MAB

The activating properties of anti-CD3 mAbs have been suggested as potentially useful for tumor immunotherapy, despite the subsequent immunosuppressive effect of the mAbs. Initial animal studies showed that treatment with low doses of activating anti-CD3 mAb reduced the incidence of tumors and provided long-lasting tumor immunity in mice injected with progressive tumor cells. Tumor rejection was initially dependent on $CD4^+$ T cells and required the presence of $CD8^+$ T cells throughout the immune response, suggesting that the early cytokine release episode induced by anti-CD3 stimulation of T cells resulted in the generation of tumor-specific cytotoxic T cells.

A phase 1 clinical trial using the activating properties of a humanized anti-CD3 mAb in cancer patients has been completed. A dose of anti-CD3 mAb that results in

T-cell activation with non–life-threatening side effects and brief TCR downmodulation was identified in this trial (141). Interestingly, malignant ascites resolved in three patients following anti-CD3 administration, suggesting the potential usefulness of this molecule for the treatment of certain cancers.

ALTERED PEPTIDE LIGANDS

The use of altered peptide ligands has been proposed to enhance T-cell responses to otherwise weak antigens. The human gp100/pmel 17 differs by 3 amino acids from its mouse homolog, expressed on melanoma cells as well as in normal melanocytes. Immunization with a vaccinia virus encoding the human but not the mouse protein elicited a $CD8^+$ T-cell response and promoted rejection of an established melanoma tumor in mice (142). The human peptide appeared to have a higher affinity for MHC class I and increased the ability of T cells to secrete IFN-g.

Mice are differentially susceptible to *Leishmania* infection, depending on their strain of origin. A normally susceptible strain with T cells transgenic for a *Leishmania*-specific antigen has been shown to become resistant to subsequent infections if pretreated with peptides that differ in one amino acid from the Leishmania immunodominant peptide (143). These altered peptide ligands appear to prevent IL-4 production by T cells from these animals, an important event for generation of Th2-type cells and susceptibility to the infection.

No data are currently available on the use of altered peptide ligands for immune enhancement in patients. These high-affinity, “superagonistic,” altered peptide ligands may be an effective therapy for enhancing T-cell responses against tumor antigens and infectious agents.

ANTIGEN/APC VACCINATION

Because T cells are optimally activated by peptides and costimulatory ligands, both of which are both present on professional APCs, another approach for immune enhancement has consisted of immunizing animals or patients with APCs that express a tumor antigen. The variables in these protocols include the source of antigen (tumor cells, tumor lysate, tumor antigen, or peptides), the type of APC, and the route of administration to ensure that relevant T cells will come in contact with the antigen-expressing APCs. The first generation of vaccines was using whole cancer cells or tumor-cell lysates as a source of antigen in combination with various adjuvants, relying on APCs of the host to process and present tumor-specific antigens. These therapies resulted in occasional clinical responses and are being tested in prospective clinical trials (144).

The second generation of vaccines utilized specific APCs incubated *ex vivo* with antigen or transduced to express antigen and subsequently reinfused into patients. Among the most promising therapeutic APCs are dendritic cells because of their expression of high levels of MHC and costimulatory molecules. Dendritic cells, generated from animals or patients following incubation of blood or bone marrow cells with GM-CSF or GM-CSF and IL-4, are the optimal APC to stimulate antigen-specific T cells *in vivo*. Preclinical studies showed that animals immunized with dendritic cells previously pulsed with MHC class I-restricted peptides derived from tumor-specific antigens develop potent antitumor CTL responses and protective tumor immunity can be generated (145). Because generation of dendritic cells from patients is time consuming and cumbersome, other types of APCs have been investigated as well. Recent data indicate that leukocytes isolated from peripheral blood and immediately pulsed with antigen can be used to immunize mice against tumors if exogenous IL-12 is provided simultaneously (146). IL-12 acts as a costimulatory molecule promoting Th1 and Tc1 differentiation, a phenotype that is more effective at promoting tumor rejection. In addition, IL-12 upregulates B7 family members on host APCs, thereby optimizing T-cell activation. This provides an easy approach in humans; a clinical trial in melanoma patients using peripheral blood cells pulsed with the HLA-A2-binding peptides MAGE-3 or MART-1 is currently being completed in patients expressing an HLA-A2 allele.

Dendritic cells pulsed with a tumor antigen elicit tumor-reactive T cells in clinical trials of patients with different types of cancers. The surface immunoglobulin expressed on B-cell lymphoma has unique idiotypes that can be recognized by T cells. Forty-nine percent of patients immunized with tumor immunoglobulin protein coupled to keyhole limpet hemocyanin (KLH) and emulsified in adjuvant generated specific immune responses against the idiotypes. This correlates with a more favorable clinical outcome because patients with antiidiotypic responses were free of disease for almost 8 years compared with 1 year in patients who did not mount an immune response (147). Multiple myeloma is another B-cell malignancy in which immunotherapy directed at idiotypes may be useful. Myeloma cells do not express immunoglobulin on their surface, but they do express idiotype-derived peptides on MHC molecules. Six patients have been treated with dendritic cells pulsed with autologous idiotype and control KLH antigen. Treatment was associated with T- and B-cell responses to KLH in all patients as well as increased antiidiotype cytotoxic T cell precursor frequency in three patients (148). Numerous melanoma-specific antigens have been identified, including MAGE-1, MAGE-3, tyrosinase, gp-100, and MART-1. The MHC class I alleles that bind these peptides have been identified, allowing vaccination of patients expressing certain HLA alleles. In a pilot study, dendritic cells generated from patients were pulsed with a cocktail of gp100, MART-1, tyrosinase, MAGE-1, or MAGE-3, depending on the patient HLA haplotype, or with tumor lysate when HLA alleles were inappropriate for binding the known peptides. Cells were directly administered into uninvolved lymph nodes by ultrasound guided injection. The majority of patients displayed a DTH response to peptide-pulsed dendritic cells and had peptide-specific CTLs at the DTH site. Five patients had objective clinical responses to the treatment including 2 patients treated with dendritic cells pulsed with tumor lysate (149). This suggests that this therapy could be applied to cancers without defined tumor antigens and to patients with HLA haplotypes unsuited to bind known tumor peptides.

Finally, the role of the route of vaccine administration in vaccine efficacy has been investigated. Using radiolabeled dendritic cells, Eggert and colleagues found that intravenous administration results in accumulation of dendritic cells in the spleen, whereas subcutaneous administration leads to homing to draining lymph nodes. In a mouse model of melanoma, these investigators showed that the subcutaneous route of administration resulted in improved survival and increased CTL activity compared with intravenous injection (150).

Immunotherapy and tumor vaccination in cancer patients thus are developing rapidly and appear promising in certain settings. One must keep in mind, however, that tumors can generate multiple mechanisms of escape from the immune system, some of which would render vaccination less effective. For example, downregulation of MHC molecules would preclude MHC/peptide recognition and lysis of tumor cells by T-lymphocytes in the effector phase of the immune response. Even in this case, however, indirect presentation of tumor antigens to T cells still may allow subsequent activation of NK cells and macrophages and tumor rejection. Thus, association of immunotherapy with chemotherapy or other antitumor approaches may be warranted.

DNA VACCINATION

A novel approach to promoting immune responses against specific antigens has taken advantage of an observation first made in 1990 that intramuscular administration of “naked” DNA encoding a specific protein antigen resulted in myocyte-specific protein expression (151). Since then, numerous articles have reported the efficacy of DNA vaccines in small and large animal models of infectious diseases and cancer (152). DNA immunization elicits humoral and $CD4^+$ T-cell responses in rodents and primates, but its major advantage at the immunologic level is its capacity to induce antigen-specific MHC class I-restricted CTL responses typical of live virus vaccination, a major mechanism of protection against intracellular pathogens. The mechanisms by which these systemic responses occur following local administration of the vaccine, such as an intramuscular injection, are not fully understood but may include presentation of peptide derived from the encoded protein by the myocytes themselves, direct transfection of local APCs, or transfer of the antigen from transfected myocytes to professional APCs.

The advantage of DNA vaccination over peptide immunization is that it permits generation of entire proteins, enabling determinant selection to occur in the host without having to restrict immunization to patients bearing specific HLA alleles. A safety concern for this technique, however, is the potential for integration of the plasmid DNA into the host genome with the possibility of disrupting important genes leading to phenotypic mutations or carcinogenicity.

In humans, DNA vaccination appears to induce cellular responses. For example, all normal volunteers who were immunized with a plasmid DNA encoding a malaria protein developed specific CTLs against ten malaria peptides tested (153). These results are encouraging, but basic immunology tenets suggest that the immunization strategy would benefit from the stronger responses afforded when the immunogen is coupled with an inflammatory response. This may be achievable by the incorporation into the DNA vaccine of adjuvants, such as CpG motifs or monophosphoryl lipid A. Unmethylated CpG dinucleotides within specific DNA sequences are detected, in bacterial or viral DNA, as inflammatory signals by the vertebrate immune system and have been shown to be potent Th1-directed adjuvants in mice (154). Administration of these DNA motifs into the immunogen results in upregulation of MHC class II and costimulatory ligands on B cells, increasing both T- and B-cell responses (155). Monkeys vaccinated against hepatitis B with a CpG adjuvant developed 15 times more antihepatitis antibody titers than those who received vaccine alone (156). The ability of these motifs to promote Th1 differentiation has been taken advantage of in animal models of asthma and vaccination. Immunomodulatory DNA sequences prevents IL-5 production, eosinophil accumulation, and airway hyperresponsiveness in mice (157) and hold promises for treatment of Th2-driven allergic diseases and asthma in patients. Monophosphoryl lipid A is another bacterial adjuvant that has been shown to potentiate T- and B-cell responses to DNA vaccines in mice and may promote Th1 differentiation (158).

Finally, studies have focused on the use of DNA to deliver antigen to APCs. One strategy has been to introduce genes encoding tumor antigens directly into dendritic cells, thereby allowing the possibility of endogenous peptides being presented both by MHC class I and class II molecules. This has been achieved most successfully by using recombinant viruses to transduce APCs; traditional ways of transfecting cells, such as electroporation or chemical treatment, have failed to induce CTL responses after reinfusion of the treated APCs (145).

VIRAL VACCINATION

Another approach to generate or enhance immune responses against specific antigens consists of infecting cells with recombinant viruses that encode the protein antigen of interest. Different types of viral vectors, such as vaccinia, avipox, or adenovirus have been used, that can infect mammalian cells. Infection can be performed *in vitro* to introduce proteins of interest, such as tumor antigens, into specific cells. For example, human dendritic cells derived *in vitro* from hematopoietic progenitors, can be infected with vaccinia virus or adenovirus to express a foreign protein (159) and then can be used for vaccination of patients. Alternatively, patients can be vaccinated directly with the replication-defective virus. In a pilot clinical trial, cancer patients were vaccinated with canarypox vector and the human CEA, a tumor antigen expressed in colon cancer cells. T cells obtained from seven of eight patients after, but not before, immunization were capable of specifically lysing cell lines expressing HLA-A2 pulsed with a CEA peptide (160). Viral vaccination also allows introduction of mutant peptides derived from tumor antigens that can induce stronger CTL responses than native peptides. Valmori et al. showed increased immunogenicity following vaccination of mice with recombinant vaccinia virus containing a minigene encoding for a mutant peptide analog of melan-A, a melanoma tumor antigen (161). Thus, recombinant vaccines provide another strategy to induce the generation of strong CTL responses *in vivo* with the potential to eradicate tumors or infectious pathogens.

TARGETING COSTIMULATORY MOLECULES

As described, activation of T cells requires not only signaling through the TCR but also a second costimulatory signal that is necessary for cell-cycle progression, survival, and maximal effector function. A number of candidate costimulatory molecules have been targeted for tolerance induction because reagents that block the binding of costimulatory receptors to their ligands are more readily available than agonistic reagents. Some costimulatory molecules are soluble, such as IL-2, and many are T-cell surface receptors, such as CD28, 4-1BB, OX40, CD30, and CD40L. Each has the ability to augment the T-cell proliferative response to antigenic stimuli. It is likely that each of these molecules acts through different mechanisms, some delivering costimulatory biochemical signals to the T cell, some enhancing adhesion to APCs, and still others mediating homing to target tissues. These therapies represent some of the most promising candidates for clinical tolerance induction in transplantation and autoimmune diseases such as systemic lupus erythematosus, diabetes, psoriasis, and MS.

Strategies for Immunosuppression

TARGETING B7/CD28 INTERACTIONS

One of the most important costimulatory pathways of T-cell activation involves the interactions of the CD28 receptor with its ligands B7-1 and B7-2. Preventing ligation of CD28 molecules during TCR stimulation *in vitro* results in T-cell anergy. In contrast, T cells that are not simultaneously activated through the TCR remain unaffected by CD28/B7 blockade, as demonstrated by normal T-cell responses following washing and restimulation of T cells. Therefore, disruption of the CD28/B7 pathway *in vivo* should result in tolerance of antigen-reacting T cells without affecting T cells of other specificities not activated at the time of treatment. Several approaches have been used to disrupt the CD28/B7 interactions *in vivo*. Initially, the field exploited the finding that CTLA-4 has a significantly higher affinity for B7 ligands than CD28 by developing a soluble molecule comprising the extracellular domain of CTLA-4 fused to the hinge and Fc portion of an immunoglobulin, resulting in a reagent termed CTLA-4Ig. This reagent, as well as anti-B7 antibodies, has been used to disrupt ligation of CD28 *in vivo*.

Extensive studies in rodent transplant models suggested that inhibition of the CD28/B7 pathway is a promising strategy to promote allograft acceptance as well as to prevent or treat autoimmune diseases (16,162,163,164 and 165). In primates, the effects of B7 blockade have been modest. Treatment with a 2-week course of human CTLA-4Ig prolonged renal allograft survival in rhesus macaques, but the two animals in this study went on to reject their allograft. Similarly, CTLA-4Ig monotherapy inhibited islet allograft rejection in cynomolgus monkeys in two of five recipients (166). Interestingly, CTLA-4Ig therapy abrogated the humoral response in all the recipients. Similar inhibition of the alloantibody response was obtained in the renal allograft model (Kirk et al., and Pearson and Larsen unpublished observations). More recently, Kirk and colleagues presented data demonstrating that an antihuman B7-1 mAb can significantly prolong renal allograft survival alone or with antihuman B7-2. Thus, whereas the initial experiences in primates with agents blocking this pathway (e.g., anti-B7-1 and B7-2 mAbs and various CTLA-4Ig fusion proteins) were somewhat disappointing, new second-generation reagents and optimized protocols are being developed that will warrant testing in human trials, particularly in conjunction with other agents.

Also, CTLA-4Ig has been shown to prevent the onset of collagen-induced arthritis (CIA) in BB rats and mice and progression of diabetes in NOD mice. Lenschow et al. found that a soluble human CTLA-4Ig could prevent diabetes in NOD mice (167). The prevention of disease was due to the blockade of B7-2/CD28 interactions. Herold et al. made similar observations in diabetes induced with multiple low doses of streptozotocin (168). In murine autoimmune thyroiditis, blockade of B7-2 inhibited priming and *in vitro* activation of effector cells (169). Furthermore, the combination of anti-B7-1 and anti-B7-2 not only prevented murine CIA, but it also suppressed clinical and histologic evidence of joint inflammation. Thus, in theory, the best strategies might include CD28 blockade to suppress T-cell expansion combined with anticytokine therapy, such as anti-TNF mAbs, to inhibit Th1 effector function, promoting quiescence in the inflamed tissue and minimizing the costimulatory effects of soluble and cell-surface accessory molecules.

CD28/B7 blockade has been used in clinical trials. First, the *in vitro* principle that TCR stimulation during CD28/B7 blockade results in T-cell anergy has been applied in a pilot clinical trial involving bone marrow allograft transplantation in an attempt at reducing GVHD. GVHD is a complication arising from donor T cells recognizing recipient cells as allogeneic and foreign. Recipient peripheral blood leukocytes were collected from 12 patients before myeloablation, irradiated, and incubated *in vitro* with donor bone marrow cells mismatched for one MHC haplotype in the presence of CTLA-4Ig to induce anergy of donor T cells specific for recipient allo-MHC. Restimulation *in vitro* showed reduced responses of donor T cells against recipient but not third party cells, suggesting that donor-specific anergy had occurred. The incidence of GVHD following transfusion of these cells into patients was relatively low, suggesting the potential efficacy of this type of treatment (170). Further studies comparing incidence of GVHD prospectively when donor cells are incubated with and without CTLA-4Ig are warranted.

In addition, human CTLA-4Ig has been used *in vivo*. Forty-three patients with stable psoriasis vulgaris received four infusions of the soluble chimeric protein CTLA-4Ig (8). Forty-six percent of the patients achieved a 50% or greater sustained improvement in clinical disease activity, with greater effects observed in the highest-dosing cohorts. Improvement in these patients was associated with quantitative reduction in epidermal hyperplasia and correlated with quantitative reduction in skin-infiltrating T cells. No markedly increased rate of intralesional T-cell apoptosis was identified, suggesting that the decreased number of T cells in the lesions was probably attributable to an inhibition of T-cell proliferation, T-cell recruitment, or apoptosis of antigen-specific T cells at extralesional sites. It is important to note that, although altered antibody responses to T-cell-dependent neoantigens were observed, immunologic tolerance to these antigens was not demonstrated. This study illustrates the importance of the CD28/CTLA-4 pathway in the pathogenesis of psoriasis and suggests a potential therapeutic use for this novel immunomodulatory approach in an array of T-cell-mediated diseases. The data also emphasizes that tolerance induction, as defined by experiments *in vitro* and in small animal *in vivo*, may not reflect functional mechanisms in humans *in vivo*.

INHIBITION OF CD40/CD40L INTERACTIONS

Blockade of CD40/CD40L interactions also has been shown to affect autoimmunity. Treatment of NOD mice with anti-CD40L can prevent diabetes if administered early in life but, in some studies, did not block recurrent insulitis (171,172). Anti-CD154 antibody treatment also reduced severity of EAE both in diseased animals and in adoptive recipients of encephalitogenic T cells. This suggests that CD40/CD154 interactions may be involved in directing the CNS migration of these cells or in their effector ability to activate CNS macrophages and microglia (173).

Studies using anti-CD154 (CD40L) mAbs in experimental rodent and primate transplant models demonstrated that the interruption of CD40/CD154 interactions represents a powerful strategy to inhibit allograft rejection. In rodents, perioperative treatment with anti-CD40L promoted indefinite survival of murine cardiac allografts (174). Studies in preclinical primate models also strongly support the hypothesis that costimulation blockade will provide a powerful means to prevent rejection and promote long-term allograft acceptance. Kirk and colleagues reported that a 2-week course of anti-CD40L monotherapy markedly prolonged (>180 days) renal allograft survival in rhesus macaques (175). Anti-CD154 therapy in nonhuman primate recipients of allogeneic islets allowed for successful engraftment, long-term maintenance of function, and preservation of islet mass at levels comparable to prepancreatectomy. Several monkeys treated with anti-CD154 maintained islet and renal allograft function long after discontinuation of therapy with no evidence of rejection (176). Whereas optimal dosing, frequency of administration, and duration of therapy remain under active investigation, subsequent studies suggested that treatment for 6 months confers more durable graft protection (>1 year). Although a loss of donor-specific reactivity *in vitro* in the presence of a maintained anti–third-party responsiveness has been shown in both models, there is as yet no convincing data in rodents or primates that anti-CD154 monotherapy can induce stable donor-specific tolerance. Thus, although the precise mechanism for *in vivo* efficacy of this therapy is unclear, the achievement of nonhuman primate allografts survival for over 3 months after discontinuation of immunotherapy, in the absence of any rejection episode, indicates that deletional mechanisms, immune deviation, or long-lasting immune unresponsiveness is operating. Industry-sponsored phase 1 and 2 clinical renal transplant trials using long-term anti-CD154 maintenance therapy in calcineurin inhibitor-free protocols are now under way. The results of these trials will be extremely helpful in the design of studies using anti-CD154 in combination with other agents (e.g., anti-B7s, CTLA-4Ig, rapamycin) for tolerance induction.

COMBINED COSTIMULATORY BLOCKADE

In several murine models, simultaneous blockade of the CD28 and CD40 pathways has been shown to inhibit synergistically immune responses. For example,

simultaneous, but not independent, blockade of the CD28 and CD40 pathways markedly prolonged the survival of allogeneic skin grafts and inhibited the development of chronic vascular rejection of primarily vascularized cardiac allografts (177). This strategy greatly prolonged the survival of rat and porcine xenogeneic skin transplants in mice (164). A similar synergistic effect between these reagents has been observed in murine aortic allograft and contact dermatitis models (178,179). Furthermore, whereas anti-CD154 monotherapy is effective in the rhesus renal allograft model, there is evidence that, unlike CTLA4-Ig, this agent failed to inhibit completely the antidonor humoral response (Alan Kirk, personal communication). This finding suggests that simultaneous blockade of the CD28 and CD40 pathways may have immunologic advantages. Finally, several investigators observed that the effectiveness of costimulation blockade is significantly enhanced by coadministration of donor cells in the form of a blood transfusion, splenocytes, or bone marrow cells (7,180,181).

TARGETING OTHER TNF/TNFR FAMILY MEMBERS

Although CD28 is constitutively expressed on naive T cells and is important for initial costimulation, differentiated T cells are less dependent on CD28 and upregulate a series of other cell surface molecules on stimulation. Therefore, other costimulatory interactions may be important in regulating ongoing immune responses. Members of the TNF/TNFR family are likely to play important roles as both effector and costimulatory molecules in conditions of chronic stimulation, such as autoimmune diseases and transplantation settings. For instance, OX40 expression has been demonstrated in T cells isolated from the CNS of mice with EAE. Blocking of OX40/OX40L interactions using soluble OX40-Ig or deleting CNS-OX40$^+$ T cells with an anti-OX40-immunotoxin mAbs results in reduced severity of murine EAE (182,183). In addition, *ex vivo* stimulation of rat MBP-specific T cells with anti-CD3 and anti-OX40 mAbs resulted in T cells capable of inducing EAE following adoptive transfer, whereas stimulation with anti-CD3 mAb alone did not (184). Similarly, expression of OX40 was observed on activated T cells following bone marrow transplantation and treatment with anti-OX40 reduced lethality induced by semiallogeneic bone marrow transplantation in mice (185). Whether efficacy in either of these models results from simple deletion of OX40$^+$ T cells or blockade of the costimulatory pathway remains to be determined.

Targeting 4-1BB may be of specific interest for certain disease models because costimulation through 4-1BB seems to affect preferentially CD8$^+$ rather than CD4$^+$ T cells. Cross-linking 4-1BB *in vivo* in mice appears to accelerate skin and cardiac allograft rejection (186), pointing to 4-1BB as an important molecule to target for immunosuppression when blocking mAbs or fusion molecules become available. In addition, a splice variant resulting in a soluble form of 4-1BB has been identified in T cells, and its concentration appears to be increased in the serum of patients with rheumatoid arthritis (187), suggesting that this molecule may be an interesting target in autoimmune settings.

Finally, the list of other potential regulators of systemic and local immunology is growing continuously. Thus, it will be critical to study each, first in preclinical models, then in humans, both as monotherapy and in combination, to maximize the potential for tolerance induction.

TARGETING COSTIMULATORY CYTOKINE/CYTOKINE RECEPTOR PATHWAYS

IL-2/IL-2R

Interleukin-2R is transiently expressed on recently activated T cells and, on IL-2 ligation, promotes cell cycle progression and expansion of Ag-specific T cells. Therefore, animal and human studies in autoimmune or transplantation settings have used anti-IL-2R Abs or IL-2 fused to different immunotoxins to target specifically and to inactivate or eliminate that subset of T cells (188). Notably, a fusion IL-2-Ig molecule that was shown to bind IL-2R on T cells, fix complement, and result in T-cell deletion reduced the incidence of diabetes by 70% in a model of adoptive transfer of diabetogenic splenocytes into non diabetic male NOD mice (189). Numerous clinical trials have been performed in transplantation and autoimmune settings, initially using mouse and rat antihuman IL-2R mAbs and then humanized mAbs. Because of the low rate of rejection episodes with current global immunosuppressive agents, new therapies in transplantation require, on ethical bases, to be added to agents like cyclosporin or corticosteroids rather than replace them. Importantly, both cyclosporin and steroids inhibit T-cell activation, cytokine production, and IL-2R expression, possibly lowering the putative efficacy of an anti-IL-2R mAb. Keeping that in mind, however, anti-IL-2R mAbs seem efficacious at preventing allograft rejection despite the concurrent presence of immunosuppressive drugs, although efficacy may not be as high as with anti-T cell preparations. Interestingly, there seems to be a trend toward a reduced rate of immunosuppression-related infections, and notably of CMV infections in patients treated with anti-IL-2R mAbs, compared with patients treated with antilymphocyte serum or OKT3, suggesting more alloantigen-specific immunosuppression (190). In a study involving patients with autoimmune Th1-mediated uveitis, cyclosporin and steroids were tapered down and discontinued before administration of a humanized anti-IL-2 mAb. This treatment alone prevented intraocular inflammation and resulted in improved visual acuity in eight of ten patients, suggesting that this approach may be effective for certain autoimmune diseases as well (191). In contrast to the potent deletion of IL-2R-expressing T cells following anti-IL-2R mAb treatment in rodent models, anti-IL-2R mAbs in humans may function by preventing utilization of IL-2 by T cells or by inactivating IL-2R$^+$-T cells. Indeed, IL-2R-expressing T cells do not appear to be eliminated from the circulation (192).

Therapies that target IL-2R may be useful to complement other strategies of costimulatory blockade. Indeed, a percentage of T-lymphocytes may escape or bypass efficient blockade of CD28 or CD40 costimulatory pathways and may progress to an activated state in which IL-2R is expressed. Although high levels of activation would not be expected in the presence of costimulatory blockade, cells that escape blockade, memory cells, and preexisting autoimmune or alloreactive-specific T cells could lead to autoimmunity or allograft rejection. Targeting IL-2R could specifically affect these cells and have additive effects to those of costimulatory blockade. A subset of IL-2R-expressing T cells has been identified as suppressive T cells capable of inhibiting responses of antigen-specific T cells (193,194) and may be important in maintenance of tolerance to autoimmune diseases (195,196). Therefore, long-term effects of targeting IL-2R$^+$ T cells will have to be investigated.

Cytokine/Cytokine Receptor Pathways that Affect T-Cell Differentiation

Interleukin-4 binding to IL-4R appears important for differentiation of T cells into Th2- and Tc2-type cells. Type-2 T cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 on restimulation and, in particular, provide help to eosinophils that have been implicated in the pathogenesis of allergic diseases. In addition, IL-4 also supports proliferation of mast cells and BCR-stimulated B cells and promotes immunoglobulin switch to an IgE isotype, making IL-4 a suitable target in IgE-mediated diseases. A naturally occurring soluble form of IL-4R is secreted and inhibits the biologic actions of IL-4 by preventing IL-4 from ligating IL-4R-expressing cells. A recombinant version of this molecule that contains only the extracellular portion of IL-4R and lacks the transmembrane and intracellular domains has been generated. Blockade of IL-4 signaling via intranasal administration of soluble IL-4R has been shown to reduce airway inflammation and eosinophil accumulation in a murine model of asthma (197). In addition, a clinical trial using aerosolized soluble IL-4R also shown to improve asthma scores in a phase 1 and 2 placebo-controlled trial (198).

Conversely, IL-12 in rodents and primates and IFN-a in humans promote Th1 differentiation, as discussed previously. Numerous infectious diseases appear to be regulated by the balance between type 1 and type 2 T-cell responses, such as leprosy, *Schistosoma mansoni*, and *Leishmania* infections. From the extensive study of a mouse model of *Leishmania* species, it appears that susceptible mice are more prone to generating Th2-type responses, whereas resistant strains preferentially develop Th1 responses. Furthermore, neutralization of IL-4 in susceptible strains of mice can result in Th1 differentiation and eradication of the pathogen, suggesting that, in the clinic, blockade of IL-4 may be appropriate in certain infectious diseases (199). Conversely, immunization of primates with killed *Leishmania* and human IL-12 promoted Th1 differentiation and conferred protection against parasite challenge (200).

Similarly, some autoimmune diseases are preferentially driven by Th1-type responses in both rodents and humans. These include, for example, experimental uveitis, diabetes, psoriasis, Crohn disease, rheumatoid arthritis, and EAE. In contrast, other autoimmune diseases, such as progressive systemic sclerosis and ulcerative colitis, seem to be promoted by Th2-type responses (201,202). Therefore, therapies that would modulate the Th1/Th2 balance by favoring one differentiation pathway over the other may have beneficial effects in these settings. Yet treatment with IL-4 or anti-IL-12, or IL-12 or anti-IL-4 to promote or limit Th2 differentiation, respectively, yielded controversial results in animal models, sometimes affecting the severity of the illness, but more often lacking efficacy. Thus, it is unclear at this point exactly how to translate experimental results to patients suffering from autoimmune diseases. For instance, it is likely that the events that skew differentiation of newly activated T cells may not equally affect already differentiated effector T cells. Nevertheless, an anecdotal case report of a patient treated with IL-12 as an adjuvant therapy for cancer who also suffered exacerbation of RA illustrates how manipulation of T-cell differentiation may affect autoimmune disease (203).

Strategies for Immune Enhancement

Positive costimulation of antigen-specific T cells is desirable for immunotherapies directed against cancers and infectious diseases. This has been achieved for different surface molecules in many ways, including induction of expression of costimulatory ligands on tumors via transfection, DNA vaccination, or viral transduction. In addition, when agonistic antibodies to costimulatory receptors exist, they can be used to promote tumor rejection or eradication of viral or parasitic infections.

CD28/CTLA-4/B7 PATHWAY

Activation of CD28 on tumor-specific T cells was achieved initially by transfecting tumors to express B7 molecules as a means for providing direct costimulation. Following B7 transfection, some tumors that otherwise grew progressively in mice were rejected in a T-cell-dependent manner, indicating that increasing the chance that tumor-specific T cells receive potent costimulatory signals improves clinical outcome in tumor models (204,205). One of the potential problems with this approach has been the inadvertent engagement of the downregulatory CTLA-4 molecule as a consequence of ectopic B7 expression. Thus, approaches have been developed to activate selectively the CD28 molecule on antigen-reactive T cells. In this regard, direct costimulation of T cells via injection of agonistic anti-CD28 mAb also was

shown to enhance antitumor responses in mice (206). The soluble form of the CD28 agonist is less effective than membrane-bound antibodies, however. Therefore, several groups generated membrane-bound single-chain anti-CD28 mAbs that can be expressed on tumors or locally in sites of infection (207,208). This approach led to a highly effective means of delivering CD28-specific costimulation.

Another strategy that takes advantage of costimulation is the *ex vivo* expansion of T cells as immunotherapy in cancer patients and HIV-infected persons. T cells from peripheral blood are stimulated *in vitro* using plastic beads coated with anti-CD3 and anti-CD28 mAbs (209). This protocol leads to a dramatic increase in both $CD4^+$ and $CD8^+$ T-cell numbers. The T cells are not antigen-restricted, but they include antigen-specific T cells if they are present in the peripheral blood of the patient. When this approach was used to expand T cells from HIV-infected patients, it was noted that only uninfected T cells proliferated and stimulated cells were rendered resistant to HIV-1 infection (210). In a pilot study, reinfusion of these cells into patients restored immune competence, although further evaluation of T-cell responsiveness *in vivo* needs to be performed. Similarly, clinical trials using *ex vivo* expansion and reinfusion of T cells in lymphoma-bearing patients is currently under way and will provide insight into the antitumor function of in vitro activated T cells (C. June and D. Liebowitz, personal communication).

Blockade of the negative regulator of T-cell function CTLA-4 has been used successfully in animal models to enhance T-cell function and promote tumor rejection (24) or eradication of pathogens (211). Treatment of mice with anti-CTLA-4 mAbs prevented tumor growth and conferred immunity against secondary exposure to tumor cells in mice. In some instances, the therapy induced rejection of preestablished tumors. In addition, treatment with anti-CTLA-4 mAbs in mice has been shown to increase the efficacy of vaccination with irradiated tumor cells engineered to produce GM-CSF or of chemotherapeutic regimens. These combined therapies induced rejection of tumors otherwise resistant to anti-CTLA-4 mAb monotherapy (212,213). In a mouse model in which tumor exposure induces hyporesponsiveness of TCR transgenic tumor-specific T cells, anti-CTLA-4 treatment was unsuccessful at reversing tolerance induction (214). In humans, tumors usually exist for long periods before being detected raising the possibility that tumor-specific T cells may have been rendered tolerant by mechanisms normally in place to prevent autoimmunity. If and when this is the case, anti-CTLA-4 mAb treatment may not have the potential to augment antitumor immunity. Clinical trials using anti-CTLA-4 mAbs are being designed and should yield important information.

CD40/CD40L AND OTHER TNF/TNFR PATHWAYS

Like B7, CD40 has been used as a transfected costimulatory molecule on tumor cells to increase T-cell activation. Implanted CD40-transfected tumors elicited a strong CTL response in mice, leading to eradication of the tumor and induction of long-lasting immunity that resulted in rejection following subsequent challenge with parental tumor (215). In addition, immunization with liposomes containing a tumor peptide induced CTL responses in mice that were greatly potentiated by coadministration of blocking anti-CTLA-4 and agonistic anti-CD40 mAbs, resulting in rejection of an antigen-expressing tumor (216). Interestingly, this response occurred also in MHC class II mice that lack $CD4^+$ T cells, indicating that $CD8^+$ can be directly activated in this manner. Using DNA vaccination to induce CD40L expression also increased T-cell responses. Mice treated with DNA encoding a trimeric complex of CD40L proteins did not die of metastases when challenged with a lethal dose of tumor. Similarly, coinjection of the CD40L-cDNA with *Leishmania* antigen resulted in resistance to subsequent *Leishmania* infection in mice. The increased T-cell response was blocked by anti-IL-12 and by CTLA4-Ig therapy, indicating that administration of CD40L-cDNA induced increased IFN-g production and CTL activity in an IL-12 and B7-dependent manner (217).

Triggering 4-1BB signaling also has been shown to potentiate immune responses as well. Administration of an agonistic 4-1BB-specific mAbs (218) or transfection of tumors with 4-1BBL (219) resulted in the rejection of different established tumors in mice, including otherwise poorly immunogenic tumors. Both $CD4^+$ and $CD8^+$ T-cell subsets were activated via 4-1BB in this tumor model.

Similarly, signaling through CD27 can result in immune enhancement. Mice immunized with a tumor infected with vaccinia virus encoding CD70, the ligand for CD27, were protected from tumor growth as well as from a subsequent challenge with CD70-negative tumor cells. In addition, vaccination with vaccinia virus encoding CD70 as well as the colon carcinoma tumor antigen CEA was more effective at preventing rejection of colon carcinoma than CEA immunization alone (220).

Finally, stimulation of CD30 has been used extensively as a direct tumor-targeting strategy rather than for T-cell activation inasmuch as CD30 is expressed on certain lymphomas. Bispecific mAbs containing anti-CD3 and anti-CD30 components have been used to stimulate T cells that would be in close proximity of CD30-expressing tumor cells. These therapies have proven largely successful (221,222). As the costimulatory role of CD30 molecule of T cells has been realized, however, the basis of this positive therapeutic outcome is unclear because treatment can either redirect T cells to tumor cells or costimulate tumor-specific T cells locally. The precise mechanism for induction of tumor-specific immunity remains to be analyzed in more detail.

CYTOKINE/CYTOKINE R PATHWAYS

The Th1/Th2 and Tc1/Tc2 balance also affects clinical outcome in tumor settings. It is believed that type 1 responses are desired for the rejection of tumors, possibly, in part, because IFN signaling is necessary for acquisition of a cytotoxic phenotype (223). Development of Epstein-Barr virus (EBV)-induced B-cell lymphoma is a classic complication of global immunosuppression in transplant settings. EBV infection induces Th2 differentiation because it stimulates B cells to produce IL-10, and it contains a gene, *BCRF1*, which encodes a protein with IL-10 activity in humans. Like IL-4, IL-10 promotes Th2 differentiation. Patients who develop EBV-related lymphoproliferative disorders following transplantation may therefore benefit from modulating the Th1/Th2 balance. Therefore, treatment with IL-12 or IFN-a theoretically has therapeutic potential. In fact, IFN-a not only favors Th1 differentiation in humans, but it also has direct antiviral and antineoplastic effects. Few patients with lymphoproliferative disorders have been treated with IFN-a, but a review of these cases showed that only 3 of 21 (14%) have died from the disease when treated with the cytokine. In contrast, the historical reported mortality in this type of patients ranges from 23% to 81% in the absence of IFN treatment. This result warrants further testing of this drug in this setting as well as investigation of T-cell phenotypes before and after treatment (224).

Interleukin-2 has been used as an adjuvant treatment to cancer patients as a means to expand antigen-specific T cells. Historically, IL-2 was first used *ex vivo* to expand tumor-infiltrating lymphocytes (TILs) and tumor-infiltrating NK cells (LAK cells) following biopsies or surgical excision of tumors. It was anticipated that those T cells were tumor antigen specific and that expansion and reinfusion into patients would result in eradication of remaining tumor cells. These therapies, however, have shown minimal clinical benefit over the addition of exogenous IL-2 alone. Hypotheses for the limited efficacy of this approach include the observations that reinfused T cells may not migrate to the tumor sites and that tumor cells develop mechanisms to evade immune responses, including the selective loss of the original tumor antigen against which TILs were directed. Administration of IL-2 in addition to other chemotherapy drugs, howeve, has shown beneficial effects in a number of cancer clinical trials, in particular for melanoma and renal carcinoma patients (225,226 and 227). Whether IL-2 in these cases exerts its effects by inducing tumor-specific T-cell expansion, promoting cytotoxicity of TILs, activating NK cells, or other mechanisms remains to be evaluated.

FUTURE DIRECTIONS

The past decade has seen a revolution in our understanding of the immune system and the basis for immune recognition and responsiveness. It is now well established that cellular adaptive immune responses are mediated primarily by T-lymphocytes, which have antigen-specific receptors that recognize foreign antigens. Antigen/MHC complex binding to the TCR/CD3 complex coupled with soluble and membrane-bound costimulatory signals initiates a cascade of signaling events that lead to productive immunity. In addition, the immune response is also regulated by a number of negative signaling events that control cell survival and expansion. For the first time, *in vitro* and preclinical *in vivo* studies have demonstrated that we can selectively inhibit immune responses to specific antigens without the associated toxicity of current immunosuppressive therapies. With these new insights comes the enormous promise of specific immune therapies to treat the vast array of immune disorders from autoimmunity, transplant rejection, and asthma, to infectious diseases and cancer. We will not have to “blow up the house to kill the fly anymore.” What will these new therapies look like? Most likely, they will include combination therapies that take advantage of a number of agents. On the tolerance side, these might include drugs that target the primary TCR-mediated signal either by blocking cell surface receptor interactions or inhibiting early signal transduction events. The drugs will be combined with therapies that effectively block costimulatory to prevent cell expansion and differentiation of those cells that have engaged antigen while maintaining a noninflammatory milieu by the use of antiinflammatory drugs such as anti-TNF mAbs. On the flip side, new cancer and infectious disease vaccines will be developed that promote T-cell expansion and differentiation, in addition to costimulatory agonists and agents that block the off signals that dampen immunity. Pharmaceutical and small biotech companies continue to refine the new therapeutics that will be used in combination. The National Institutes of Health (NIH) has invested in immune tolerance by creating the Immune Tolerance Network (www.immunetolerance.org), a collaborative effort that will develop new clinical strategies for the purposes of inducing, maintaining, and monitoring tolerance in humans for kidney and islet transplantation, autoimmune diseases, and allergy and asthma. A new vaccine center has been established on the NIH campus to discover new ways to promote immunity in HIV infection, emerging infections, and cancer. The future is bright as we enter the new millennium.

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88 CYTOKINES AND CANCER IMMUNOTHERAPY

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Although the field of cancer immunology attracted considerable skepticism during the past century, it now stands firmly positioned upon a rich scientific foundation. William Coley's intriguing finding that the administration of bacterial toxins could elicit tumor regressions in some patients with advanced sarcoma continues to inspire a wealth of studies into the host/tumor relationship (1). Although the mechanisms underlying the striking antitumor effects of bacterial toxins have yet to be fully delineated, investigative efforts in this area now encompass cytokine biology, tumor antigen discovery, antigen-presenting cell function, CpG oligonucleotide chemistry, immune effector activity, and tumor angiogenesis. The clinical acumen of Coley may ultimately spawn a new generation of cancer immunotherapies based on the insights of molecular genetics, pathologic analysis, cellular technologies, and informed clinical investigation.

The scientific breadth of cancer immunology at the beginning of the new millennium is impressive. Techniques have been developed that allow the discovery of immunogenic gene products either overexpressed or selectively expressed in cancer cells. Molecularly defined cancer antigens can be incorporated into several novel immunotherapeutic strategies likely to enhance antitumor immune reactivity. Sensitive and specific methods for the quantification and characterization of specific antitumor effector cells in tumor-bearing hosts are available. Together, these technical advances provide powerful opportunities for gleaning an improved understanding of the therapeutic potential and limitations of cancer immunotherapy.

Numerous clinical trials of cancer vaccination based on these recent insights have been initiated. These early studies primarily are aimed at determining the immunogenicity, toxicity, and feasibility of the new treatment approaches. As these critical characteristics become more fully defined, a limited number of promising strategies will advance to definitive efficacy testing in the setting of minimal residual disease. The most productive selection of immunotherapies is likely to depend on a combination of clinical intuition and the demonstration of biologic activity, because it may prove difficult to elicit meaningful clinical responses in patients with advanced, multiresistant disease.

In an effort to help clarify the scientific foundation and challenges confronting the development of cancer immunotherapy, I highlight in this chapter some of the genetic and cellular advances emerging from current laboratory studies and some of the intriguing patient data emerging from early clinical investigations.

ENDOGENOUS ANTITUMOR IMMUNITY

The central problem cancer immunology must resolve is whether an antitumor host response can modulate the natural history of neoplasia. One important way to address this issue is to determine whether endogenous immune responses protect against the development of cancer. Several congenital and acquired forms of clinical immunodeficiency confer a substantial risk for particular malignancies, including Kaposi sarcoma, non-Hodgkin lymphoma, and skin cancer (2). However, these tumors are closely associated with viral infection, and their elevated incidence most likely reflects a compromise in antiviral immunity, rather than antitumor immunity *per se.*

The notion that selective defects in immunity can lead to enhanced cancer development is a more contentious issue. Whereas Paul Ehrlich (3) first formulated the concept of cancer immunosurveillance nearly 100 years ago, Lewis Thomas (4) and MacFarlane Burnet (5) subsequently elaborated on the idea and proposed that T lymphocytes play decisive roles in the process. Although Stutman (6) challenged the validity of immunosurveillance, based on the failure to find an increased incidence of spontaneous or chemically induced tumors in nude mice, the generation of immunodeficient gene-targeted mice has provided compelling support for the existence of a host defense system against carcinogenesis.

Kaplan et al. (7) presented persuasive evidence that interferon-g is involved in tumor surveillance in several murine models. These investigators showed that mice rendered interferon-g deficient (by deletion of the gene encoding the interferon-g receptor or the STAT-1 molecule) developed a greater number of tumors after exposure to 3-methylcholanthrene than did wild type litter-mate controls. Moreover, these tumors formed with a shorter latency in interferon-g–deficient mice than in control animals. To test whether interferon-g also influenced the development of spontaneous tumors, Schreiber et al. introduced a mutant p53 allele into the interferon-g–deficient background. Strikingly, doubly mutant animals developed a much broader spectrum of cancers than did mice mutant in p53 alone. Analysis of the mechanisms underlying the enhanced carcinogenesis in both of these systems revealed important roles for both the tumor cells and host innate immunity. The involvement of T lymphocytes in cancer surveillance was suggested by other experiments, which delineated an inverse association between the development of contact hypersensitivity to polycyclic hydrocarbons (a reaction dependent on CD4-and CD8-positive T lymphocytes) and the incidence of tumors induced by these agents (8).

Although these studies in murine systems strongly suggest the existence of a cancer-immunosurveillance system, the evidence for a comparable defense network in humans currently is less complete. One informative approach to unveiling such a system involves identifying characteristics of progressive cancers that may underlie escape from immune control. Analogous investigations of successful pathogens have revealed a plethora of clever strategies to undermine immune defense (9). In this context, cancer cells are highly reminiscent of both viral and parasitic agents. For example, alterations in major histocompatibility complex (MHC) molecules and other components of the antigen-presentation pathways are frequently found in progressive tumors (10) and intimate that cytotoxic T lymphocytes likely mediate some tumor destruction. In support of this idea is the finding that tumor cells generally produce soluble factors, such as transforming growth factor-b, which antagonize T-cell function (11). Moreover, tumor cells release other molecules, such as vascular endothelial growth factor, which inhibit the development and function of dendritic cells (12). Indeed, recent studies have illustrated that only immature dendritic cells reside within most breast adenocarcinomas (13).

A second method to delineate whether endogenous host immunity can play a cancer-surveillance function involves the characterization of antitumor immune responses in long-term-surviving patients. Highly provocative findings in this regard have been gleaned through the pathologic analysis of the host response to early-stage tumors. Clark et al. (14) and Clemente et al. (15) established a tight association between the generation of dense T-lymphocyte infiltrates in the vertical growth phase of primary malignant melanoma and a reduced rate of recurrent disease and tumor mortality.

An intriguing result in this work was the failure to find any survival benefit associated with lymphocytes that circumscribe, but do not infiltrate into the tumor; they are functionally equivalent to the absence of an infiltrate. Studies aimed at understanding the differences between infiltrating and circumscribing T lymphocytes are likely to reveal important new information about abortive antitumor immune responses. In this context, it is interesting to note that analogous findings have been made in studies of autoimmune diabetes, in which a periislet cell accumulation of T cells is not sufficient to mediate disease; additional events are required to trigger T-cell infiltration and destruction of the islets (16). Genome-wide mapping studies directed at elucidating checkpoints in autoimmune diabetes in mouse and human (17) may thus provide insights into the development of antitumor immunity. Mihm et al. (18) also demonstrated that these same patterns of T-lymphocyte/melanoma interaction in regional lymph node metastases similarly predict differences in recurrence and survival.

T-lymphocyte infiltrates in early-stage adenocarcinoma of the colon are associated with a favorable clinical outcome as well; their presence can be used as effectively as the standard Duke's staging system for stratifying patient risk of recurrence (19). The incidence of lymphocyte infiltrates in these patients is inversely correlated with tumor stage, underscoring the idea that progressive colon cancer may be linked to escape from host defense. In this context, those tumors in which significant CD4- and CD8-positive T-cell infiltrates are found also harbor activated macrophages expressing high levels of the B7-1 and B7-2 costimulatory molecules, suggesting effective antigen presentation and T-cell activation (20).

Although additional clinicopathologic studies remain to be performed, lymphocyte infiltrates have been variably reported with many cancers, including lymphoma, glioblastoma, and carcinomas of the stomach, breast, bladder, testes, and ovary. It will be of interest to see whether similar patterns of lymphocyte/tumor interaction will have prognostic importance in these tumors as well. If so, then cancer immunosurveillance may prove to be a general phenomenon, and a deeper understanding of these “spontaneous” host reactions may provide the foundation for crafting efficacious immunotherapy.

TUMOR ANTIGENS

Because T-lymphocyte infiltrates in early-stage tumors are associated with prolonged patient survival, an important goal of investigation is the identification of the target

antigens stimulating these responses. The discovery that T lymphocytes recognize processed peptides, derived from cellular proteins, inserted into the grooves of MHC molecules (21) revolutionized the ability to accomplish this goal. Boon et al. (22) exploited this understanding to devise the first productive approach to characterize T-cell antigens; in this strategy, tumor-specific cytolytic T-cell clones were used to probe cDNA expression libraries (derived from tumor samples) transfected into target cells expressing the relevant MHC class I molecules. The recent demonstration that vaccination with either P1A or mutated mitogen-activated protein kinase (murine cancer antigens identified with these techniques) engendered protective immunity against tumor challenge validated the power of the Boon approach (23,24). Further, early clinical trials have shown that immunization with peptides derived from the MAGE-3 and gp100 gene products (human cancer antigens identified with these methods) can evoke at least partial tumor destruction in some patients with metastatic melanoma (25,26). An alternative, highly related strategy developed by Cox et al. (27) involves the use of tumor-specific T cells to identify peptides antigens acid-eluted from MHC molecules on tumors; candidate peptides are then sequenced with reverse-phase high-performance liquid chromatography or mass spectrometry.

A different approach to tumor antigen discovery was developed by Sahin et al. (28), who used patient sera to screen tumor-derived cDNA expression libraries. The rationale underlying this strategy is that in cancer patients, coordinated humoral and cellular antitumor responses may develop; consequently, at least some of the antigens stimulating high-titer immunoglobulin (Ig)G antibodies (whose synthesis requires CD4 T cells for isotype switching) also will be targets for CD8-positive T cells (29). This hypothesis was initially validated by the finding that antibody-based expression cloning detected the tyrosinase and MAGE-1 gene products, antigens previously discovered with T cell–based methods. More important, though, was the demonstration that NY-ESO-1, a gene product first identified through serologic screening, could also be a target for MHC class I–restricted cytotoxic T lymphocytes (30).

These provocative findings illustrate the striking ability of antibody-based cloning strategies to define the repertoire of cancer antigens. In contrast to the difficulties involved in establishing tumor-specific T-cell clones, patient serum is readily available and can be applied to expression cloning without any extensive manipulations. Recently, several studies of this approach in colon carcinoma, breast carcinoma, renal cell carcinoma, and melanoma have been published (31,32,33,34 and 35). One intriguing result from this work is the identification of a limited set of gene products that stimulate antibody responses only in cancer patients; this finding suggests that serologic tests might be used effectively in cancer diagnosis.

Together, the three cancer antigen–discovery strategies have begun to clarify the rules determining immune recognition of cancer. Four classes of targets have been unveiled thus far [reviewed in (36)]. The first group, termed “cancer-testis” antigens, consists of oncofetal proteins aberrantly expressed in tumors. These gene products are broadly expressed during fetal development (their functions have not yet been elucidated), but in the adult, their expression is limited to the testis and placenta; members of this group include NY-ESO-1 and the MAGE, BAGE, and GAGE gene families. A second group of antigens is composed of mutated cellular proteins, some of which may contribute to the transformed state, such as cyclin-dependent protein kinase 4, b-catenin, and caspase 8. A third category consists of short peptides derived from intron sequences abnormally expressed in cancer cells; these include MUM-1, p15, *N*-acetylglucosaminyltransferase V, and a novel form of TRP-2.

Although the mechanisms by which these gene products can serve as cancer antigens may be readily understood, a fourth class of targets presents a conceptual challenge. This last group consists of nonmutated cellular proteins; the most thoroughly studied to date are proteins in the melanin biosynthetic pathway, including tyrosinase, gp75 (tyrosinase-related protein-1, TRP-1), TRP-2, Melan A/MART-1, and gp100/Pmel 17. These targets highlight the close connection between autoimmunity and tumor immunity (37). The variables that underlie the ability to distinguish tumor cells and normal host cells remain to be clarified. Nonetheless, some level of autoimmunity might be an acceptable toxicity in those cases in which normal tissues are not essential for survival, such as the prostate, breast, thyroid, and melanocyte.

STRATEGIES TO ENHANCE ANTITUMOR IMMUNITY

A useful hypothesis framing much current immunotherapy research is that cancer cells generally are inefficient in stimulating immune responses. This idea receives considerable support in light of recent insights into the mechanisms underlying tumor antigen presentation. Extensive studies have revealed critical roles for dendritic cells in the initiation of effective immunity (38). These rare cells function to stimulate antigen-specific T- and B-cell responses because they capture and process antigens efficiently into both MHC class I and II pathways and express a full repertoire of costimulatory molecules. Indeed, these features may explain in part why tumor cells secrete factors that inhibit the local maturation and activation of dendritic cells. Tumor cells themselves, moreover, are typically compromised in antigen presentation and lack most of the key costimulatory molecules. As a consequence, it is unlikely that T and B lymphocytes encounter tumor antigens in an optimal immunologic environment; instead, tumor-specific lymphocytes remain ignorant of a progressing mass or undergo abortive responses (39).

Based on this model of how tumor cells may evade optimal host recognition, investigators are exploring a number of approaches to improve tumor antigen presentation. One widely pursued strategy involves the direct intermingling of tumor cells and dendritic cells. The discovery of techniques to expand large numbers of dendritic cells from hematopoietic precursors by culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) (40, 41) has spawned many investigations showing that dendritic cell–based vaccines substantially augment antitumor immunity in murine tumor models. Several schemes involving the *ex vivo* manipulation of dendritic cells have demonstrated activity (42,43,44 and 45), including dendritic cells pulsed with tumor antigen–derived peptides or whole tumor cell lysates and dendritic cells genetically engineered to express tumor antigens.

In addition to these strategies involving the *ex vivo* handling of dendritic cells, other approaches attempt to modify dendritic cell function *in vivo*. The cytokine flt3-ligand is a potent growth factor for dendritic cells, and its administration effectuates significant tumor destruction in multiple murine models (46,47). Immunization with plasmid DNA encoding tumor antigens can enhance augment antitumor immunity, and this involves either the transfection of dendritic cells *in vivo* or the processing by infiltrating dendritic cells of protein translated in skin or muscle (48). Recent studies have revealed that the unmethylated CpG oligonucleotides present in plasmid DNA molecules stimulate the growth and activation of dendritic cells (49). Tumor-derived heat-shock proteins, which chaperone peptide fragments, also show promise for increasing antitumor immunity, and the mechanism underlying their vaccination potential involves uptake by dendritic cells *in situ* (50).

The generation of an inflammatory focus filled with necrotic debris is a potent stimulus for dendritic cell activation (51,52). This insight provides the foundation for a number of immunization strategies that aim to elicit an inflammatory nidus at the site of tumor antigen administration. A widely studied method involves the inoculation of recombinant viral vectors expressing tumor antigens; these systems have been engineered from adenovirus, herpes simplex virus, and vaccinia and fowlpox viruses (53). It is hoped that the immune response directed against viral components will elicit a favorable mixture of cytokines, resulting in the “bystander” generation of tumor antigen–specific responses. A limitation of this strategy is that the immune response may remain directed toward viral targets, with inefficient epitope spreading to the cancer antigen.

To overcome this difficulty, several groups are evaluating a variety of adjuvants administered with tumor antigen–derived proteins or peptides. QS-21 and DETOX appear to be among the most potent of the group of saponin/microbial product complexes. These agents function as depots for sustained antigen delivery and as stimuli for cytokine production (54). Recombinant cytokines also are being administered with tumor peptides/proteins, and encouraging preliminary results have been found with GM-CSF and interleukin (IL)-12 (55,56).

The application of recombinant cytokines as vaccine adjuvants represents an extension of the pioneering studies of Forni et al. (57), who showed that the peritumoral injection of specific cytokines, particularly IL-2, could elicit tumor destruction through the local activation of neutrophils, eosinophils, macrophages, natural killer cells, and lymphocytes. This orchestrated response could in some cases generate protective immunity against wild-type tumor challenge. These striking findings stimulated a large number of investigations exploring the antitumor effects of various immunostimulatory molecules. These studies were significantly advanced with the application of gene-transfer techniques for the engineering of tumor cells, as this approach resulted in superior cytokine delivery to that which could be achieved by the injection of recombinant proteins [reviewed in (58)].

Retroviral vectors were most commonly used in these investigations because of their ability to achieve stable, high-level gene expression without the generation of replication-competent virus (59). Many immunostimulatory molecules have been evaluated for the ability to abrogate tumorigenicity; IL-12 and IL-2 are the most consistently active cytokines in this assay (60,61 and 62). The antitumor effects of these molecules remain to be fully delineated, but include the coordinated functions of CD4- and CD8-positive T lymphocytes, natural killer cells, macrophages, neutrophils, and eosinophils. The destruction of the tumor vasculature and inhibition of angiogenesis are decisive components as well (63).

An important result in this work was that the development of systemic immunity could be dissociated from the reduction in tumorigenicity of gene-modified cells (64). Although this paradox remains poorly understood, it has stimulated exploration of a second assay in which the relative abilities of different molecules to stimulate systemic immunity were compared. Because most cytokines do not inhibit tumorigenicity, this assay required the use of replication-incompetent tumor cells for vaccination. Irradiation was an appropriate tool for this purpose, for although it induces a G_2 cell-cycle arrest, it does not reduce transgene expression *in vitro* for several days.

Our own group has tested the immunization activity of more than 30 different gene products in this manner. We have found that vaccination with irradiated tumor cells engineered to secrete GM-CSF stimulates potent, specific, and long-lasting antitumor immunity in multiple murine tumor models (65). GM-CSF–based vaccinations depend on both CD4- and CD8-positive T lymphocytes and likely involve the enhanced phagocytosis and digestion of irradiated tumor cells by activated dendritic cells

and macrophages recruited to the immunization site.

EXPLORATORY CLINICAL TRIALS

The development of vaccination strategies targeting antigen-presenting cells and the identification of candidate tumor antigens have provided clinical investigators with the complex problem of evaluating the relative immunogenicity and toxicity of these new treatment approaches. Because the safety profiles of these strategies have yet to be fully defined, patients initially receiving these treatments will harbor advanced disease. It is likely that tumor cells at this stage of illness are multiresistant and markedly heterogeneous. The immune system at this point also has been compromised by the toxicity of prior cytoreductive therapies and the suppressive effects of advanced cancer. These formidable obstacles may render it difficult for immunotherapy to effectuate substantive antitumor effects in these individuals, and thus the primary goal of this phase of testing should be a preliminary assessment of immunologic activity.

The use of defined antigens for cancer vaccination presents substantial advantages in terms of feasibility and immunologic monitoring. In this context, it is encouraging that several different approaches already have revealed some degree of antitumor activity. Immunization with melanoma antigen–derived peptides alone can elicit tumor regressions; seven of 25 patients receiving a human leukocyte antigen (HLA)-A1–presented peptide encoded by the MAGE-3 gene product manifested objective tumor responses (25). Intriguingly, the presence of antigen-specific cytotoxic T lymphocytes could not be detected in the peripheral blood of these patients, suggesting that either other antitumor effector mechanisms were stimulated by vaccination or that the activated cells had been recruited to metastatic tumor deposits.

Recombinant cytokines have been added to free peptides in an effort to enhance immunogenicity. GM-CSF has been shown to stimulate antigen-specific cytotoxic T cells and delayed-type hypersensitivity reactions in patients receiving mixtures of peptides derived from Melan A, tyrosinase, and gp100; moreover, some tumor regressions have been observed as well (66). GM-CSF also appears to enhance vaccination strategies using the idiotypes present in multiple myeloma (67) and follicular lymphoma (68). This treatment approach is based on the idea that the unique immunoglobulin molecule expressed on malignant B cells can serve as a tumor-rejection antigen. A significant proportion of lymphoma patients immunized with this scheme after autologous bone marrow transplantation achieved molecular remissions, and antigen-specific CD4- and CD8-positive T-cell and antibody responses developed. High doses of IL-2 also may serve an adjuvant function, as has been suggested by recent studies of a modified peptide derived from the gp100 (26). Early trials of bone marrow–derived dendritic cells loaded with melanoma antigens or B-cell idiotypes have disclosed antitumor effects as well, although the effector mechanisms remain to be clarified (69,70 and 71).

The analysis of antigen-specific–based vaccines has been considerably advanced by the recent development of peptide epitope/MHC molecule tetramers. Tetramers are complexes of synthetic peptide epitopes and bacterially produced MHC molecules conjugated to a fluorochrome (72). Their multimeric nature overcomes the inherent low affinity and fast off-rate of T-cell receptor/MHC-peptide interactions, permitting these complexes to be used in fluorescein-activated cell sorter (FACS) analysis of bulk T-cell populations. These reagents also can be used to sort tetramer-binding cells for detailed functional studies, including antigen-induced cytokine production, proliferation, and cytotoxicity.

Although peptide and protein targets are the most widely studied targets in antigen-specific vaccine strategies, carbohydrate molecules also have elicited promising results. Furthest in development are those schemes based on the GM2 ganglioside (73); phase III trials have been initiated of vaccination with this antigen admixed with QS-21 in stage III malignant melanoma patients after lymph node dissection. Immunization with globo H also has been shown to stimulate antibody responses and modify the rate of prostate specific antigen (PSA) elevation in metastatic prostate carcinoma patients (74).

The selection of antigen loss variants may ultimately limit the overall efficacy of antigen-specific vaccines. Indeed, this already has been observed in melanoma peptide vaccine trials, where some progressive tumors were shown to lose expression of the immunizing antigen (75). The use of polyvalent vaccines may be one strategy to obviate this difficulty, and, in this context, whole tumor cells are a particularly attractive source. Allogeneic tumor cells offer many logistic advantages and could be readily adapted for large-scale clinical testing. Allogeneic melanoma cells have been used in a variety of forms, including intact cells or shed antigens with bacille Calmette-Guérin (BCG), virus-modified cell lysates, and cell lysates admixed with complex adjuvants (76,77,78 and 79). Some of these strategies have recently entered phase III testing in settings of minimal residual disease. Vaccination with allogeneic tumor cell lines engineered to express immunostimulatory molecules has been initiated as well. A pilot study of immunization with IL-2–secreting melanoma cells disclosed the generation of inflammatory reactions in metastases (80); trials of GM-CSF and B7-1–expressing allogeneic cells also have been initiated.

Although allogeneic cell vaccines are easily prepared, substantial data in murine systems suggest that tumor-specific antigens may be the most potent (81). An intriguing phase III study in support of this idea was recently reported; patients with Duke's B colorectal carcinoma showed a 61% reduction in the risk of recurrent disease after vaccination with irradiated, autologous colorectal carcinoma cells admixed with BCG as postoperative therapy (82). The administration of autologous, hapten-modified melanoma cells admixed with BCG and cyclophosphamide also has stimulated the development of inflammatory reactions in metastases (83).

Vaccination with autologous tumor cells genetically modified to express immunostimulatory molecules has recently entered clinical testing. Two small trials have examined the biologic activity of lethally irradiated, autologous melanoma cells engineered to secrete interferon-g (84,85). These studies have revealed the stimulation of antimelanoma IgG antibody responses and some minor tumor regressions. A small study of immunization using irradiated, GM-CSF–secreting autologous renal cell carcinoma cells has been published (86). Vaccination sites demonstrated an infiltrate of macrophages, dendritic cells, eosinophils, and lymphocytes. After, but not before vaccination, injections of nontransfected tumor cells elicited strong delayed-type hypersensitivity reactions consisting of lymphocytes, eosinophils, and macrophages. Similar results were found in a phase I study of vaccination with irradiated, GM-CSF–secreting autologous prostate carcinoma cells in patients with increasing PSA levels (87).

We recently reported the results of a phase I trial of 21 metastatic melanoma patients who were vaccinated with irradiated, autologous melanoma cells engineered to secrete GM-CSF (88). Although metastases resected before vaccination were minimally infiltrated with cells of the immune system in all cases, metastases resected after vaccination were densely infiltrated with T lymphocytes and plasma cells and showed extensive tumor destruction (at least 80%), fibrosis, and edema in 11 of 16 patients examined. Lymphocytes isolated from the infiltrated metastases showed marked cytotoxicity and broad cytokine production in response to autologous melanoma cells. High-titer antibodies recognizing intracellular and surface melanoma antigens could be demonstrated by Western blot and FACS analysis with postvaccination sera.

CONCLUSION

Cancer immunology has been reinvigorated by the infusion of molecular genetics, advanced cell-culture techniques, and careful clinicopathologic study. The recognition that antitumor immune responses are commonly stimulated, and that specific kinds of immune responses (those defined pathologically by brisk intratumor T-cell infiltrates) are associated with prolonged survival provides a strong intellectual foundation for the crafting of novel immunotherapies. The design of several vaccination schemes that dramatically augment tumor antigen presentation represents an important first step in this regard. Immunotherapies are likely to find their most effective application in early-stage disease, however, and thus appropriate clinical testing will take considerable time. Fortunately, the rigorous assessment of immunogenicity can now be the basis for mapping out the long-term course.

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89 ANTI-TNF-a THERAPHY IN RHEUMATOID ARTHRITIS AND OTHER DISEASES

Marc Feldmann, Ph.D., F.R.C.P., and Ravinder N. Maini, F.R.C.P.

THE ROLE OF TUMOR NECROSIS FACTOR-a IN THE PHYSIOLOGY OF IMMUNITY, INFLAMMATION, AND PATHOLOGY

Basic Properties

Tumor necrosis factor-a (TNF-a) is a key mediator of inflammation, immunity, and pathology (1,2). This is now clear from the analysis of more than a decade of the properties of TNF-a *in vitro*, in animal models, from the use of anti–TNF-a biologicals in clinical trials, and lately, from observations in routine clinical practice (3,4).

TNF-a in its biologically active state is a homotrimer, which exists in two forms. Initially TNF-a is membrane bound, but subsequently the membrane form is cleaved by a surface metalloproteinase, known variously as TNF-a–converting enzyme (TACE), also known as a disintegrin and matrilysin (ADAM)17 (5) (Fig. 89.1). TNF-a is produced chiefly by monocytes/macrophages and T lymphocytes, but to a lesser extent by many other cells including mast cells, neutrophils, natural killer (NK) cells, dendritic cells, endothelial cells, and malignant cells (1,2).

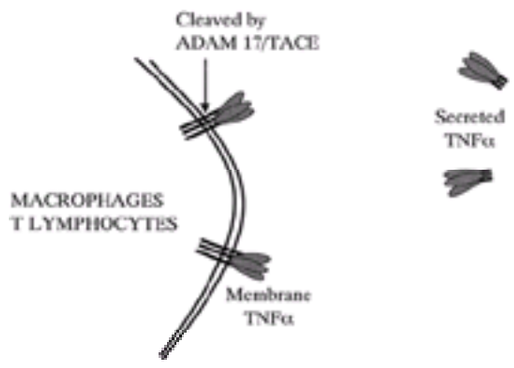

Figure 89.1. Tumor necrosis factor (TNF)-a is shown as a homotrimer on the cell surface. The soluble form of TNF-a is released by the action of TNF-a cleavage enzyme, ADAM17/TACE.

All cells of the body express TNF receptors, and thus are susceptible to its actions. There are two distinct receptors, with the p75 receptor expressed chiefly on hemopoietic cells, preferentially binding membrane TNF-a and involved in immunity, whereas the p55 receptor is ubiquitous and is involved mainly in proinflammatory and apoptotic signals (6,7). The mechanism by which the TNF receptor mediates signals is complex, but has been partly unraveled with the help of gene knockouts and other techniques.

Physiology of Tumor Necrosis Factor-a

TNF-a is one of the most rapidly released cytokines, with abundant blood levels detected within 30 minutes of lipopolysaccharide (LPS) stimulation. This is quicker than other proinflammatory cytokines such as interleukin (IL)-1 or IL-6 (8), and together with *in vitro* data, suggests that TNF-a has a special role in coordinating the response to stressful proinflammatory stimuli, such as LPS stimulation or exposure to ultraviolet light, or chronic inflammation such as rheumatoid synovitis (9). The initial source of TNF-a is probably from the cleavage of preexisting membrane TNF-a on monocytes, neutrophils, and T lymphocytes, and the release of TNF-a from mast cell granules. Subsequently, newly synthesized TNF-a by macrophages and T lymphocytes is the major source.

The actions of TNF-a on almost all cells are too numerous to describe here (for useful further details on this and other cytokine topics, the “Cytokine Reference” is useful) (10). The most important actions of TNF-a include (11)

T lymphocytes: Upregulation of CD25 (IL-2Ra chain);

B lymphocytes: Growth factor;

Macrophages: Activator, induces other proinflammatory mediators such as IL-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, induces chemokines (e.g. IL-8, degradative enzymes);

Endothelium: Augments adhesion molecule expression [vascular cell adhesion molecule (VCAM)-1, E-selectin], induces chemokines, upregulates NOS;

Fibroblasts: Induce production of matrix metalloproteinases, prostaglandin (PG)E_2;

Chondrocytes: Induce production of matrix metalloproteinases, reduce proteoglycan synthesis;

Osteoclasts: Mediate osteolysis.

Lymphotoxin a (LT-a) is a very closely related molecule, also a homotrimer, which although sharing ~30% sequence identity, has essentially the same three-dimensional structure, and so activates the same two receptors as TNF-a. It is produced by fewer cell types, chiefly lymphocytes (T and B), and has a role in the immune system (12).

TNF receptors are clustered on the cell surface, with an association domain at their terminal region (13) (Fig. 89.2). This keeps the intracytoplastic domains apart inside the cell. Aggregation of the TNF-R is an essential step, which initiates the signaling cascade. Physiologically this is accomplished by the TNF-a (or LT-a) trimer, and the importance of aggregation is demonstrable by the agonist effects of most monoclonal antibodies to the p55 TNF-R (14). Receptor aggregation brings the intracytoplasmic domains close together, permitting the signaling process to be initiated.

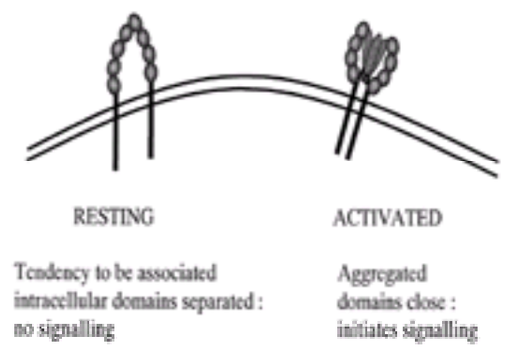

Figure 89.2. Resting and activated forms of tumor necrosis factor (TNF) receptor molecules on the cell surface. The binding of the soluble TNF-a molecule to the TNF receptors initiates signaling.

Once receptors are aggregated, a complex series of events follow and eventuate in the activation of a number of cascades. NFkB is activated, after IkB, the inhibitor of NFkB, is phosphorylated by Ik kinases and degraded by the proteosome. All three of the mitogen-activated kinase cascades, p42/44 ErK, p38 stress-activated kinase (SAPK) and p54 JUNK, are also induced. The p55 TNF-R can activate caspases involved in apoptosis very effectively, and induces apoptosis if antiapoptotic factors are not generated, as is the case in TNF-a bioassays, by blocking protein synthesis with cycloheximide or actinomycin D. Further details of TNF signaling can be found in recent reviews (14,15).

The outcome of TNF signaling is the induction of multiple genes and their products, including cytokines, chemokines, adhesion molecules and PGE_2. Some aspects of TNF signaling are still controversial, such as the role of sphingomyelinases. TNF-R signaling is summarized in Fig. 89.3.

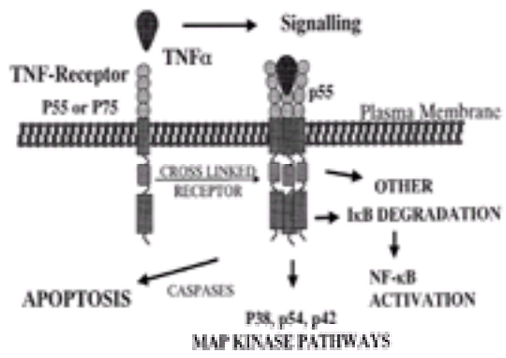

Figure 89.3. Activation of tumor necrosis (TNF) receptors, p55-TNF-R or p75-TNF-R by TNF-a induces multiple cell-signaling pathways leading to cell and gene activation.

The strongly proinflammatory actions of TNF-a are modulated by a number of mechanisms. These include the TNF-R–associated inhibitory molecules, cellular inhibitors of apoptosis (cIAPs), and the silencer of death domain (SODD). These increase the threshold of activation and are displaced when the TNF-R is cross-linked (16). A second mechanism is dependent on the shedding of the extracellular domain of the TNF-R, which leads to a reduction in receptor density, thus reducing TNF signaling efficiency. It also results in the generation in body fluids of appreciable levels of soluble TNF-Rs, which act as competitive inhibitors and reduce access of free TNF-a to the membrane receptors (17,18 and 19). Third, TNF-a synthesis, especially by macrophages, can be downregulated by a number of cytokines, including IL-4, IL-10, IL-13, IL-11, and tumor growth factor (TGF)-b.

One of the clearest ways of investigating the physiology of TNF-a is to study targeted mutations in murine models, commonly known as knock-outs (20,21). TNF-a knock-out mice have a reduced response to inflammatory stimuli such as LPS, and importantly, are unable to mount an adequate defense against intracellular parasites, such as *Listeria*, *Mycobacteria*, or fungi. However, they also are unable to control noninfectious granulomatous stimuli, including exposure to heat-killed *Corynebacterium parvum*, which result in severe liver damage, thus emphasizing the complexity of the role of TNF-a, which under certain conditions can suppress the immune response *in vitro* and *in vivo* (20,21 and 22). Lack of TNF-a is implicated in the pathogenesis of systemic lupus erythematosus in NZB/W mice (see later). Appropriate but controlled production of TNF-a is beneficial in host defense.

TNF-a–overproducing transgenic mice also have been useful in defining the pathophysiology of TNF-a. Kollias et al. (23) made a number of these, which have emphasized aspects of TNF-a pathophysiology. Some of these transgenics are created by deleting the endogenous 3' region of the TNF-a DNA, which has numerous AU-rich motifs, involved in the binding of inhibitory proteins to this important regulatory region of the TNF gene. One of these proteins is tristetraprolin (abbreviated TTP) knock-outs, which have augmented TNF-a concentrations (24).

Transgenic mice overexpressing human TNF-a develop arthritis, and some have psoriasis-type rashes and inflammatory bowel disease (21,23,25,26). The capacity of human TNF, which binds to the murine TNF-R p55 but not murine TNF-R p75 to induce arthritis, indicates that the p55 signaling pathway is essential for the development of arthritis, whereas the p75 pathway is not. Murine TNF transgenics also induce arthritis, indicating that the p75 does not have an essential “protective” role.

In most animal models of arthritis, it is clear that T cells are an important part of the pathogenesis, for example, in DBA/1 mice injected with type II collagen in complete Freund's adjuvant, or rats injected with *Mycobacteria* (adjuvant arthritis). However, this is not the case in TNF-a transgenics, which if backcrossed to lymphocyte-deficient RAG2 knock-outs, still develop arthritis, but interestingly, do not develop inflammatory bowel disease (26). Thus it appears that in the context of arthritis, the immune activity takes place upstream of TNF-a production, which can thus be viewed as a final common pathway leading to chronic inflammation.

TNF-a transgenics also have cachexia, emphasizing the role of TNF-a in the early observations of Cerami and Beutler (1), who cloned mouse TNF-a as “cachectin.”

BIOLOGIC AGENTS THAT BLOCK TUMOR NECROSIS FACTOR-a

These are diagramatically illustrated in Fig. 89.4.

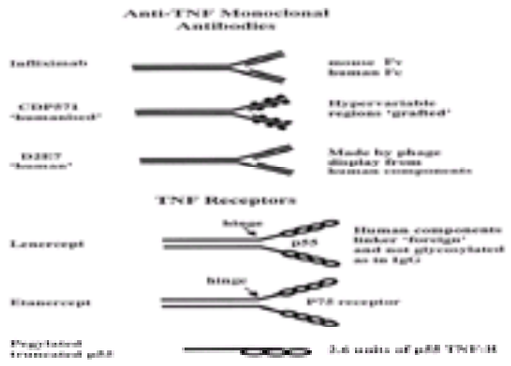

Figure 89.4. Anti–tumor necrosis factor (TNF) monoclonal antibodies and TNF receptors biologicals that have been developed for therapeutic use.

Monoclonal Antibodies

These were the first agents produced to inhibit TNF-a actions specifically. Murine monoclonals were first generated and were extensively used in sepsis trials, without success.

CHIMERIC MONOCLONAL ANTIBODY, INFLIXIMAB

Subsequently Centocor, Inc., generated a “chimeric” monoclonal by replacing three fourths of the mouse gene with human constant regions of light and heavy chains of immunoglobulin (Ig)G, but leaving the murine Fv region, producing a protein that is 75% “human,” and has the human Fc region and hence effector functions on human cells. This was used for anti TNF-a therapy for rheumatoid arthritis (RA) and Crohn disease (CD) and is now generically designated infliximab, and is sold by Centocor/J&J as Remicade® for RA and CD in the United States, and by Schering-Plough in Europe.

OTHER MONOCLONAL ANTIBODIES

Newer entrants to the field have produced other antibodies (e.g., Celltech generated a “humanized” hypervariable complementary determining region (CDR) grafted antibody CDP571, which has been used in trials. Cambridge Antibody Technology (CAT) and BASF have been using an antibody generated by phage-display technology (D2E7), which in 2000 was in phase III development.

A schematic representation of these antibodies is shown in Fig. 89.4. The logic of using humanized antibodies, to various degrees, is to reduce the immunogenicity of the “murine” determinants, and also to introduce the human Fc, which would increase circulating half-life, as well as permit the effector function of the Fc region.

Tumor Necrosis Factor Receptor Immunoglobulin G Fc Fusion Proteins

There are two TNF receptors, p55 and p75, both of which bind both TNF-a and LT-a. Thus the fusion proteins composing the Fc region of IgG linked to the hinge region, and two TNF receptors have a different specificity from the monoclonal antibodies. At present it is not known what added benefits or risks are conferred by the blocking of LT-a and TNF-a over TNF-a alone.

P75 TUMOR NECROSIS FACTOR RECEPTOR FC ETANERCEPT, ENBREL (IMMUNEX/AHP)

This dimeric TNF-R p75 human Fc molecule produced by Immunex neutralizes TNF effectively *in vitro*, and in animal models, and has been highly successful in the clinic.

p55 TUMOR NECROSIS FACTOR RECEPTOR Fc LENERCEPT

The first reported TNF-R Fc fusion protein molecule was a dimeric p55 TNF-R IgG Fc that was strongly neutralizing *in vitro*, and in animal models, but surprisingly was not very successful in clinical trials, perhaps because of immunogenicity. It has thus been abandoned by Roche.

PEGYLATED TRUNCATED p55 TUMOR NECROSIS FACTOR RECEPTOR Fc

This is a monomeric TNF-R p55 (produced by Amgen) trimmed to remove potentially immunogenic sites, and pegylated to increase its circulating half-life.

ANTI–TUMOR NECROSIS FACTOR-a THERAPY IN RHEUMATOID ARTHRITIS

Rationale

Cytokine expression studies in rheumatoid synovium and synovial fluid in the 1980s revealed a very complex pattern of cytokines, with virtually all of assayable cytokines expressed, with the exception of IL-4 (27). This led to a common belief that cytokines were not good therapeutic targets because of their “redundancy,” a term used to reflect the markedly overlapping properties of various cytokines, with, for example, TNF-a, IL-1, and GM-CSF having very similar effects. It was believed that blocking a single cytokine, from a group with overlapping properties, was unlikely to be clinically beneficial, as the remaining ones would still drive the pathologic process.

Fortunately, the redundancy of the cytokine system is much more apparent *in vitro* than it actually is *in vivo*, and this pessimistic prediction has not proven true.

The first clues that TNF-a may be a good therapeutic target came from an analysis of IL-1 regulation in mixed rheumatoid synovial cell cultures, which we had found to be a good model of the rheumatoid synovium *in vitro*. The enzymatically dissociated cells (30% T, 30% macrophages, with B cells, fibroblasts, endothelium, B lymphocytes) rapidly reaggregate into clumps containing of T cells/macrophages/dendritic cells. These cultures produce cytokines over a prolonged period (e.g., 6 to 7 days), reflecting the prolonged cytokine production found in active rheumatoid synovium. Thus high and relatively stable levels of IL-1 mRNA and protein are released by these cultures (9,28) and provide an opportunity to explore why cytokine production is perpetuated and does not switch itself off, as it does in cultures of normal tissue after stimulation with mitogens or antigens.

The tools we used to dissect the phenomenon of the problem of the prolonged expression of cytokines were antibodies, and it was found that anti–TNF-a (but not anti–LT-a) antibody markedly reduced the production of IL-1 protein, within 24 hours if measured at the mRNA level, or 2 to 3 days if measured at the protein level (Fig. 89.5) (9). This was the first clue that TNF-a has a special role in the proinflammatory cytokine network and “orchestrates” the proceedings. This concept was established by evaluating the effects of TNF or IL-1 blockade on the production of other proinflammatory cytokines, including GM-CSF, IL-6, and IL-8 (3,29). An oversimplification of our current concept of the proinflammatory cytokine network is illustrated in Fig. 89.6. It is an oversimplification to have all the signals from the immune system going via TNF and IL-1, and of course, this scheme ignores the various feedback loops and the effects of genetic polymorphisms in cytokine function. Furthermore, the longer-term influences of cell recruitment and of control of cell survival and death cannot be summarized schematically.

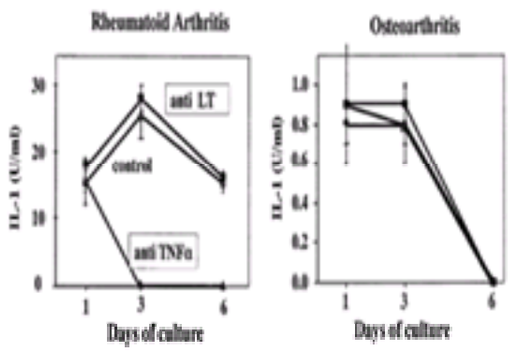

Figure 89.5. Synovial cells from patients with rheumatoid arthritis (RA) or osteoarthritis (OA) were cultured for 6 days with anti–tumor necrosis factor (TNF) or antilymphotoxin (anti-LT) monoclonal antibody. *In vitro* synthesis of interleukin-1 was inhibited by the anti-TNF monoclonal antibody in the RA but not the OA cell cultures. *Circles,* control cultures; *triangles*, with anti–TNF-a; *squares*, with anti-LT. (Reproduced from Brennan R, Chantry D, Jackson A, et al. Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet* 1989;2:244–247, with permission.)

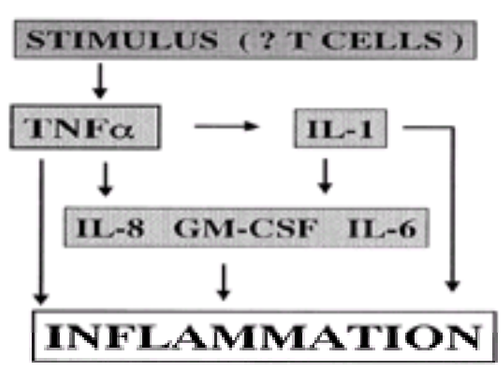

Figure 89.6. The dominance of tumor necrosis factor (TNF)-a in the cytokine cascade. TNF-a affects target cells either directly or indirectly through interleukin (IL)-1; a cascade of other cytokines is induced, each of which induces other inflammatory activities.

This analysis of the role of TNF-a in regulating other cytokines suggested that blocking TNF-a alone would be of potential therapeutic benefit. However, before that could be attempted, it was important to confirm some of the key predictions in *ex vivo* and *in vivo* situations. The presence of upregulated TNF and TNF-R expression *in vivo* was demonstrated by an *ex vivo* immunohistologic approach with fresh frozen (~1 minute) tissue, a time far too short to induce the *de novo* production of TNF *in vitro* (30).

Most important is the effect of anti–TNF-a antibody in animal models of RA. These studies have been very clear cut, with multiple groups all finding that TNF-a blockade after disease onset in animal models is effective at reducing inflammation and disease spread, as well as protecting joints from destruction (31).

Clinical Results of Anti–Tumor Necrosis Factor-a Antibody Therapy in Rheumatoid Arthritis

Overall, excellent and highly consistent results have been obtained by treating chronic active RA patients for whom all existing therapies have failed. Multiple agents that block TNF-a (+LT-a) have yielded highly significant and clinically relevant results, with the consequent approval by the Food and Drug Administration (FDA) of two of these agents (etanercept and infliximab) for clinical use in severe RA, and more on the way. The benefit and utility of these biologic agents blocking TNF-a has exceeded most people's expectations, with more than 150,000 RA patients treated by April 2001. A recent editorial reviewing anti–TNF-a therapy in RA reflects on the place and future of the therapy (32).

CLINICAL BENEFIT WITH ANTI–TNF-a INFLIXIMAB, REMICADE

This was the agent first used, and so the largest body of data exists with this drug. Phase I studies were initiated in May 1992, at the Charing Cross Hospital/Kennedy Institute of Rheumatology, on a cohort of active RA patients at the late stage of disease, for whom all prior therapy had failed. They were given a large dose of antibody, 20 mg/kg in two or four divided doses, over a 2-week period. The results were notable: many patients felt less tired and weak within hours, and diminution of pain and stiffness was marked. Swollen joints and tender joints diminished within 2 weeks, and plateaued at 6 to 8 weeks. All 20 patients improved markedly, with a duration of benefit ranging from 8 to 26 weeks before relapse (33).

This phase I trial was “open,” not blinded or placebo controlled, but the magnitude of the therapeutic benefit in patients resistant to all existing drugs made it likely to be a treatment, rather than a placebo effect. This was confirmed by assays on the serum acute-phase protein, C-reactive protein (CRP), which was reduced to the normal range within a few days. A summary of this trial is shown in Fig. 89.7.

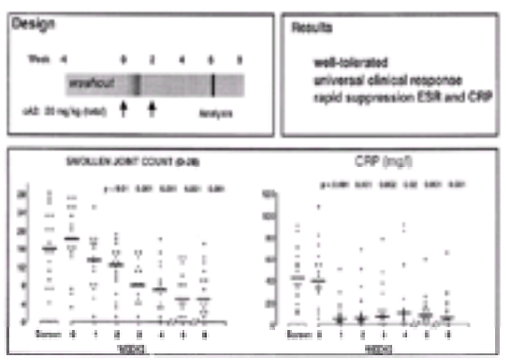

Figure 89.7. Open-label treatment with infliximab in rheumatoid arthritis (RA). Patients with active RA were infused with a total of 20 mg/kg infliximab. Mean serum C-reactive protein levels were close to the normal range, and swollen joint counts decreased significantly over the 8-week period. (Reproduced from Elliott MJ, Maini RN, Feldmann M, et al. Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha. *Arthritis Rheum* 1993;36:1681–1690, with permission.)

Randomized, double-blind, placebo-controlled trials, are the currently required standard of proof that a therapy is effective. The first such trial of anti—TNF-a conducted in Europe was reported in 1994, and the results were unequivocal. The assessments used at this time were the criteria devised by Harold Paulus, a composite index comprising of numeric assessments of swollen joints, tender joints, duration of morning stiffness, and pain, as well as laboratory indices of inflammation such as erythrocyte sedimentation rate (ESR). A 20% degree of improvement at 4 weeks was the predetermined point for evaluating success. As summarized in Fig. 89.8, this 20% improvement level was reached in 8% of the placebo-infusion patients, 44% of those receiving a single infusion of 1 mg/kg, and 79% of those receiving a single infusion of 10 mg/kg. This was a very clear-cut demonstration of benefit (34). Figure 89.8 summarizes this trial.

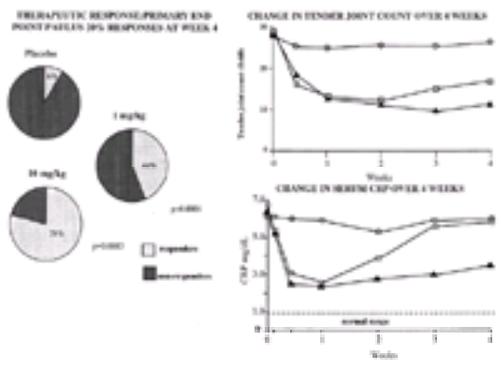

Figure 89.8. Randomized trial comparing infliximab and placebo. Patients with active rheumatoid arthritis (RA) received a single infusion of either placebo or infliximab at 1 mg/kg or 10 mg/kg body weight. At week 4, only 8% of placebo patients responded, compared with 44% and 79%, respectively, of these treated with 1 mg/kg or 10 mg/kg. Tender joint counts and serum C-reactive protein levels also decreased in the treatment groups, the effects being more pronounced in the 10-mg/kg patient group. (Reproduced from Elliott MJ, Maini RN, Feldmann M, et al. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis. *Lancet* 1994;344:1105–1110, with permission.)

The reports of these phase I and phase II trials led other companies, which had developed their TNF inhibitors for use in the then fashionable but difficult area of sepsis, to transfer their anti-TNF reagents to use in RA trials (e.g., lenercept, etanercept, CDP571).

As RA is a long-term disease, it was important to address the questions that the former short-term studies did not. For example, if TNF-a is blocked, will other pathways take over to drive the disease process? Could a chimeric antibody be used safely and effectively over the longer term? Would TNF-a blockade predispose to infections, malignancy, or autoimmunity?

The next trial lasted 6 months, and five infusions were given over this period. This demonstrated that efficacy could be maintained over this duration, with a good safety profile. As this trial incorporated infliximab with and without methotrexate (MTX), and a dose/response of infliximab (35), it helped define effective doses and demonstrated the benefit of even very low dose (7.5 mg/week) MTX as a supplement (Fig. 89.9).

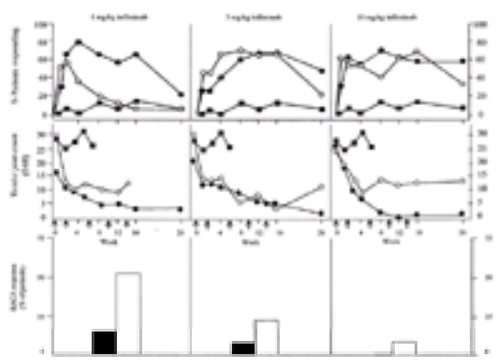

Figure 89.9. Synergy of anti–tumor necrosis factor-a and methotrexate (MTX). **Top panels:** Duration of response to therapy as defined by 20% Paulus criteria at three doses of infliximab and without MTX and placebo plus MTX. Results shown are the proportion (%) of patients responding at weeks 1, 2, 4, 8, 16, and 26. The Paulus response is achieved by 20% improvement in four of six of the following: tender-joint and swollen-joint scores, duration of morning stiffness, erythrocyte sedimentation rate, and a two-grade improvement in the patient's and observer's assessment of disease severity. **Middle panels:** Serial measurements (median values) of the tender-joint count, before (day 0), during (weeks 1–14), and after (weeks 14–26) treatment. Results are included only up to the point at which £50% of patients remained in the trial (up to week 6 for the placebo-plus-MTX group and up to week 14 for the infliximab, 1-mg/kg group). *Arrows,* the timing of infusions of infliximab at weeks 0, 2, 6, 10, and 14; *solid circles*, infliximab with MTX; *open circles*, infliximab without MTX; *squares*, placebo plus MTX. **Bottom panels**: Incidence of human antichimeric antibody (HACA) in the patient groups receiving infliximab, with (*shaded*) or without (*clear*), MTX. The rate of HACA responses is inversely proportional to the dose of infliximab. (Reproduced from Maini RN, Breedveld FC, Kalden JR, et al. Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis (see comments). *Arthritis Rheum* 1998;41:1552–1563, with permission.)

The assays for immunogenicity of infliximab revealed that there was a high frequency of anti-infliximab responses at 1 mg/kg in the absence of MTX. However, this was markedly reduced by both increasing the dose and adding MTX. These results indicate that in humans, as previously shown in experimental animals, there are mechanisms of downregulating antibody response to injected IgG, by using an immunosuppressive (MTX) or a sufficient dose of deaggregated gamma globulin. There is an extensive literature from the 1960s and 1970s that deaggregated gamma globulin (like infliximab), given intravenously, induces “high zone” tolerance, at higher doses (36). Although this appears to be the only plausible interpretation of the data, there is no proof of this mechanism, which would require challenge with aggregated IgG to prove tolerance formally. The reduction in antiidiotype antibody at higher doses is illustrated in Fig. 89.9.

A phase III study with infliximab, the ATTRACT trial, has been decoded, after 2 years of anti–TNF-a therapy given every 4 or 8 weeks. Efficacy was maintained for the 2 years. It has confirmed that antibodies can be used repeatedly in a chronic disease, with good clinical benefit (37,38). Most important, the longer-term duration of this study permitted the analysis of the effects of infliximab on joint destruction, as assessed by radiograph, using a modified Sharp score (Van der Heijde modification). The results were presented at the November 1999 American College of Rheumatology (ACR) meeting and have recently been published (38). Two key points emerged. First was that by 12 months, the progression in joint damage was arrested in more than 50% of patients, as assessed by two independent experts blinded to the treatment and sequence of the radiographs. The results were clear at all four dose regimens of infliximab used, with arrest in progression noted at all doses. When the patients were analyzed according to their clinical response, by using the 20% ACR criteria, it was observed that the patients who did not benefit at the 20% level still benefited at the radiographic level. This interesting result needs further analysis, but at face value suggests that the widely held view based on animal and *in vitro* experiments that joint destruction depends on IL-1 and not TNF-a should be reevaluated.

OTHER ANTI–TUMOR NECROSIS FACTOR-a ANTIBODIES

Much less has been published with other antibodies. Celltech reported trials with CDP571, a humanized IgG4 antibody. This was effective at 10 mg/kg but not at 1 mg/kg and so is less efficacious than infliximab (39). A second-generation anti–TNF-a is being tested by Celltech, a pegylated anti–TNF-a Fab fragment, which appears efficacious compared with placebo (40).

D2E7, a human IgG1 anti–TNF-a antibody produced by phage display by Cambridge Antibody Technology (CAT) and BASF/Knoll, is currently in phase III trials. The data reported so far indicate that it is efficacious at a wide range of doses and treatment regimens. The results as presented appear to be comparable to those reported with infliximab, although in many of the trials, D2E7 is used subcutaneously rather than intravenously. Joint protection also has been reported in an abstract. The safety profile appears to be good (41,42).

Anti–Tumor Necrosis Factor Receptor Biologicals

p75 TUMOR NECROSIS FACTOR–RECEPTOR Fc ETANERCEPT (ENBREL)

This fusion protein is administered subcutaneously, twice a week, which means that self-administration is possible. The results are comparable to those obtained with infliximab, with minor differences, possibly linked to the dose and route of administration. The recommended dose of 25 mg twice a week, s.c., was the maximum used in trials, so it is not certain yet whether it is the optimal dose.

The efficacy of etanercept was first established in a phase II trial comparing 0.25, 2, and 16 mg/m^2 over a 3-month period. There was a 61% reduction of swollen and tender joint counts at the highest doses, compared with a 25% reduction with placebo, with intermediate results at 2 mg/m^2 (43).

Phase III studies compared 10 and 25 mg, s.c., twice per week, with placebo, over a 6-month period. There was a 59% ACR 20% response at the 25-mg dose compared with 11% in the placebo-treated group (Fig. 89.10) (44).

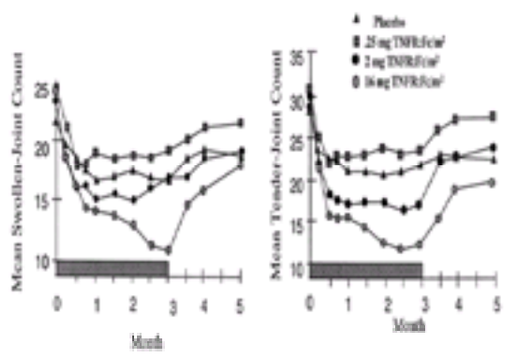

Figure 89.10. Treatment of rheumatoid arthritis with recombinant human p75 tumor necrosis factor (TNF)-R-Fc fusion protein (etanercept). Patients received subcutaneous injections of placebo or etanercept at 0.25 mg, 2 mg, or 16 mg per square meter of body-surface area, twice weekly for 3 months. Mean swollen-joint counts and tender-joint counts are shown over a 5-month period. *Shaded bar,* the treatment period; *triangles*, placebo; *squares*, 0.25 mg/m^2; *solid circles,* 2 mg/m^2; *open circles*, 16 mg/m^2. (Reproduced from Moreland LW, Baumgartner SW, Schiff MH, et al. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N Engl J Med* 1997;337:141–147, with permission.)

Like infliximab, etanercept has been used in clinical trials in conjunction with MTX. This may also augment efficacy, with a 71% ACR response rate in the combination therapy, compared with 27% with MTX plus placebo. As there was no etanercept-alone arm in this trial, it is not clear if the combination of etanercept and MTX was more effective than etanercept alone (45).

Etanercept also has been approved for juvenile RA, of the severe polyarticular type, which is not responding to other drugs. At a dose of 0.4 mg/kg (twice a week, s.c., for 90 days), 74% showed a clinical response by the predefined criteria (46).

TUMOR NECROSIS FACTOR–RECEPTOR P55 Fc (LENERCEPT)

This was the first fusion protein to be extensively tested. The results were positive, but were somewhat variable (47). The reasons for this are not fully known, but two hypotheses for the inconsistent results have been discussed. One is that there were batch-to-batch variations due to manufacturing problems. Another is that lenercept was more immunogenic than would have been predicted. Lesslauer et al. (48) reported that the extended peptide linker between the immunoglobulin Fc region and the TNF-R was the immunogenic part of the molecule, and with time, antibodies extended to the TNF-R itself (48). If the latter hypothesis is correct, then agonistic effects due to cross-linking cell-surface p55 receptors also may have contributed to variable results. It is noteworthy that Lesslauer et al. reported agonistic antibodies in a small cohort (three of seven) of patients given subcutaneous lenercept. Changes in half-life of lenercept were noted at the second injection compared with the first, which supports the concept that it was immunogenic. However, in some trials, many patients did very well, over long periods.

PEGYLATED p55 TUMOR NECROSIS FACTOR RECEPTOR

This product, developed by Amgen, has been shown to be effective in rodent and primate models, and there also is preliminary evidence in phase I/II trials in RA (49). This was a dose-ranging study, not powered for efficacy, which showed a trend to reduction in disease activity.

Biologic agents are much more costly to manufacture than are small chemical drugs, and current market costs of the anti-TNF drugs on the market are significantly higher than other anti-rheumatoid drugs. However, the multiplicity of anti–TNF-a biologicals that are being developed is likely to lead to competitive pricing in the future and make them more cost effective.

MECHANISM OF ACTION OF ANTI–TUMOR NECROSIS FACTOR-a THERAPY (INFLIXIMAB) IN RHEUMATOID ARTHRITIS

Overview

This section refers to infliximab specifically, as the only published data we have access to concerns studies with this agent. It is possible, even likely, that the pharmacodynamics of intravenous infliximab therapy at the doses achieve high serum levels and differ from those occurring with lower-dose administration by subcutaneous injections and hence lower concentrations of other anti-TNF agents. In the former, there is a rapid and virtually total TNF blockade and hence TNF clearance (“washout”), with consequent reduction in many other “downstream” cytokines. The mechanism of action of etanercept have many similarities.

Anti–Tumor Necrosis Factor-a Downregulates the Cytokine Cascade *In Vivo*

As the effect of anti–TNF-a on other downsteam cytokines was an important part of the rationale for anti–TNF-a therapy, it was of interest to evaluate whether this also was the case *in vivo*. The easiest cytokine to assay *in vitro* is IL-6, as there are elevated levels in RA patients, averaging about 100 pg/mL.

It was found that within a day of infliximab therapy, serum IL-6 levels reverted to the normal range (50). This was not unexpected, as it was already known that CRP, believed to be controlled chiefly by IL-6, returned to normal levels within a few days of treatment. However, the IL-6 result is important, as it is a formal proof that TNF-a regulates other proinflammatory cytokines. The speed and magnitude of the effect makes it likely to be a direct consequence of TNF blockade on the cytokine network, rather than due to reduction in numbers of cells producing IL-6 (for example, due to indirect effects on leukocyte trafficking, or by killing of TNF-a–producing cells).

There is abundant other evidence for a downregulation of the cytokine “cascade.” Our own group has documented a reduction in various cytokines and chemokines including IL-8, MCP-1, and vascular endothelial growth factor (VEGF) (51), and the group of Kalden (52) has reported downregulation of serum IL-1 (52).

Infliximab Diminishes Leukocyte Trafficking into Joints

As it was known that TNF-a regulates adhesion molecule expression on endothelial cells, the changes in adhesion molecule expression were monitored in the infliximab trials. Two approaches were used. The most quantitative was to measure serum concentrations of all three adhesion molecules, intercellular adhesion molecule (ICAM)-1, E-selectin, and VCAM-1, among which E-selectin, an endothelium-specific molecule, most probably reflects the adhesive properties of blood vessels. It was found that there were significant reductions in serum E selectin and ICAM-1 (53). Less quantitative, but more relevant to the joint disease, was semiquantitative analysis of synovial biopsies. This had demonstrated, with blinded observers, that the expression of adhesion molecules in the joint was diminished after anti–TNF-a (54).

Already discussed is the observation that serum levels of many chemokines are reduced after infliximab. Together with the adhesion molecule data, it strongly suggests that leukocyte trafficking will be reduced, and it was possible to verify this directly by using ^{111}In-labeled granulocytes, reinfused into the patient before and 2 weeks after infliximab treatment (55).

Whereas these radiolabeled cell-uptake results strictly apply only to granulocytes, it is very likely that other leukocytes also enter joints more slowly after infliximab. Posttreatment synovial biopsies are much less cellular, with numbers of T cells and macrophages reduced. Because T lymphocytes and macrophages also use the same spectrum of adhesion molecules as neutrophils and the relevant chemokines are downregulated, it is likely that there is reduced trafficking of all leukocyte

subsets. Increased apoptosis noted in T lymphocytes also may contribute to reduced cellularity (P. Taylor, unpublished data).

Infliximab Reduces Vascular Endothelial Growth Factor and Angiogenesis in Inflamed Joints

Angiogenesis is a prominent feature of chronic rheumatoid joints, and so it was pertinent to investigate whether infliximab therapy was associated with reduced angiogenesis. Initial studies focused on measurement of VEGF, a potent and endothelium-specific growth factor that promotes angiogenesis. Based on previous work of Fava et al. (56) and Koch et al. (57), who had demonstrated high VEGF levels in synovium, we assayed longitudinal blood samples from patients in the infliximab trials on the assumption that increased serum levels might reflect enhanced synovial synthesis. Pretreatment serum levels were indeed elevated and significantly reduced after infliximab therapy in two separate trials (51).

The partial reduction in serum VEGF levels led us to explore the possibility that angiogenesis was reduced. Computerized image analysis of endothelium, for multiple markers of endothelium, has shown a reduced vascularity after infliximab therapy (58,59).

Infliximab Restores Hematologic Abnormalities in Rheumatoid Arthritis

A tendency to low hemoglobin and a normocytic normochromic anemia is common in RA patients. In view of the profound effects of cytokines on hemopoiesis, it was of interest to investigate the effects of infliximab in this system.

In the phase II trial, with an end point at 4 weeks, hemoglobin levels decreased over a 4-week period in the placebo-treated group, possibly because of blood loss for experimental analyses. At the 1-mg/kg infliximab treatment, it stayed level, but at 10 mg/kg, it was significantly elevated over baseline. This result suggests that the anemia of RA, and by inference probably the anemia of other chronic inflammatory diseases is cytokine dependent. Whether the anemia is an effect of TNF-a or IL-6 or both is not clear, because both are reported to diminish red cell production experimentally (60).

Elevated platelet counts are a potentially dangerous consequence of RA, as in excess they may promote atherosclerotic and thrombogenic complications. Infliximab reduced the elevated levels to the normal range (61). However, there is as yet no evidence to support the hypothesis that infliximab is protective against thrombosis. The tendency of RA patients to exhibit a moderate neutrophilia was normalized.

ANTI–TUMOR NECROSIS FACTOR-a THERAPY IN OTHER CHRONIC INFLAMMATORY DISEASES

Crohn Disease

Crohn disease is a chronic inflammatory disease that affects any portion of the alimentary tract. The granulomatous inflammatory lesions can extend right through the full thickness of the gut, and can extend beyond it, causing fistulas.

CD was the first disease indication for which anti–TNF-a therapy was approved, for severe CD with or without fistula. This rapid approval was due to the capacity of infliximab to close more than 50% of the fistulas within 4 weeks of a single infusion (and all the fistulas in 30% to 40% of infliximab-treated patients). Because no other effective therapy is available for this distressing condition, this was a most impressive result (62).

CD trials began after it became evident that infliximab was effective in clinical trials of RA, and the results have been closely analogous (63). Patient populations treated were those doing badly on existing therapy, as judged by the CD activity index (CDAI). Single doses of infliximab were sufficient to put ~50% of the patients receiving 5, 10, or 20 mg/kg infliximab into remission for the 12-week duration of the trial. Retreatment was possible and led to further benefit. Endoscopy also was used to demonstrate the benefit of infliximab in CD (64). A maintenance of remission study was conducted as a follow-up, and four more doses given, with follow-up period of 44 weeks. This showed that remission was maintained in 60% at the 10-mg/kg dose, compared with improvements in 35% receiving placebo treatment. At the 1-mg/kg dose, remission was maintained in 51% of patients compared with 21% receiving placebo treatment (65).

Other anti-TNF reagents also have been used in CD. CDP571 also was efficacious, but the duration of benefit was shorter (66). There are no published data on etanercept in CD. Trials are in progress. A detailed review of anti–TNF-a treatment in CD has been recently published (67).

Sepsis

Anti–TNF-a therapy with a number of antibodies and fusion proteins has been in clinical trials with more than 4,000 patients treated, with no significant success. The reasons for lack of success in patients, in stark contrast to many studies in experimental animals, are not yet clear. There is no consensus for the discrepancy, but possibilities include that to be successful, anti–TNF-a therapy must be very early, as it is in experimental models. In most human patients, sepsis supervenes on grave underlying diseases, and the diagnosis is often delayed. These factors may obviate a positive result. In anti-TNF antibody trials there have been trends toward benefit, noticeable in subset analyses, but no conclusive results. The negative result may reflect on the complex role of TNF-a *in vivo*, with some protective, as well as potentially pathogenic, effects (68,69).

Trials with etanercept, which also blocks LT-a, yielded different results in a sepsis study, as there was a dose-dependent increase in mortality (69). The trial was not repeated, and at present it is not clear what mechanisms were operating. It is possible that LT-a blockade was important; alternatively, it is possible that TNF-a binding to etanercept was unstable, and its release by “ligand passing” may have been detrimental.

Congestive Heart Failure

TNF-a is produced in the heart, and has been found in the failing myocardium. A small trial of etanercept in CCF has been performed, and the results were very encouraging, with multiple parameters of cardiac function statistically significantly improved. The mechanism may be due to the effects of TNF-a on myocyte contractility, and/or in cardiac remodeling (70). Larger studies have recently been reported to be negative.

SAFETY OF ANTI–TUMOR NECROSIS FACTOR THERAPY

During clinical trials, safety is closely monitored. As the populations used in the trials are late severe RA patients, it is anticipated that complications would occur in the trials.

However, the overall safety profile has been good. There were initial scares about a possibly increased incidence of lymphoma. However, this or other cancer risks have not been established in subsequent studies, which have revealed the numbers expected by comparison with populations of this age.

There appears to be a minimal risk of drug-induced lupus, the mechanism of which may be different from that in other drug-induced lupus, as anti–TNF-a appears to be able to make some lupus models (e.g., NZB/W) worse (71). Increases in anti–double-stranded DNA antibodies occur in ~14% of patients given anti-TNF antibody or fusion proteins, but only about 0.2% develop symptoms of systemic lupus erythematosus (SLE), which responded well to discontinuation of the antibody treatment. This tells us that SLE is at a different end of the spectrum of cytokine-dependent disease from RA, and the risk of induced SLE by anti-TNF is not a clinical problem.

There is a concern that infections may be increased in anti-TNF–treated patients. Thus far there are no statistically augmented risks of serious infection during the trials, although increased antibiotic use suggests that there may be a difference. What will happen on a longer time scale of treatment is not clear, but as etanercept has been on the market for more than a year, the infectious risk does not appear to be a major issue. Whether different anti–TNF-a agents have a different propensity to infection is not known. Safety is discussed in more detail in other reviews (3,4). A recent review of TNF therapy has been published (76).

OTHER ANTICYTOKINE THERAPY

Much less has been reported concerning other anticytokine therapy. There have been attempts to block IL-1 and IL-6, with anti–IL-8 only just beginning.

Interleukin-1 Receptor antagonist

This is a receptor antagonist that binds to the signaling membrane type I IL-1R, but does not signal, and it prevents the binding of either IL-1a or IL-1b, and thus acts as

a competitive antagonist. This entity has been used in clinical trials of RA, only one of which is published in any detail.

In this study, 440 patients with active RA of varying duration were repeatedly injected with three different doses of IL-1Ra subcutaneously (30, 75, and 150 mg) daily for 24 weeks (72).

There was a moderate antiinflammatory effect at the higher doses, although not comparable with the effect of TNF-a blockers. The authors have highlighted the results in radiographic progression, which was statistically significant at some but not all doses. These results are puzzling, as there was not a clear dose/response curve or maximal benefit at the highest dose, which would have been expected from its known mechanism of action. Further studies are needed to know exactly how it compares with TNF blockers, and whether there is an additive or synergistic effect with the latter. This possibility has been shown in experimental models of arthritis (73). In this context, it is worth pointing out that IL-1Ra has a very short half-life, and so the results with this agent do not necessarily reflect on the relative roles of IL-1 and TNF in RA.

Anti IL-6 Therapy

Two studies are known. First a murine anti–IL-6 antibody was used, with transient benefit in active RA patients. The immunogenicity of this antibody precluded its long-term use (74).

A humanized antibody to the IL-6 receptor also was reported by Kishimoto et al. (75). This had a good clinical effect, apparently matching that of anti–TNF-a in preliminary trials, although the kinetics of onset was much slower, over 4 weeks rather than a few days. Whether this difference will be consistent in larger trials is not known.

Anti–Interleukin-8

A trial has been reported in psoriasis with a humanized antibody made by Abgenix with benefit.

CONCLUSION

Anticytokine therapy has arrived in clinical medicine, and the omens are that this field will grow very rapidly in future years. This is because the indications for the undoubted success of anti–TNF-a will expand, and many more cytokine inhibitors will be assessed. As cytokines are molecules produced in small quantities in time of stress, they are rate-limiting steps and are thus likely to be good therapeutic targets.

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90 The Use of Intravenous Immunoglobulin

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Intravenous immunoglobulin (IVIG) is used in the treatment of a wide variety of immunologic diseases. There are now five Food and Drug Administration (FDA)-approved clinical indications for IVIG therapy and numerous others currently under investigation. In some cases, the effect on disease progression is very dramatic, but the mechanisms of action and the indications for IVIG therapy remain unclear.

HISTORICAL OVERVIEW

The cold ethanol fractionation method developed in the 1940s by E. J. Cohn and J. L. Oncley (1,2) to separate plasma proteins into stable and biologically active fractions initiated the modern use of immunoglobulin preparations. Cohn fraction II was found to contain more than 95% gamma globulin as well as high antimeasles activity, and the intramuscular administration of small amounts of this plasma protein fraction was found to have prophylactic activity against measles, tetanus, and diphtheria (3,4). As a result, a 16% solution of Cohn-Oncley fraction II (immune serum globulin, ISG) became the first commercially available gamma globulin preparation. ISG proved to be effective in the prevention and attenuation of measles and infectious hepatitis (hepatitis A), and later became indicated for prophylaxis of tetanus and rabies (5,6 and 7). However, there were disadvantages associated with intramuscular administration, including slow and incomplete absorption into the circulation, pain at injection site, and limits in the volume that could be administered (8). Attempts were made to administer larger amounts of ISG intravenously for the treatment of children with severe symptoms of measles, but the children experienced serious reactions (e.g., fever, convulsions, restlessness, chills, and vasomotor collapse) (4,5). Further attempts at intravenous use were discouraged until Bruton's discovery of agammaglobulinemia in 1952 and the emergence of antibody-replacement therapy (9). Again, intravenous injections proved unsuccessful, and Barandun (10) reported that the intravenous administration of gamma globulin induced adverse reactions in 93% of agammaglobulinemic children with severe acute infections compared with 13% of normal children. Intramuscular injections of ISG could be used as prophylaxis in children with agammaglobulinemia, but clinically acceptable doses gradually lost the ability to increase serum immunoglobulin levels effectively and to reduce the occurrence of infection as the patients became older (11). The subsequent discoveries of other primary and secondary antibody-deficiency syndromes during the 1960s solidified the need of a safe high-dose gamma globulin preparation and escalated many efforts to produce immunoglobulin concentrates with intravenous application and few adverse side effects.

Initial experiments were aimed at lowering the immunoglobulin aggregates in intravenous preparations that were believed to activate complement and induce anaphylaxis-like reactions. Enzymatic and filtration techniques were used to prevent immunoglobulin aggregation during concentration yet retain the ability of immunoglobulin to bind antigen. An early intravenous preparation consisted of $F(ab')_2$ fragments of IgG, prepared by pepsin digestion (12). Although this preparation was well tolerated and did not activate complement, it proved to be ineffective without Fc activity and was rapidly eliminated from the circulation by urinary excretion (13). Other modifications included the use of b-propriolactone, reduction and alkylation, reduction and sulfonation, and trypsin (14). These procedures also affected the integrity and the activity of the immunoglobulin (Ig)G molecule. Milder treatments aimed at eliminating impurities and unwanted activities were silica gel adsorption, polyethylene glycol (PEG) precipitation, and diethylaminoethyl (DEAE) Sephadex chromatography. All final preparations were initially at a neutral pH until ISG preparations at pH 4 in the presence of trace amounts of pepsin were found to contain decreased anticomplement activity but to retain normal functional activity (10). Imbach et al. (15) used this IVIG preparation to treat patients with hypogammaglobulinemia and thrombocytopenia and observed that patient platelet counts increased significantly 1 to 2 days after infusion. This important finding led to the successful treatment of children with idiopathic thrombocytopenic purpura (ITP) with IVIGs, and led to attempts to treat a diverse spectrum of inflammatory and autoimmune diseases (16). In 1983 Tenold (17) demonstrated that Cohn-Oncley fractions II and III are stabilized at an acid pH, eliminating the need for further chemical modification or enzymatic treatment. Consequently, several relatively nontoxic preparations of immunoglobulin concentrates have been produced for intravenous use (IVIG) in the United States and are now available for general clinical therapy.

CHARACTERISTICS OF INTRAVENOUS IMMUNOGLOBULIN PREPARATIONS

There are currently nine IVIG preparations available in the United States (Table 90.1) (3,18). Standard IVIG preparations are derived from large donor pools of human plasma, typically consisting of more than 10,000 individual donations. Manufacturers use the Cohn-Oncley fractions of gamma globulin or a modification by Kistler and Nitschmann as the starting material for batches of IVIG. The gamma globulin fraction is further modified by using various combinations of enzymatic treatment (plasmin, pepsin) incubation at low pH with or without low concentrations of porcine pepsin, PEG precipitation, ultrafiltration and diafiltration, and ion-exchange chromatography. Final preparations may be at low pH or a neutral pH, liquid or lyophilized, and all contain one or more of the following stabilizers: maltose, glucose, sucrose, mannitol, albumin, and PEG. The final product is more than 95% IgG with intact Fc effector functions. IVIG preparations contain low but variable amounts of IgA (0.005% to 1.5%) and negligible amounts of IgM, IgD, and IgE. The distribution of IgG subclasses tends to vary according to the characteristics and subclass distributions of the donor pools, but each batch usually contains normal subclass ratios. IVIG preparations also have a broad range of antibody activity for various antigens, again reflecting the population and exposure of the donor pool. In 1981, in an attempt to standardize partially preparations of IVIG, the World Health Organization (WHO) established a list of criteria for production of IVIG (19).

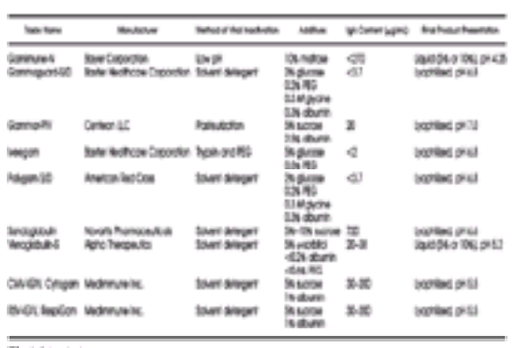

TABLE 90.1. Intravenous Immunoglobulin Products Licensed in the United States

IVIG preparations should

Be derived from human plasma of at least 1,000 donors;

Be free of prekallikrein activator, kinins, plasmin, preservatives, and essentially free of aggregates;

Contain at least 90% intact IgG, with a normal distribution of IgG subclasses;

Maintain biologic activity (e.g., complement fixation and opsonic activity);

Contain a wide spectrum of antibodies to various antigens, including tetanus and measles; and

Be free of infectious agents.

Two hyperimmune immunoglobulin preparations are currently available for intravenous application. Respiratory syncytial virus immune globulin intravenous (RSV-IGIV) contains a high titer of anti-RSV antibodies and is used to induce prophylaxis in pediatric patients susceptible to RSV infections. Cytomegalovirus immune globulin intravenous (CMV-IGIV) is indicated for prophylaxis of CMV associated with patients receiving organ transplants. Presently, only unmodified, intact IVIG preparations are available in the United States. Other preparations outside the United States include several modified preparations as well as one preparation enriched with significant amounts of IgM and IgA (Pentaglobin, Biotest Pharma, GmbH). This combination of the three major classes of immunoglobulins is used in the treatment of bacterial infections and reported to be highly opsonic and more potent in immunomodulatory activity when compared with standard IgG preparations (20,21).

The Safety of Intravenous Immunoglobulin Preparations

Viral Safety

Transmission of viral infection has been a major concern in all preparations of plasma proteins prepared by plasmapheresis of multiple donors. In 1993 and 1994, there was an outbreak of hepatitis C (HCV) from batches of IVIG predominantly but not exclusively prepared by ion-exchange DEAE Sephadex chromatography (22). HCV was transmitted to an estimated 450 patients worldwide. Approximately 115 cases were reported in the United States, leading to the establishment of stringent donor exclusion criteria and a reevaluation of production methods. Traces of HCV particles were detected in the immunoglobulin fraction after the Cohn-Oncley fractionation process, and HCV has been found in lyophilized IVIG products (23,24). The exclusion of donors with anti-HCV antibodies from the donor pool may affect neutralization or precipitation of HCV particles and how they partition during primary fractionation (25). In addition to screening all individual donations and plasma pools for evidence of infection by HBV, HCV, human immunodeficiency virus (HIV)-1, and HIV-2, manufacturers of IVIG now use various viral inactivation and elimination methods. These include the use of solvent detergents, low pH, pasteurization, and trypsin, pepsin, or PEG addition. Risk of viral transmission is now extremely low, and there has not been another reported case of HCV transmission. The cold-ethanol fractionation process has been shown to inactivate HIV, and although HIV-infected plasma had been used in the early 1980s, there have been no reports of HIV transmission by IVIG (26). The overall safety record of IVIG preparations is exceptionally good, but there is no 100% safe product. Some considerations concerning the viral safety of IVIG are listed in Table 90.2.

- HIV-1, HIV-2, HBV, and HCV screening of individual donors, plasma pools, and final preparations
- Donor exclusion criteria
- Viral partitioning during cold ethanol fractionation
- Viral inactivation treatment of Cohn fraction II
- Neutralizing antibodies present during fractionation/purification procedures
- Segregation and containment after viral inactivating procedures
- Surveillance of patients receiving IVIG: follow-up of adverse reactions

HIV, human immunodeficiency virus; HBV, hepatitis B virus; IVIG, intravenous immunoglobulin.

TABLE 90.2. Viral Safety Considerations

There is no evidence that the rare neurologic disorder, Creutzfeldt-Jakob disease (CJD), or other prion-related diseases are transmitted via blood products, although transmission has occurred from tissue transplantation (27). If a plasma donor is later diagnosed with CJD, manufacturers are required to recall the corresponding production lots from the market. A shortage of IVIG has occurred in the United States in parallel with a 10% per year increase in demand, as manufacturing techniques have been made more stringent to avoid viral contamination. Physicians are now reevaluating the necessary and effective conditions for IVIG therapy to conserve IVIG supplies and to meet the rising costs of IVIG therapy.

ADVERSE EFFECTS OF INTRAVENOUS IMMUNOGLOBULIN THERAPY

IVIG is one of the safest biologic products on the market. Severe adverse reactions have been documented, but they occur seldom and may be the result of a preexisting immunologic condition unknown at the time of infusion. Reports of such conditions are crucial to the overall safety of IVIG administration.

Mild and moderate adverse reactions during and after infusion occur in approximately 10% of patients receiving IVIG therapy (28,29). Signs and symptoms usually occur within the first hour of infusion. The most common side effect is headache; other symptoms include chills, facial flushing, dyspnea, back pains, and myalgia. Slight changes in blood pressure and tachycardia also may be detectable. Postinfusion reactions such as headache, nausea, vomiting, diarrhea, fever, and malaise develop within 24 hours and may last from several hours to several days. Suggested mechanisms are possible aggregates and dimers in IVIG preparations (30). Aggregates in IVIG preparations and acute antibody/antibody complex formation in some patients may induce complement activation and the production of the complement anaphylatoxins C3a and C5a. They also may interact with Fc receptors and directly or indirectly stimulate mast cells and polymorphonuclear granulocytes to release their contents, including histamine, prostaglandins, platelet-activating factor (PAF), and other granular mediators (11). Proinflammatory cytokine release also has been suggested, but other studies show that IVIG is able to downregulate proinflammatory cytokine release (31,32). Certain stabilizers in IVIG preparations also may be sources of rare adverse reactions, and preparations may contain low-molecular-weight peptides or compounds that may activate the fibrinolytic system (29). Most of these adverse effects subside with an interruption in therapy followed by a slower rate of infusion. If symptoms disappear, the regular infusion rate may be resumed. Other forms of management include the use of analgesics, antihistamines, or hydrocortisone, which may prevent or reduce the risk of known adverse effects of IVIG in certain disease states. Patient histories are vital to risk management, and initial treatments should be carefully monitored and documented. IVIG therapy should not be administered for the first time in a home-care environment.

Anaphylactic Reactions

True anaphylactic reactions are very rare and almost exclusively occur in patients with low or absent levels of serum IgA. Most of the reported reactions have occurred in patients with common variable immunodeficiency (33). Some of these patients have anti-IgA antibodies, which may react with the variable amounts of IgA in IVIG preparations. These complexes may consist of IgG, IgE, and IgM anti-IgA antibodies and may lead to anaphylaxis within seconds to minutes after beginning an infusion. Symptoms include facial swelling, shortness of breath, and hypotension. Determination of patient serum IgA concentrations before starting IVIG therapy and use of IVIG preparations containing the lowest possible concentration of IgA to treat patients with selective IgA deficiency markedly reduce the risk of anaphylaxis.

Acute Renal Failure

Approximately 40 cases of acute renal failure have been reported in association with IVIG therapy (34). In approximately half of these patients, there has been preexisting kidney disease. It was noted that sucrose, a stabilizing agent in some IVIG preparations, was responsible for renal tubular injuries in affected patients (35). Unlike glucose and maltose, sucrose given intravenously must be enzymatically hydrolyzed to glucose and fructose before filtration occurs at the glomerulus. The resulting increased sucrose exposure of the renal tubules potentially leads to acute renal failure. There are rare reports of fatalities, thus emphasizing the need to

assess renal function before the advent of IVIG therapy and, although it is not an exclusive indicator of renal damage, to monitor serum creatinine levels after administration.

Thrombotic Events

Serum viscosity is increased in IVIG patients up to 0.5 cp above the normal range, and patients with preexisting elevated viscosity levels are prone to potential thrombotic reactions (36). Such conditions as ITP, cryoglobulinemia, hypercholesterolemia, or hypergammaglobulinemias, especially in the elderly, increase the risk of stroke, myocardial infarction, and pulmonary embolism after IVIG treatment. Older patients with vascular disorders such as hypertension or cerebrovascular or cardiac disease are at risk for IVIG-induced thrombotic events, especially myocardial infarction (37). Careful consideration should be given to justify the use of IVIG therapy in such patients. More important, slow infusion rates will reduce the risk of impaired blood flow. It has been reported that pediatric HIV patients with high serum immunoglobulin levels also may be at risk of hyperviscosity syndrome and neurologic complications (38). Recently the FDA ruled that prophylaxis with RSV-IGIV is contraindicated in pediatric patients with cyanotic congenital heart disease (39).

Neurologic Complications

Some patients with a history of migraine are susceptible to migraines after IVIG administration (40). This can be prevented with the use of propranolol. These patients appear to have a risk for recurrent aseptic meningitis as well, but this reaction has not correlated with the IVIG product, the infusion rate, or the daily dose (28). One study reported that six of 54 patients with neuromuscular disorders experienced severe adverse effects including migraine, fever, lethargy, photophobia, painful eye movements, nausea, and vomiting (41). These symptoms continued for 3 to 5 days, and seemed to mimic bacterial meningitis. Cerebrospinal fluid contained increased protein levels and polymorphonuclear pleocytosis, but no bacterial growth was observed. The mechanism of aseptic meningitis is still unclear, and its association with IVIG therapy is equally perplexing. Although the number of reported cases is very low, reduced dosages and extended infusion times (1 g/kg over 24 hours) for patients with a history of migraine are recommended.

Hemolytic Anemia

IVIG is often used for treatment of autoimmune hemolytic anemia; however, there are some conflicting reports of efficacy (42). Paradoxically, it also has been reported that *in vivo* hemolysis and, in rare cases, the onset of hemolytic anemia can follow IVIG treatment (43). IVIG as well as enriched-IgM preparations have been shown to contain antibodies to major and minor blood group antigens and to Rh factors that cross-link red cells and increase the tendency to rouleux formation (29,44). “Immune complex–like moieties” in IVIG preparations also have been reported to activate complement, bind to erythrocyte CR1, and enhance erythrocyte sequestration and opsonization (45,46). Hemolysis has been described in both patients and healthy volunteers, and some patients show a positive Coombs test after infusion.

Dermatologic Effects

IVIG-treated patients have experienced allergic reactions to IVIG, the most common being a transient rash, either urticarial or maculopapular (47,48). One case involving an elderly patient with Sjögren syndrome developed lichenoid dermatitis after IVIG therapy with cutaneous eruptions on the trunk and upper extremities (49). Immunofluorescence of skin biopsies showed nonspecific fibrinogen deposition and significant lymphocyte infiltration, suggesting a type IV cell-mediated allergic reaction. Other dermatologic adverse reactions associated with IVIG treatment include eczema, erythema multiforme, purpuric erythema, and alopecia. The mechanisms for these effects are not well understood.

One case of arthritis has been reported after IVIG administration, and symptoms of severe joint pains have been documented to be transient, lasting approximately 10 days (50). The formation of immune complex aggregates at the site of inflammation may be responsible for these effects. IVIG-induced neutropenia has been reported in several pediatric patients with ITP and appears to be transient as well (51,52).

Vaccine and Serologic Interactions

IVIG preparations can interfere with live viral vaccinations for up to 6 months after IVIG therapy and 9 months after RSV-IGIV prophylaxis (53). This is not a contraindication for IVIG treatment for patients who have been recently vaccinated against measles, mumps, and rubella, but physicians should be aware of this interaction. An increase in serologic titers to viruses also may be seen in some patients receiving IVIG infusions, the result of passive infusion of antiviral and antibacterial antibodies present in preparations.

MECHANISMS OF ACTION

The immunologic effects of IVIG have been studied in many *in vitro* and *in vivo* models. Many of these are outlined later. The positive effects of IVIG in these models are striking, but no simple model integrates these diverse observations.

Fcg Receptor Modulation and Blockade

The rapid increase in platelet counts observed in ITP patients administered IVIG suggests a direct interference with the phagocytic destruction and reticuloendothelial clearance of antibody-coated platelets associated with the disease (54,55). Immunoglobulins interact with Fcg receptors (FcgRs; CD16, CD32, CD64) on phagocytes, lymphocytes, and natural killer (NK) cells and may subsequently influence Fc-mediated functions and the immune response (56,57). Monomers or dimers in IVIG preparations or immune complexes formed after IVIG infusion may interact with FcgRs with varying affinity, potentially saturating receptor sites or altering receptor affinity for disease-related immune complexes (30,32). The most convincing evidence for an IVIG-induced FcgR blockade occurs in patients with ITP. It has been reported that IVIG administration causes a decrease in the rate of clearance of anti-Rh (D)-coated autologous erythrocytes followed by an increase in the number of circulating platelets in adult patients with ITP (58). It was later shown that rosette formation between peripheral blood mononuclear cells (PBMCs) of IVIG-treated patients and IgG-coated erythrocytes is significantly inhibited and that the administration of anti-FcgRIII (CD16) antibodies are able to increase platelet counts in patients with refractory ITP (59,60). Infusions of Fcg fragments also have been demonstrated to induce platelet increases in children with acute ITP, and it was suggested that IVIG may exert its immunomodulatory effects by increasing the level of soluble FcgR in the circulation that is free to bind antibody-coated cells and prevent phagocytic destruction (61). FcgR modulation by IVIG also may play a role in the treatment of certain autoimmune-related neuromuscular disorders [Guillain-Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP), inflammatory myopathies], affecting macrophage-mediated phagocytosis and demyelination (28).

Effects on Lymphocyte Function and Proliferation

Many reports demonstrate a regulatory role of IVIG on lymphocyte function and proliferation. *In vitro* experiments using various models of cellular immune function show suppressive effects by IVIG and both Fc and $F(ab')_2$ fragments of IgG. Intact IgG has been shown to inhibit in a dose-dependent manner the mitogenic responses of human PBMCs to phytohemagglutinin A (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM) (57,62,63 and 64). This suppressive effect is inhibited by the addition of antibodies to the FcgRIII or the Fcg fragment and is reversed if IVIG is removed. IVIG inhibition of IgM production by Epstein-Barr virus (EBV)-transformed B lymphoblastoid cells also has been reported to be dependent on the Fcg fragment. It was suggested that IVIG is able to suppress Ca^{++}-dependent signal transduction via Fcg receptors on the surface of B lymphocytes and interfere with cell differentiation and antibody synthesis (65). Another study shows that $F(ab')_2$ fragments of IgG inhibit IgG and IgM production of spleen cells stimulated with the polyclonal B-cell activators lipopolysaccharide (LPS) from *Escherichia coli* and PWM, whereas no inhibition was observed with Fc fragments (66). This result may reflect the ability of the $F(ab')_2$ fragment of the IgG molecule, perhaps via antiidiotype interactions, to bind B- and T-cell surface receptors (TCRs) and other molecules [CD4, CD5, and human leukocyte antigen (HLA) class I molecules] that influence lymphocyte proliferation and antibody production (57,66). IVIG inhibition of IgE synthesis also has been reported in patients with severe asthma receiving long-term steroids (67).

IVIG induced a 35% decrease in $CD4^+/CD8^+$ lymphocyte ratio and a 50% increase in circulating $CD8^+$ T lymphocytes in hypogammaglobulinemic patients (68). However, there was no increase detected in levels of soluble CD8 antigen in the circulation associated with $CD8^+$ lymphocyte activation. High plasma levels of neopterin strongly correlated with *in vitro* inhibition of PMA- or ConA-stimulated lymphocyte proliferation, indicating a possible IVIG-induced release of monokines that downregulate lymphocyte function and proliferation. Other reports demonstrate that IVIG inhibits cytokine-dependent T-cell proliferation and the generation of cytotoxic T lymphocytes *in vitro*, and a posttranscriptional interference with cytokine production has been suggested (69,70). IVIG supplementation also was shown to affect NK cell cytotoxicity. Women at high risk for recurrent spontaneous abortion (RSA), a condition associated with high NK cytotoxic activity, show significant decreases in NK activity as well as decreased interleukin (IL)-12 and increased IL-10 expression on peripheral lymphocytes in response to IVIG treatment (71). Because these effects are not observed in healthy pregnant women, the authors suggested that these results imply an IVIG-related shift from a cytotoxic T-helper (Th)1 immune response to a more protective Th2 immune response. Whether IVIG can downregulate an overactive immune system and restore balance between Th1 and Th2 immune responses needs further elucidation.

Modulation of Cytokine Production and Release

IVIG therapy may selectively regulate cellular cytokine production and release, and, as a result, numerous proinflammatory and antiinflammatory cytokines may be affected. The ability of IVIG administration to modulate IL-1 production is an important observation because excessive IL-1 production is thought to be detrimental in certain immune-mediated disorders and to contribute to tissue damage in various organs (72,73). For example, IL-1 release decreases to normal levels in children with acute Kawasaki disease treated with IVIG, although patients with chronic symptoms do not respond (74). Other studies demonstrate that IVIG induces monocytic release of IL-1 receptor antagonist (IL-1ra), which may subsequently block IL-1 binding to receptors of various cell types (32,75,76). PBMCs and plasma from IVIG-treated hypogammaglobulinemic patients show increases in IL-1ra production and levels of soluble IL-1 receptor type II correlated with moderate decreases in production of IL-1a, IL-1b, and levels of soluble IL-1 receptor type I (77). This blockade in turn affects the ability of IL-1 to stimulate PBMC production and release of tumor necrosis factor (TNF)-a and other pro-inflammatory cytokines. In patients with GBS, IVIG therapy decreased the circulatory levels of IL-1b and TNF-a, whereas antiinflammatory cytokines were unaffected (78). Clinical improvement was then correlated with a reduction in unbound TNF-a during the acute phase of the disease. Increased TNF-a production also is associated with HIV-1 infection in children, and one bolus injection of IVIG has been shown to decrease TNF-a levels and increase levels of TNF receptors in plasma and LPS-stimulated PBMCs (79). This suppression of TNF-a activity also correlated with a significant increase in circulating CD4 T lymphocytes.

Evidence against an antiinflammatory role of IVIG was observed in patients with common variable immunodeficiency (CVID) (80). IVIG administration induced an increase in IL-2 expression in CD4+ lymphocytes and TNF-a expression in CD8+ lymphocytes without an influence on interferon (IFN)-g expression. These effects were not observed in patients with X-linked agammaglobulinemia (XLA). An IVIG-induced elevation of TNF-a production also has been shown in rheumatoid arthritis patients, but the dose of IVIG was much higher than the dose used in CVID and XLA (81).

Another important *in vivo* study shows that IVIG downregulates transforming growth factor-b1 (TGF-b1) mRNA expression in patients with dermatomyositis (82). TGF-b1 expression is normally high in these patients and may play a pleiotropic role in chronic inflammation and tissue fibrosis. An IVIG-induced decrease in TGF-b1 expression strongly correlated with clinical improvement and repair of the intramuscular cytoarchitecture, but this effect was not observed in patients with inclusion body myositis (IBM).

In vitro reports have shown that IVIG is capable of inducing a wide range of effects on cytokine activity produced by normal and patient PBMCs, depending on the system used. Anti-CD3 activation of normal PBMCs in the presence of IVIG is associated with a decrease in IL-2, IL-10, TNF-b, IFN-g, and TNF-a production, as well as a decrease in IL-2 receptor expression (83). A significant increase in IL-8 production also is observed. PMA/ionomycin-stimulated cells, however, show a reduction of IL-2, IL-3, IL-4, IL-5, IL-10, TNF-b and granulocyte-macrophage colony-stimulating factor (GM-CSF) production in the presence of IVIG, whereas IFN-g and TNF-a are not affected (84). With the bacterial superantigen streptococcal pyrogenic exotoxin-A (SPE-A), IVIG can dramatically inhibit toxin-induced synthesis of IFN-g and TNF-a, even if added 24 hours after the incubation has begun (85). In this model, monokine production (IL-1a, IL-1b, IL-1ra, IL-6, IL-8) is either unaffected or increased. If staphylococcal enterotoxin-B (SEB) is the superantigen, cytokine IL-4 is selectively inhibited, but IFN-g and TNF-a production remain unchanged (86). With an LPS-activated system, IL-6 synthesis is selectively downregulated by IVIG in a dose-dependent response (87). Because IL-6 is required for IgG secretion by B lymphocytes, this negative-feedback mechanism appears consistent with the IVIG-mediated effects on lymphocyte responses that regulate antibody synthesis. It is clear that IVIG can affect numerous cytokine functions, depending on which experimental model is used. However, one important study suggests that the ability of IVIG to modulate cytokine production and lymphocyte function is highly dependent on the accessory cells present in the system studied (88). Stabilizing agents in IVIG preparations also must be considered and have been shown to interfere with mononuclear cell proliferation and contribute to the immunomodulatory effects of IVIG (89).

Inhibition of Complement-mediated Immune damage

IVIG has been shown to inhibit the deposition of activated complement fragments on target cells and tissues *in vitro* and *in vivo*. This effect is thought to be important clinically, interfering with complement-mediated tissue damage associated with disease. Initial studies were performed in two *in vivo* models in the guinea pig. A decrease in the rate of clearance of IgM-coated erythrocytes from the circulation and the prevention of Forssman shock demonstrated the inhibitory effects of IVIG on complement-dependent immune injury (90,91). Several mechanisms have been postulated for the IVIG effect, and in almost all systems studied, IVIG exerts its effects by inhibiting the classic pathway of complement activation (92,93 and 94). It has been proposed that IgG and IgM molecules act as preferred acceptors or “scavengers” for fluid-phase complement components C3b and C4b, thereby inhibiting complement deposition on target membranes (95,96 and 97). No evidence has been presented thus far showing an IVIG effect on the alternative pathway of complement, although C3 is a major component of this pathway (98). A recent report found no influence of IVIG on antibody-independent complement attack on oligodendrocytes, but IVIG did interfere with antibody-mediated complement deposition (99). The authors concluded that IVIG exerts its effects via antibody binding and does not directly interfere with complement activation.

IVIG administration given to nonhuman primates during xenotransplantation has been shown to inhibit complement deposition in porcine cardiac xenografts and to prevent complement-mediated hyperacute rejection (100). One group reported that IgG natural antibodies specific for Gala1-3Gal directly interfere with complement activation mediated by host IgM antibodies bound to the foreign xenograft; however, IVIG preparations contain very low levels of this natural antibody, indicating the importance of other mechanisms (101). Several groups have used a guinea pig-to-rat cardiac transplantation model, each demonstrating that IVIG can successfully delay hyperacute xenograft rejection. Both Fc and $F(ab')_2$ fragments of IVIG have been shown to mediate this effect (102,103 and 104).

In patients with dermatomyositis, IVIG has been shown to inhibit complement deposition and membrane attack complex (MAC) formation on endomysial capillaries *in situ* and to prevent complement-dependent microangiopathy (105). Again, IVIG is hypothesized to form complexes with activated C3, thus reducing the amount of C3 available to bind to target tissues and affecting subsequent C5 convertase assembly and MAC formation. This IVIG-induced effect may extend to other immune disorders, such as GBS and myasthenia gravis, which involve complement-mediated immune damage (106).

Inhibition of Endothelial Cell Proliferation

In vitro activation of healthy umbilical cord endothelial cells was significantly and reversibly inhibited by the presence of IVIG in a dose-dependent manner (107). This effect also was observed with either $F(ab')_2$ fragments or Fc fragments of the IgG molecule. IVIG also downregulated mRNA expression of various adhesion molecules, chemokines, and proinflammatory cytokines induced by TNF-a and IL-1b. This suggests a profound effect of IVIG that may control endothelial cell activation and the generation of microvessels characteristic of inflammation and neoplasia. IVIG has been shown to be beneficial in such vascular disorders as Kawasaki disease, Wegener granulomatosis, or vasculitides in which the endothelium plays a major role in disease pathogenesis (108).

Enhanced Immunoglobulin G Autoantibody Catabolism

Under normal conditions, IgG binds to a protective receptor, FcRn, in recirculating endocytotic vesicles to avoid uptake in phagocytic endosomes and intracellular degradation, thus enabling the intact IgG to return to the circulation. This receptor is located in many tissues, including skin, muscle, and intestinal epithelium, and is highly expressed in vascular endothelial cells, thought to be the predominant site of IgG catabolism (109). It is hypothesized that IVIG administration may lead to a saturation of FcRn receptors on the endothelium and an increase in catabolism of endogenous pathogenic IgG autoantibodies (110,111). This depleting effect may participate in the overall clinical efficacy of IVIG in patients with antibody-mediated immune disorders.

Fas-mediated Apoptosis

In the severe adverse drug reaction, toxic epidermal necrolysis (TEN, Lyell syndrome), keratinocytes undergo Fas (CD95/APO-1)-mediated apoptosis via the lytically active Fas ligand (FasL). IVIG was shown to inhibit keratinocyte death *in vitro*, possibly interfering with Fas/FasL interactions and preventing apoptosis (112). However, another study demonstrated *in vitro* an IVIG induction of apoptosis in human lymphoid cell lines due to anti-Fas antibodies in IVIG triggering the Fas apoptotic pathway (113).

Antiidiotype Modulation

IVIG is prepared from large plasma pools with a wide spectrum of low-titer antibodies that react with various human protein antigens or antigenic sequences on the variable regions of immunoglobulins, or idiotypes. Idiotypic determinants are thought to play a role in autoantibody pathogenesis and thus are potential sites of immunoregulation and autoreactivity control by antiidiotypic antibodies (56,114,115 and 116). Antiidiotypic antibodies have been found during remission in autoimmune diseases associated with pathogenic autoantibodies [GBS, systemic lupus erythematosus (SLE), anti–factor VIII, myasthenia gravis, and anti-fibrinogen autoimmune disease], supporting their role in antibody-mediated immunomodulation (114). IVIG therapy is thought to transfer passively to patients these immune-modulating antiidiotypic antibodies that can react with idiotypic regions of disease-related autoantibodies. The formation of idiotype/antiidiotype complexes may interfere with autoantibody binding to antigen and facilitate Fc-mediated clearance of the autoantibody. These complexes also may bind Fcg receptors on B cells and downregulate

autoantibody synthesis.

IVIG preparations contain a large percentage (up to 40%) of dimeric pairs or idiotypic/antiidiotypic antibody complexes, depending on the number of donors in the plasma pool (28,30,117). Increasing the number of donors increases the percentage of dimeric pairs and the probability of an antiidiotypic antibody in an IVIG preparation specific for the idiotype of a pathogenic autoantibody, although a large donor pool may dilute the activity of a specific antiidiotypic antibody. IVIG has been shown to inhibit the activity of autoantibodies to factor VIII, thyroglobulin, DNA, intrinsic factor, peripheral nerve, neutrophil cytoplasmic antigen (ANCA), platelet glycoprotein IIb/IIIa, and retinal autoantigens in a dose-dependent manner (118,119). The $F(ab')_2$ fragments of IVIG were demonstrated to have high affinity for the variable regions of these autoantibodies, but no antibodies have been found against the allotypic regions of the IgG molecule. Investigators also have found antibodies to the Fc fragment of IgM, cytokines (IL-1a, IFN-a2a, GM-CSF), cytokine receptors, determinants on ab TCR, CD4 and CD5, major histocompatibility complex (MHC) class 1 molecules, and adhesion molecules in IVIG preparations (57,116,120). All of these findings suggest that there are many direct and indirect mechanisms of IVIG immunomodulation that can influence both antibody-mediated and cell-mediated functions, and as a result, may induce a transient or long-term suppression of pathogenic autoantibody production.

One general hypothesis to explain the diverse actions of IVIG is an effect on the immune network regulation of autoreactivity (114,116). It is suggested that autoreactivity is a physiologically normal but highly regulated immune function, and a normal immunoglobulin repertoire downregulates autoimmune reactivity to avoid the occurrence of a pathologic autoimmune disease. Because IVIG antibodies are thought to neutralize some circulating pathogenic autoantibodies in autoimmune patients, it is suggested that this may downregulate lymphocyte production of pathogenic autoantibody clones and induce lymphocyte production of immunoglobulins representative of the immunoglobulin repertoire in an IVIG preparation. IVIG may exert selective pressures that stimulate and restore normal repertoires of circulating immunoglobulin in IVIG patients and an immune network capable of preventing potential pathologic autoimmune conditions.

Clinical Uses of IVIG Therapy

There are numerous suggested indications for IVIG use, including primary and secondary antibody deficiencies, treatment of specific infections, and numerous autoimmune and inflammatory conditions. Few are FDA approved, and most remain at the experimental level. Clearly, more controlled trials are needed to clarify a role for IVIG therapy in many of these disease states. Discussion of individual disease indications is beyond the scope of this chapter; however, a list of FDA-approved indications, generally accepted indications, and those that come from small clinical trials or anecdotal reports is provided in Table 90.3.

TABLE 90.3. Clinical Uses of Intravenous Immunoglobulin

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91 MONOCLONAL ANTIBODY THERAPY

David W. Thomas, Ph.D., and James W. Larrick, M.D., Ph.D.

HISTORICAL PERSPECTIVE

During the past 100 years, antibodies have evolved into a remarkable platform technology for generating therapeutic molecules to benefit human and animal health. In 1998 more than 700 antibodies were in clinical and preclinical development with more than 250 companies worldwide exploring monoclonal antibodies (mAbs) for human therapeutic and diagnostic uses. The recognized potential of antibody therapy was evident early this century when Von Behring and Kitasato received the first Nobel Prize for their demonstration that passive administration of immune sera could prevent or treat certain infectious diseases (264). Based on this pioneering work, passive “serum immunotherapy” using rabbit or horse immune sera was developed and widely used for the treatment of pneumococcal pneumonia, meningococcal meningitis, diphtheria, scarlet fever, measles, tuberculosis, and so on (35,36 and 37). Although serum therapy was remarkably effective for certain conditions, a number of problems (see Table 91.1), primarily serum sickness, limited the extent of its use. With the advent of antibiotics in the 1930s (sulfonamides, 1935) and 1940s (penicillin, 1942), the use of immune serum was largely abandoned. However, toxin-mediated diseases such as tetanus, botulism, diphtheria, and snakebites (271), as well as digitalis drug toxicity (243) still continue to be treated with antibody preparations (278). Despite the recognized potential and usefulness of antibodies, the modern renaissance in antibody therapy awaited the development of mAbs and recombinant DNA technology.

Serum sickness
Immediate and delayed allergic reactions
Lot to lot variation in potency
Need for parenteral administration
High expense

TABLE 91.1. Problems with Heterologous Serum Therapy

DEVELOPMENT OF MONOCLONAL ANTIBODY TECHNOLOGY

Development of Monoclonal Antibodies Rests on a Firm Understanding of Clonal Selection and the Molecular Basis of Antibody Structure and Diversity

Two major scientific advances led to the development of mAb technology. The first was the firm establishment of the Clonal Selection Theory proposed by Talmage (249) and Burnet (29) in the late 1950s, in which it was shown that each B cell produced an antibody of only a single specificity. The second was an understanding of the genetic formation of antibodies and creation of binding diversity from an assembly of discontinuous gene segments by Tonegawa et al. (254), which provided the basis for genetic manipulation of antibodies. Thus the stage was set for the generation of mAbs through the immortalization of B cells, producing an antibody of single antigen-binding specificity. In the first demonstration, Kohler and Milstein (135) created hybridomas by fusing the spleen cells of mice immunized with sheep erythrocytes (SRBCs) with an immortal murine plasmacytoma cell line that grew continuously in culture. A fraction of the immortalized cells were single-cell cloned and shown to produce only monoclonal anti-SRBC antibody. This basic hybridoma technology has since been used to generate mAbs to a vast number of antigens and has led to wide application in discovery and characterization of antigens, diagnostics, and to a new family of therapeutic drugs. Within a few years of the Kohler-Milstein *Nature* article, enthusiasm created by this technology supported the foundation of many antibody-based companies. However, 20 years later, the clinical potential of mAbs is just being realized. Over the past decade, there has been a major renaissance in antibody-based drugs, as evidenced by the recent registration of several important therapeutic antibodies and the large number of mAbs in clinical development.

Recombinant Monoclonal Antibodies

Although murine mAbs provided powerful new tools for antigen characterization, discovery, and diagnostics, their use in humans as therapeutic drugs was limited by several problems (148,149). For example, murine antibodies injected into humans had a relatively short circulating half-life of only about a day as compared with up to 3 weeks for human antibodies. Second, murine antibodies were highly immunogenic, and patients rapidly produced antibody responses against the murine antibody that neutralized its binding activity and caused rapid clearance (143,235). The immune response elicited by the human antimurine antibody antibodies (HAMAs) also was potentially dangerous because, as with heterologous antisera, subsequent injection could lead to severe allergic reactions. For these reasons, molecular engineering of mAbs to manipulate their properties became important for creating antibody drugs better suited for use in humans (24,25).

Ideally, a mAb drug should be nonimmunogenic, have a long-lived *in vivo* retention, and in some cases, retain effector functions for the elimination of the antigenic target (e.g., cancer cells or pathogenic organisms). In this respect, a human-derived antibody would be expected to be superior to a rodent antibody (145). However, generation of human mAbs from immortalized human antibody-producing B cells proved much more difficult than that with rodent cells, and the resultant mAbs were usually of the lower-affinity immunoglobulin (Ig)M isotype. In addition, with B cells derived from normal or immune human donors, the repertoire of antibody-binding specificities was limited, and it was difficult to generate therapeutic antibodies recognizing human targets. Recently the production of fully human antibodies has been achieved by using recombinant DNA technologies and transgenic mice.

The Polymerase Chain Reaction Greatly Facilitates Antibody Engineering

With the elucidation of the genetic organization of antibodies came the use of recombinant DNA technology both to capture rare antibodies and to manipulate the structure of mAbs. Application of recombinant DNA technology has been an important advance in the evolution of mAb drugs for several reasons. First, it was no longer necessary to immortalize the antibody-producing cell, because isolation of an individual antigen-binding B cell was sufficient to isolate the relevant antibody-binding variable (V) region through use of polymerase chain reaction (PCR) (71,189) by using oligonucleotide primers to amplify antibody V regions (130,150,151,195). Second, the V region of mAb-producing B-cell hybridomas could easily be isolated and genetically fused to different immunoglobulin constant regions to produce the desired antibody isotype and subclass. Finally, this method provided the means to capture the entire antibody repertoire in the form of recombinant antibody libraries (281).

Chimeric Mouse/Human Antibodies: Molecular Engineering Reduces Immunogenicity

The creation of chimeric mAbs with an antigen-binding V region from the original murine mAb and a human constant region was the first major advancement in recombinant mAb drugs (186). Human/mouse chimeric mAbs (Fig. 91.1) in part solved some of the problems inherent in fully murine antibodies. They had a longer circulating half-life and reduced immunogenicity because of the human constant regions. Many of the initial chimeric antibodies used the human IgG1 constant region, which also preserved the effector functions of complement fixation and Fc receptor binding. These effector functions were important because many of the initial therapeutic uses of chimeric mAbs were to bind and eliminate tumor cells. However, where therapeutic applications use a chimeric antibody that binds to human cells, these effector functions are potentially destructive. For those uses, chimeric mAbs were generated with a human IgG4 constant region, which has greatly reduced complement activation and Fc receptor binding compared with human IgG1.

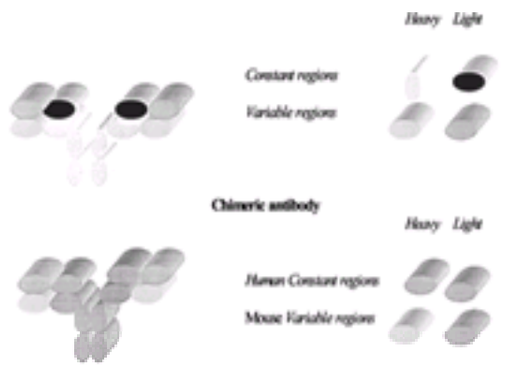

Figure 91.1. Chimeric murine/human antibody created by linking murine antigen-binding variable regions to human heavy- and light-chain constant regions. (See Color Figure 91.1.)

Despite the improved features of chimeric antibodies, immunogenicity generated by repeated administration to patients remained a problem for many antibodies because of the murine V region. In particular, antibody responses were generated against both murine framework determinants and idiotypic determinants contained within the complementarity-determining regions (CDRs) that form the antigen-binding site. The immunogenicity of chimeric antibodies, however, is still determined empirically. For example, IgG1 chimeric anti-IgE antibodies injected into patients with allergic diseases did not generate antibody responses in most patients (53), a situation similarly observed with IgG1 chimeric anti-human immunodeficiency virus (HIV) envelope antibodies (236). A chimeric anti–tumor necrosis factor (TNF-a) antibody (Remicade) has shown dramatic therapeutic benefit and is marketed for treatment of Crohn disease and rheumatoid arthritis (RA) (5,164,188). Antibody responses against this chimeric antibody have occurred in only 6% of the treated patients and have not prevented readministration of chimeric antibody (226). These results demonstrate that chimeric antibodies can be successful drugs and have reduced the problems with immunogenicity of fully murine antibodies. Nevertheless, the relative immunogenicity of chimeric antibodies cannot be unequivocally determined before injection into humans, because all of the rules governing immunogenicity of antibodies remain to be established.

Complementarity-determining Region Grafting Demonstrates That Antigen-binding Activity Can Be Transferred between Antibodies

To reduce further the potential for immunogenicity of chimeric antibodies, Winter (261,281) developed a new technology in which the murine CDRs were genetically grafted onto human V-region framework sequences. Initially, human V-region frameworks were identified that most closely resembled the original murine V region to maximize the chance that the grafted CDRs would be positioned in the proper orientation to retain the original binding specificity. Although this worked well for some CDR-grafted antibodies, in most, the binding activity was substantially reduced. To resolve this problem, key V-region framework amino acids were identified through analysis of antibody crystallographic structures to define additional framework amino acids that were important for orienting the CDRs properly to retain binding (49,124,197). Thus in most CDR-grafted mAbs generated today, several murine framework amino acid residues are retained, and in many cases, the recombinant antibodies exhibit approximately the same binding specificity and properties as the original murine antibody. Antibodies generated by this process are commonly referred to as “humanized” antibodies because the resultant antibody is mostly of human origin and retains only murine CDRs and a few murine framework residues (Fig. 91.2). Because humanized antibodies have murine CDRs, there remains a potential problem with immunogenicity and generation of antiidiotype responses in treated patients. In fact, 60% of RA patients treated with a humanized antibody designated CAMPATH-1 generated a neutralizing antiidiotype antibody response precluding further treatment (272). By contrast, a large number of patients with allergic diseases treated with humanized anti-IgE E25 antibody failed to produce any detectable antibody response (120), and only about 0.1% of patients treated with the humanized anti-HER2 antibody, Herceptin, developed antibody responses (Product insert, Physicians' Desk Reference). Thus, as is the case with chimeric antibodies, the relative immunogenicity of humanized antibodies remains empiric, and predictions about potential immunogenicity in humans cannot yet be made.

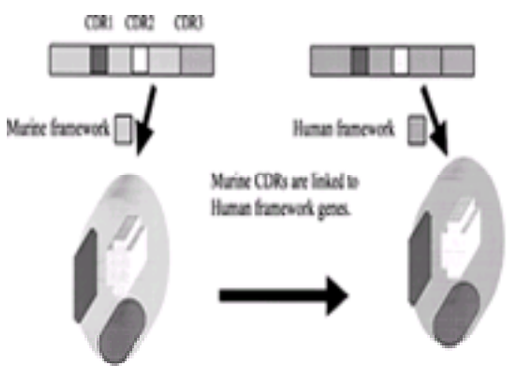

Figure 91.2. Humanization of antibodies using complementarity-determining region (CDR) grafting. CDR loops transfer antigen binding to variable region constructed with human framework regions. (See Color Figure 91.2.)

In part, the problem of potential immunogenicity may be based on the nature of the antigen bound by the administered antibody. Using a transgenic mouse model, Waldman (85) showed that CAMPATH-1 was much more immunogenic when the antigen to which it bound was expressed on the surface of cells. If the CAMPATH antigen was expressed in soluble form, then the immunogenicity of the CAMPATH antibody was greatly reduced. Results from a number of humanized mAbs recognizing a diverse set of targets, which are currently in clinical testing, should yield important information to help define the variables that contribute to the immunogenicity of antibody drugs.

TECHNOLOGIES TO PRODUCE FULLY HUMAN ANTIBODIES

Most mAbs are isolated products of normal antibody responses after immunization of mice with antigen. The repertoire of antibody specificities is determined in part by the strain of mouse immunized and by the type of antigen and adjuvant used. On repeated boosting, the mice produce high-affinity antibodies with improved antigen-binding properties through the process of affinity maturation. For therapeutic antibodies directed against human targets, this has worked well, because mice

recognize many human antigens as foreign, and high-affinity antibodies can be generated. As mentioned before, the use of human B cells for mAb production was limited mainly to those antigens to which the donor was immune, and it was difficult to generate high-affinity antibodies (150,151). It was therefore very challenging if not impossible to produce therapeutic human antibodies against a large number of targets, particularly if the target was a human antigen. Two technologies were developed to overcome these limitations and to generate fully human mAbs: transgenic mice and phage-displayed antibody libraries.

Transgenic Mice Producing Human Antibodies

Genetically engineered mice have been generated to produce human antibodies instead of murine antibodies (Fig. 91.3) (28,76,89,118,159,176). Major sections of the chromosome encoding the human antibody genes have been introduced into the germline of mice by using yeast artificial chromosomes (192). In addition, the endogenous mouse loci have been knocked out by using site-specific recombination and embryonic stem cell technology. These mice can be immunized and their human antibody-producing spleen cells immortalized by conventional hybridoma methods. Thus far, these mice have generated antibody responses against most antigens with which they have been immunized, including human antigens, such as CD4. Apparently the human antibody responses are regulated in a near-normal manner as affinity maturation takes place, generating high-affinity antibodies equivalent to those produced in unmodified mice (76,90). The clinical testing of the human antibodies from engineered mice is just beginning and includes an anti-CD4 antibody for treatment of RA (Medarex.com) and an anti–interleukin (IL)-8 antibody for treatment of psoriasis (Abgenix.com). Initial reports indicate that these human antibodies have not elicited an antiantibody response in the treated patients.

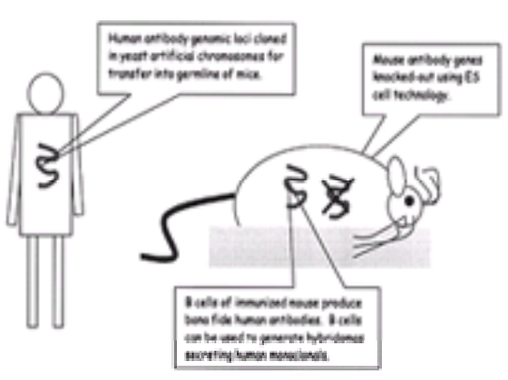

Figure 91.3. Human antibodies generated from transgenic mice.

Human Antibody Libraries Expressed on Filamentous Phages

Another approach to generation of human antibodies has taken advantage of a technology called phage display (Fig. 91.4), in which single-chain antibodies or F(ab) fragments are expressed on the surface of bacteriophages, which can be propagated by infection of host bacteria (12,13,109,167,171,172,281). To generate a library of human single-chain antibodies, the antibody V regions from B cells isolated from normal human donors are cloned into the bacteriophage. In this way, each phage expresses a single antibody-binding specificity and essentially replaces the B cell as the source of the monoclonal specificity. Instead of immunization to generate antigen-binding antibodies, the library of single-chain antibody–expressing phages is selected based on antigen binding (91,201). Those phages binding to the antigen are isolated and propagated in bacteria. Several rounds of antigen selection followed by propagation of binding phages yields human single-chain antibodies specific for the selecting antigen. The single-chain antibody V regions can then be genetically engineered into a complete human antibody. However, unlike immunization with antigen, the phage-expressed antibodies do not undergo affinity maturation and often exhibit lower binding affinities. To remedy this, mutational approaches have been taken to substitute amino acid residues in the CDRs to select for those mutations that lead to better antigen binding (206). This approach has been successful, and the antibody affinities improved as much as 1,000-fold over the starting antibody (15,286). Another way to generate the antigen-binding repertoire by using phage display is to prepare a library of antibodies in which the CDRs have been mutated before antigen selection. This is a “semisynthetic” approach to generate novel binding specificities of higher affinity through creation of new CDRs that may never occur naturally (14).

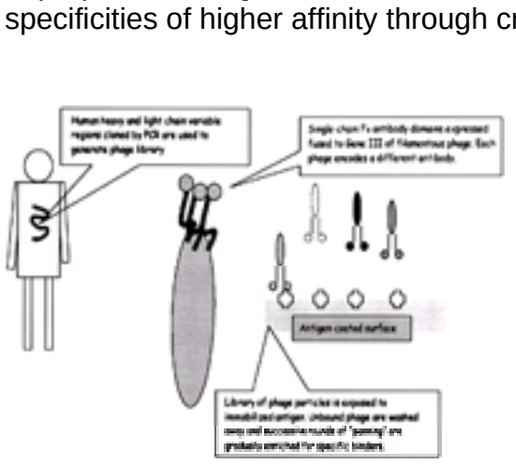

Figure 91.4. Human antibodies generated by using phage display technology.

Despite the potential to reduce the immunogenicity of antibodies by making them fully human, theoretically it is still possible that they could elicit neutralizing antiidiotype antibody responses. The reason for this is that the newly created human antibody contains novel joining regions that can be immunogenic because they are essentially “foreign.” More extensive testing of human antibodies in clinical trials will determine the extent to which fully human mAbs have solved the problem of immunogenicity.

Deimmunization: Understanding of Antigen Presentation Can Reduce Immunogenicity

The immunogenicity of therapeutic antibodies is controlled by helper T lymphocytes. In the absence of helper T cells that recognize peptides derived from the therapeutic antibody, those B cells whose surface Ig antigen receptors recognize the therapeutic antibody epitopes are not sufficiently activated to generate an antibody response. Thus to reduce the immunogenicity of therapeutic antibodies most effectively, the helper T-cell epitopes should be removed. This can be accomplished through several approaches that rely on knowledge of antigen processing by antigen-presenting cells (APCs) (98,122). Helper T cells recognize only antigens presented on the surface of the APC in association with a type of major histocompatibility antigens, termed class II human leukocyte antigens (HLAs). Class II HLA molecules consist of heterodimers of two transmembrane glycoproteins that form a groove at the top of the extracellular structure that binds a peptide containing the antigenic epitope recognized by the T-cell receptor. The loading of HLA molecules with peptides takes place inside the APC within endosomes that contain proteolytic fragments of the ingested antigen. In the case of a therapeutic antibody, the antibody is internalized by the APC, proteolytically cleaved to generate a series of derivative peptides, some of which contain the appropriate amino acid motifs to be bound in the HLA groove. The peptide-loaded HLA molecules are then transported to the APC surface to be recognized by a T cell bearing the receptor specific for that HLA/peptide complex. To render a therapeutic antibody nonimmunogenic for T cells, it can be engineered to change (a) those amino acids that are bound by the HLA proteins, (b) those amino acids serving as contact residues for the T-cell receptor, or (c) those amino acids that serve as the cleavage sites for the proteases creating the immunogenic peptides. The databases on which these strategies are based are still being compiled, particularly for determination of the structure of peptides recognized by T cells and bound to different HLA alleles. A Scottish company, Biovation Ltd., has used this information to reduce the T-cell epitopes of therapeutic antibodies based on the composition of known T-cell antigenic peptides and their HLA-binding motifs. Their strategy is to first remove B-cell epitopes through a process of “veneering,” as described by Padlán (198), in which potential B-cell epitopes are identified from structural information, and such regions are modified to eliminate the epitope. The antibody H and L variable-region sequences are then “threaded” through numerous HLA alleles, and the potential HLA-binding peptides are identified. Site-directed mutagenesis is then used to change residues responsible for HLA binding. This and related procedures (169) represent a rational design to eliminate immunogenicity of proteins. Although animal studies of such deimmunized antibodies are promising, these reengineered antibodies have yet to enter clinical trials.

MONOCLONAL ANTIBODIES: A FLEXIBLE DRUG PLATFORM

MAbs are an exceptionally flexible platform (Table 91.2) allowing a great deal of novelty in creating new drugs. As a result, mAbs have become an exciting new drug

modality for treatment of cardiovascular and infectious diseases, cancer, and allergic and autoimmune diseases, in addition to prevention of rejection of transplanted organs. The features that make mAbs such attractive drugs are their exquisite specificity in target binding and their limited inherent toxicity. In addition, mAbs can have a half-life of up to several weeks in circulation, requiring only infrequent treatment, and yet have good penetration into many body tissues.

Half-life (minutes to weeks)
Biodistribution (intravascular vs. extravascular)
Effector functions (via cells, complement, clotting, etc.)
Valency (monovalent vs. bivalent vs. multivalent)
Binding properties (affinity)
Carriers of toxins and radionuclides
Diverse structure (antibody fragments, fusion proteins, bispecificity, etc.)

TABLE 91.2. Flexible Properties of Antibodies

Genetic manipulation of mAbs can generate a variety of forms ranging from small monovalent fragments (22) to unique proteins that use the antibody constant region fused to other receptors, ligands, or other antibody fragments. MAb drugs either can be inhibitory through neutralization of the biologic activity of the target antigen, or can be agonists that cause cellular activation, acting as growth and differentiation factors. These extraordinarily diverse features allow creation of new antibody-based drugs to treat diseases in quite novel ways unmatched by small organic compounds.

THERAPEUTIC ANTIBODIES: DIVERSE TARGETS

Too many antibodies are in preclinical and clinical development to be covered in detail here. What follows is a sampling of the major classes of antibody therapeutics.

Anti–infectious Disease Antibodies

Many acute infectious diseases are successfully prevented by vaccination that generates protective antibodies and are therefore good targets for passive antibody therapy. Antibodies can directly neutralize pathogens through their high-affinity variable regions, or activate and coordinate other host defense mechanisms such as enhanced phagocytosis and complement activation through their constant regions. As long ago as the 1930s, convalescent human sera were shown to prevent mumps and measles virus infections in children (156). Recently, preparations of human polyclonal antibodies specific for rabies (146), hepatitis B (e.g., H-BIG, NABI), varicella-zoster, respiratory syncytial virus (RSV; e.g., RespiGam, MedImmune, Gaithersburg, MD) and cytomegalovirus (CMV; e.g., CytoGam, MedImmune) have been developed for treatment and postexposure prophylaxis. Pooled intravenous immunoglobulin preparations (IVIG) often contain protective titers of antiviral and antibacterial antibodies, which are effective for passive immunotherapy (e.g., Gamimune, Bayer) (23,66,275). For example, chronic red cell aplasia caused by persistent infection with parvovirus B19 has been successfully treated with IVIG (187), and human serum globulin is given to travelers to prevent hepatitis infection (240). Because highly specific antibodies can be generated rapidly, they are the preferred therapeutic modality for new, exotic, and emerging diseases. For example, a high-titer Ebola virus–neutralizing polyclonal horse serum was reported that could have therapeutic potential (117).

One recent success of mAbs to prevent RSV infection has been the clinical application of a humanized anti-RSV mAb (246) marketed as Synagis. Additional mAbs are in phase II studies for protection against infections with hepatitis B virus (PDL.com), cytomegalovirus (9), and varicella-zoster (116). Several clinical trials have been performed with mAbs directed against the envelope of HIV (39,236,289). However, the rapid mutation of the HIV envelope and the inability of these antibodies to react with all HIV clades limited the success of this approach. This result illustrates one problem in using mAbs directed against pathogens; it is easy for the epitope to modulate/mutate such that the mAb no longer binds. This is why polyclonal antibodies have an advantage in preventing infectious diseases. In the future, many infectious diseases will likely be treated with a mixture or “cocktail” of mAbs. Such “oligoclonal” drugs, comprising mAbs recognizing several different antigens, will be more effective at preventing infection and more likely to limit the emergence of resistant organisms. A recent example of this concept used anti-HIV monoclonals (10).

As noted, before antibiotics, several toxin-mediated bacterial diseases, such as tetanus and diphtheria, were successfully treated with immune sera (47,104,129). Recently, toxin-mediated diseases such as botulism and diarrhea caused by overgrowth of *Clostridium difficile* in hospitalized patients treated with antimicrobial agents have been targeted with mAbs (84). In the 1980s, numerous attempts were made to attenuate the deleterious effects of endotoxin released by gram-negative bacteria contributing to the sepsis syndrome. At least three companies developed and carried out phase III clinical trials of antiendotoxin/lipopolysaccharide mAbs in the early 1990s (270). The outcome of these trials was disappointing because of a failure to appreciate the complexity of the sepsis syndrome (40). Nevertheless, using mAbs to treat toxin-mediated diseases will likely remain an important bastion for antibody therapy because of its high specificity and ability to neutralize toxins rapidly that is unmatched by most other drugs.

In the area of infectious diseases, antibodies are expected to continue to provide potent therapeutics to neutralize pathogens. The development of low-cost production systems (i.e., transgenic plants or animals) combined with an improved understanding of microbial pathogenesis and genomics to provide novel targets will promote the application of mAb technology for prophylactic and therapeutic uses.

Antibodies for Cancer: Magic Bullets?

Unlike the application of mAb therapy to infectious diseases, in which the history of vaccination and passive immunotherapy provides reason to expect success, application of mAb technology in oncology rests on a less secure foundation. Based on the perceived promise of an mAb-based magic bullet that would specifically identify and eliminate cancer cells, an enormous amount of work has been devoted to generation and characterization of anticancer antibodies over the past 25 years (74,274). Indeed, as of this writing, anticancer antibodies are the largest group of therapeutic mAbs under development.

Antitumor antibodies have been generated to a number of targets, most of which reside on the cancer cell surface. In theory, this should be an excellent therapeutic approach based on the capacity of autoreactive antibodies to bind self-tissue antigens and mediate tissue damage through antibody-dependent cellular cytotoxicity (ADCC), complement, or natural killer (NK) cell activation. Autoimmune diseases would in this light predict that the “firepower” of the humoral immune system might successfully destroy tumor cells. However, positive clinical results have been limited by antigenic variation in tumors (174,217), lack of identification of suitable tumor-specific antigens, and poor penetration into solid tumors (191). Despite these limitations, there have been some major recent successes in treatment of cancer with mAbs, two of which were recently approved for clinical use, Rituxan and Herceptin (Table 91.3).

Name	Molecular Target	Clinical Target	MAb Form	Company
Orthoclone®, OKT3	CD3	Organ transplantation	Murine	Ortho Biotech
ReoPro®	GP IIb/IIIa	Percutaneous coronary intervention; chronic unstable angina	Fab fragments of Murine Mab	Centocor/Lilly
Synagis®/Palivizumab	65-kD F protein	Respiratory syncytial virus	gp24 humanized Mab	MedImmune
Zenapax®/Daclizumab	IL-2 receptor, CD25	Organ transplantation	gp24 humanized Mab	Protein Design/Roche/Hoffmann-La
Rituxan®/Rituximab	CD20	Non-Hodgkin's lymphoma	gp24 Chimeric Mab	IDEC/Genentech/Roche
Herceptin®/Trastuzumab	ErbB2/Her2	Breast Cancer	gp24 humanized Mab	Genentech
Remicade®/Infliximab	TNF alpha	Crohn's disease; Rheumatoid arthritis	gp24 Chimeric Ig Mab	Centocor/Lilly
Panorex®/Edrecolomab	EpCAM	Post-operative colorectal Ca (USA BLA not active filed)	Murine Mab	Centocor/Glaxo Wellcome

TABLE 91.3. Therapeutic Monoclonal Antibodies: A Major Class of FDA Approved Products

Growth factors and their receptors are promising targets for antitumor antibodies (27). The anti–breast cancer Herceptin mAb binding the erb-b2 (HER2/neu) growth-factor receptor is one such example. Treatment of patients with advanced breast cancer with Herceptin leads to objective responses in a modest fraction of patients with overexpression of the HER2/neu oncoprotein (approximately 30% of breast cancer patients are HER2/neu positive). As first-line therapy combined with anthracyclines/cyclophosphamide, Herceptin increased 1-year survival of breast cancer patients from 68% to 79% (87). Other examples of this approach include antibodies blocking epidermal growth factor (EGF) and its receptor (285) and anti–gastrin-releasing peptide (GRP) antibodies that inhibit this autocrine growth factor for small-cell lung cancer cells (128). Positive phase II studies have been reported for antibodies neutralizing each of these targets.

Another approach to treatment of solid tumors targets angiogenesis, the formation of new blood vessels. Because angiogenesis plays an important role in the metastasis and growth of tumors, a number of antiangiogenic approaches are currently being tested with both small-molecule drugs and mAbs. One such antibody in phase II clinical development is directed against vascular endothelial cell growth factor (VEGF), which plays a major role in tumor neovascularization (212).

Efficacy has been seen in clinical trials using antibodies that target tumor cell surface antigens such as B-cell idiotypes, CD20 on malignant B cells, and CD33 on leukemic blasts (4,153). Rituxan, an unconjugated anti-CD20 antibody, induced partial and complete responses in up to 50% of patients with advanced, indolent non-Hodgkin lymphoma. When such antibodies are conjugated to radionuclides, overall response rates increase, and two different radionuclide-conjugated (one with ^{131}iodine and ^{90}yttrium) anti-CD20 antibodies are currently in phase III clinical testing (123). Conjugates composed of anti-CD33 antibodies and the potent chemotherapy agent, calicheamicin, have shown promising activity in patients with relapsed or refractory acute myelogenous leukemia (6). In Europe an anti-EPCAM antibody, Panorex, has been approved for adjuvant therapy for colon cancer, and phase III trials of this antibody are under way in the United States (210,276).

These exciting results in mAb treatment of cancer provide a basis for further refinement of the existing approaches to develop new antibody-based cancer therapy strategies (125,131,207). Results from the Human Genome Project combined with gene-chip profiling of cancer cells will improve future target selection. A multitude of other mAbs have been generated for use in flow cytometry, histopathology, and diagnostics, and these have greatly contributed to our understanding of cancer cell biology and pathogenesis. Based on past performance, mAbs will increasingly become an important therapeutic modality in oncology.

Immunomodulatory Monoclonal Antibodies

In addition to using mAbs for the specific treatment of infections and cancer, many antibodies are being developed to modify immune responses. Indeed, the first mAb approved for clinical use, Orthoclone (OKT3), is an antibody directed against T-lymphocyte antigen CD3 that abrogates immune rejection of tissue grafts (44,112,279). OKT3 may inhibit T-cell responses by cellular depletion as well as by suppression of T-cell activation (242). Sometimes OKT3 stimulates T cells to release cytokines, causing what is called the “cytokine release syndrome” (265). Several humanized versions of anti-CD3 antibodies that show reduced toxicity are in clinical development for transplantation and autoimmune disorders (52,82). Several other antibodies inhibiting T-cell activity are in clinical development, including a humanized anti-CD2 antibody to prevent transplant rejection (214) and anti-CD4 antibodies to treat autoimmune diseases (287). Clinical testing of some anti-CD4 antibodies was problematic because of lack of efficacy and depletion of CD4-positive T cells (34,136,182,183,221,259,260). Despite this setback, a fully human anti-CD4 antibody is currently in phase I/II trials for RA (Medarex.com).

Whereas all of the antibodies described earlier directly prevent T-cell activation, several other antibodies are directed against cell-surface antigens to prevent cellular migration or to block cellular interactions. Antibodies directed against adhesion structures reduce the emigration of cells out of circulation into the tissues and thereby inhibit inflammatory responses. Examples of this class of antibody include humanized antibodies in phase II clinical trials directed against CD11a for treatment of psoriasis (Genentech/Xoma), anti-CD18 antibodies to prevent reperfusion injury (Leukosite), anti-VLA4 antibodies to treat multiple sclerosis (Athena/Elan), and anti-intercellular adhesion molecule (ICAM) antibodies to prevent graft rejection (228). CD40/CD40 ligand-mediated cellular interactions provide a key immunoregulatory pathway that is essential for production of IgG, IgA, and IgE antibodies and for regulation of cellular immune responses (31,62,69,144). Several humanized antibodies directed against CD40 ligand are in phase II clinical trials for treatment of autoimmune diseases, including systemic lupus erythematosus, and to prevent transplant rejection (Biogen, IDEC), and an inhibitory anti-CD40 antibody has recently entered a clinical trial for treatment of Crohn disease (v).

Soluble protein cytokines or their receptors represent yet another group of promising therapeutic targets for antibody therapeutics. Several cytokine-specific antibodies have already been approved for clinical use including the chimeric anti–tumor necrosis factor-a antibody, Remicade, for treatment of severe Crohn disease and RA (11,163,164,229,258). Zenapax, a humanized mAb directed against the IL-2 receptor, has been approved for prevention of transplantation rejection (18,262,267) and is in early clinical trials for treatment of uveitis and psoriasis (67). Other cytokine- or chemokine-directed antibodies in clinical testing include a humanized anti–IL-5 antibody for treatment of asthma (Schering Plough/CellTech), which blocks the activation and influx of eosinophils into the lungs, and a fully human anti–IL-8 antibody for treatment of psoriasis (Abgenix.com).

One exciting new mAb drug for treatment of allergic asthma and rhinitis is the humanized mAb called E25 directed against human IgE (61,120). The concept behind this novel therapeutic strategy is to prevent IgE from binding to the high-affinity IgE receptors expressed on the surface of mast cells and basophils. This therapeutic strategy has been successful in preventing the symptoms of allergic rhinitis in patients with seasonal allergies (38), as well as in decreasing the disease symptoms of patients with chronic moderate to severe allergic asthma (26,73,178).

Ironically, products of the immune system, mAbs, are emerging as a major class of drugs to regulate or modify immune responses for therapeutic benefit. A large number of antibody-based products are in development to attenuate immune responses to treat diseases caused by inappropriate or unwanted immune responses, as well as to stimulate the immune system to protect against infection and cancer (106,107,248). We anticipate that immunomodulation will be an increasingly important niche for antibody drugs in the future. Difficulties in discovering small-molecule drugs that inhibit protein/protein interactions involved in cellular recognition or cytokine/receptor binding that are key components of immune responses support development of antibody-based drugs.

Antibody Treatment of Cardiovascular Diseases

In contrast to the use of antibodies to treat infectious diseases, many mAbs are being used in “innovative” roles that are very different from their traditional protective function. This is perhaps best illustrated by the success of mAb therapy to treat cardiovascular diseases. The most widely used and commercially successful antibody on the market today is ReoPro, a monovalent chimeric mAb fragment (Fab) that prevents platelet aggregation and clotting. ReoPro can successfully prevent complications after angioplasty, acute myocardial infarctions, and unstable angina (33,168,170,180,251,255). Several other humanized antibodies are in clinical trials to prevent ischemia/reperfusion myocardial damage and stroke by interfering with the immigration of inflammatory cells into the site of tissue damage (e.g., anti-CD18, anti-ICAM, anti-CD11a antibodies). Because activation of the complement system may be one of the early triggers of cardiovascular tissue damage, another approach to treating ischemia/reperfusion injury and complications after surgery involving cardiopulmonary bypass is to block the production of proinflammatory peptides released by complement activation. A humanized single-chain antibody directed against complement component C5 has shown promise in phase II clinical trials for treatment of complications after cardiopulmonary bypass and is in early clinical trials for treatment of acute myocardial infarction as well as inflammation associated with RA and lupus (77,253,257,258). In summary, there is a plethora of clinical applications of mAbs. A subset of these is summarized in Table 91.4.

TABLE 91.4. Representative Antibodies in Preclinical and Clinical Development

NOVEL COMPOSITIONS OF ANTIBODY-BASED DRUGS

Bispecific Antibodies

Totally new binding moieties exhibiting two or more distinct antigen-binding specificities can be created with recombinant DNA technology (230). Two different mAbs or their Fab fragments can be chemically coupled, or two single-chain antibodies can be genetically linked to form a so-called diabody. Theoretically these bispecific constructs can create novel biologic functions. Many bispecific antibodies are being developed to treat a wide range of cancers (59,108,134,181,233,237,241). A bispecific construct being developed by Medarex has one binding specificity directed against the Fcg receptor I (CD64) expressed on the surface of macrophages and monocytes. Binding of antibody to CD64 results in cellular activation for ADCCs and other properties that help destroy cancer cells (45). To target this activity specifically against the tumor cells, the humanized anti-CD64 Fab is coupled to the Fab of a second antibody directed against either the EGF receptor (EGF-R, MDX-447) or HER2/neu (MDX-210), which are overexpressed by some tumor cells. Targeting the tumor cells expressing EGF-R or HER2/neu thus focuses the anti-CD64 to bind and activate macrophages for cancer cell destruction (56,211,215,222). Early clinical studies of MDX-447 in cancer patients showed tumor stabilization in a fourth of those treated. A phase II clinical trial in renal cell carcinoma with MDX-447 showed antitumor effects in about 40% of the treated patients, with some patients showing a 50% reduction in tumor volume. In a phase II trial of patients with breast, ovarian, colorectal, or prostate cancer, MDX-210 also showed antitumor activity.

A number of additional bispecific constructs have been created to specifically activate T cells at the tumor site (32,140). In most of these constructs, an anti-CD3 antibody known to trigger T cells is linked to an antibody directed against antigens overexpressed by the cancer cells. One construct using anti-CD3 linked to an anti–erb-B2 (HER2/neu) antibody stimulated Th1 cells and showed antitumor activity against human breast cancer cells implanted into mice (194). Anti-CD3 also has been coupled with anti-CD19, a B-cell antigen, for treatment of malignant lymphomas (132) and to anti-CD30 for therapy of Hodgkin lymphoma (100). In one study, immunodeficient mice were implanted with Hodgkin lymphoma cells and human T cells. When these mice were treated with bispecific constructs of anti-CD3 and anti-CD28 antibodies coupled to anti-CD30 antibodies, human cytotoxic T lymphocytes were generated, and these cells specifically infiltrated the sites of the tumors (16). In a related approach, NK cells were targeted by using a bispecific construct comprising anti-CD16 and anti-CD30 antibodies. Treatment of mice implanted with Hodgkin lymphoma cells with the anti-CD16/anti-CD30 diabody caused tumor regression (8). A phase I/II clinical trial used a murine bispecific anti-CD16/anti-CD30 construct, and antitumor activity was observed in some patients with Hodgkin lymphoma; however, the HAMA response precluded further treatment (220).

An alternative to using bispecific antibodies to focus cellular anticancer activity on the cancer cells is to use these agents to target radiotherapy to the tumor cells (113,266). In a phase I/II clinical trial, patients with small-cell lung carcinoma (SCLC) who had experienced a relapse from chemotherapy were initially treated with intravenous injection with a bispecific antibody comprising an anti–carcinoembryonic antigen (CEA) antibody coupled to an antihapten (diethylenetriaminepentaacetic acid, DTPA) antibody. Because CEA is overexpressed by many SCLC cells, the anti-CEA antibody localized on the SCLC cells. After several days to allow the unbound bispecific antibody to be cleared, leaving mainly SCLC-bound antibody, the patients were injected with radioiodinated DTPA that bound to the SCLC cell surface through the anti-DTPA. Initial results of this study were reported to be encouraging (266).

Although the primary clinical application of bispecific antibodies to date has been cancer, this type of drug offers a wide range of exciting possibilities for treatment of other diseases. Not only can bispecific antibodies mediate cellular interactions that would not normally occur, but they can also be used to modify cellular activities. As the biology of interacting receptors on the cell surface becomes better established, unique opportunities will arise to use bispecific antibodies to alter cell biology by creating novel receptor interactions. This is an exciting application of antibody technology to create new biology to treat diseases.

Immunoglobulin Fusion Proteins

Fusion of the antibody constant region to ligands or receptors can create multivalent binding molecules with improved *in vivo* bioavailability and biologic activity (17,41,101,105,110,119,158,204,288). One of the earliest antibody fusion proteins tested clinically was a construct comprising a human IgG1 constant region fused with the extracellular domain of CD4, which retained binding activity for HIV (42,83). The rationale for this therapeutic was to provide a multivalent form of soluble CD4 that would act as a decoy receptor to bind HIV virions to prevent viral spread. More recently, Progenics Inc. has tested a similar construct in HIV-infected patients in a phase I clinical trial reporting a dose-dependent reduction in HIV load (Progenics.com).

An immunoglobulin fusion protein designed to inhibit T-cell responses contains the immunoglobulin constant region of IgG1 fused to CTLA4 called CTLA4Ig. Both CD28, which provides a major T-cell costimulatory signal, and CTLA4 bind to B7.1 and B7.2 on APC. The soluble CTLA4Ig fusion protein binds to the APC B7 molecules to prevent CD28-mediated T-cell activation. A large number of preclinical models using CTLA4Ig have shown it to be very effective in blocking antibody- and cell-mediated immune responses, including those causing autoimmune diseases (55,115,205,232). CTLA4Ig also has been widely used in murine and primate organ transplantation studies, in which it has been shown to delay organ rejection, which was even more effective when combined with an anti-CD40 ligand mAb (69,133,152,154,202). T-cell anergy was apparently achieved in a clinical trial to prevent graft-versus-host disease (GVHD) in bone marrow transplantation (96). In this study, donor bone marrow was cultured *ex vivo* with recipient leukocytes in the presence of CTLA4Ig. CTLA4Ig blocked the second costimulatory signal given during recognition of the recipient antigens on the APC, resulting in anergy of the T cells in the donor bone marrow. The “anergized” bone marrow was then transfused into the recipients, who showed a dramatic long-lived reduction or elimination of GVHD. Recently, in a phase I clinical trial in patients with severe psoriasis, CTLA4Ig caused a dramatic reduction in skin lesions (1).

Another T cell–directed immunoglobulin fusion protein contains the human IgG1 constant region fused to the extracellular region of leukocyte function–associated antigen (LFA)-3 that binds CD2 expressed by T cells (175). Because CD2 can be costimulatory for T-cell activation, the LFA-3Ig was thought to block T-cell activation through interference with the CD2 activation signal (126,179). However, further studies showed that the mechanism by which the LFA-3Ig construct inhibits T-cell responses is through depletion of T cells through ADCCs (48,165). A completed phase II clinical study of LFA-3Ig in psoriatic patients demonstrated a dramatic reduction in psoriatic skin lesions (Biogen.com).

TNF-a is a major inflammatory cytokine that binds to either of two specific receptors expressed by numerous cell types. Immunex has created Enbrel, an Ig fusion protein containing the extracellular domain of the low-molecular-weight TNF-a receptor. Enbrel inhibits TNF-a–mediated inflammation and has been approved for the treatment of RA. Treatment of RA patients with twice-weekly subcutaneous injections of Enbrel caused a dramatic reduction in disease symptoms that was sustained for up to 6 months (86,121,184,185,273). Enbrel is in clinical trials for a number of additional clinical indications, including Crohn disease (65).

The clinical successes described demonstrate the therapeutic power of antibody-based drugs using properties of the flexible antibody drug platform: valency and improved bioretention. In addition, Ig fusion proteins with receptors and ligands are providing an important laboratory reagent for investigating biologic pathways. Some of these new antibody-based fusion proteins will have tremendous therapeutic value as novel clinical applications emerge.

Immunotoxins: Antibody-directed Warheads

Nature has provided a number of potentially lethal cellular toxins, such as the plant toxins ricin and gelonin, and bacterial toxins *Pseudomonas* exotoxin and diphtheria toxin. The question is how to target these proteins specifically to cancer or virally infected cells. To accomplish this goal, most of these toxins have been modified to destroy their ability to bind independently to cells, and then chemically coupled or genetically fused to mAbs to provide specific cellular delivery. Although a large number of laboratory and animal models have shown promising results with this approach (7,19,20,60,78,79 and 80,158,199,216,227,247,252,256), successful treatment of cancer in humans has thus far been limited (2,43,70,75,142,196,234). In one recent clinical trial, patients with refractory hairy cell leukemia were treated with an anti–IL2 receptor–binding fragment (Fv) fused with a modified form of *Pseudomonas* exotoxin (137,138). Of the four treated patients, one showed complete remission, and the others had a dramatic reduction in the numbers of circulating leukemia cells. Encouraging results also were found in a phase II trial in which patients with B-cell non-Hodgkin lymphoma were treated with a conjugate of a B cell–specific antibody, anti-B4, and a modified ricin toxin (94,190). However, this same immunotoxin conjugate was less successful in treatment of patients with multiple myeloma (95).

Toxic side effects remain a major problem with immunotoxin therapy, and problems exist with the maximally tolerated dose that will selectively eliminate the malignant cells (244). Although mAb delivery of toxins can be highly specific, often the cellular antigen is expressed by cells other than those targeted for destruction. For example, treatment of cancer patients with a fusion product between the Fv of an anti–erb-B2 antibody and modified *Pseudomonas* exotoxin showed unexpected liver toxicity. Subsequently, erb-B2 was found to be expressed at low levels on hepatocytes (200).

In addition to difficulty in finding an acceptable therapeutic window for immunotoxins with tolerable side effects, immunogenicity remains a major problem (70,137,263). Because these toxins are of plant or bacterial origin, they are highly immunogenic in humans, and treated patients often generate antitoxin antibody responses that preclude further treatment. Even if further treatment is possible, another problem is the emergence of resistant cancer cells no longer expressing the target of the immunotoxin. These problems present difficult hurdles for the broad therapeutic application of immunotoxins. Nevertheless, there are some clinical indications for which immunotoxins will be effective drugs, particularly when used locally rather than systemically. One such application is in the prevention of secondary cataracts in the eye (50). This therapeutic application consists of a modified ricin toxin conjugated to an epithelial cell–specific mAb to kill the epithelial cells that overgrow the lens to form the cataract, resulting in a loss of vision. A phase I/II trial in which the immunotoxin was applied to the eye at the conclusion of cataract surgery showed a significant reduction in the recurrence of cataracts. Based on these results, this promising immunotoxin has now entered phase III clinical testing.

Gene Therapy Using Antibody Genes and Fusion Products

In the future some mAbs may be delivered through antibody gene administration (203,213). Indeed, this may be an attractive way to reconstitute protective antibody responses where the immune system has been damaged or cannot produce the appropriate protective antibodies. Most experience with gene-delivered antibodies to date has been with the intracellular expression of single-chain antibody fragments, called intrabodies (46,166,282). The major advantage of this technology is that the intrabody can bind and neutralize a large number of intracellular antigens inaccessible to extracellular antibodies. Intrabodies can be localized to different subcellular compartments by using specific peptide tags engineered into the molecules. This strategy has been used successfully in cell-culture studies to prevent HIV infection with intrabodies directed against several different HIV antigens (155,177). In other laboratory studies, intrabodies neutralizing proteins that regulate cell growth were delivered to cancer cells to interfere with cell growth as an approach to creating intracellular "knockouts" (51,127,223). For example, an anti–erbB-2 intracellular scFv localized to the endoplasmic reticulum of cancer cells overexpressing erbB-2, causing the cells to undergo apoptosis and die (63,64,92,93,245,284). This anti–erb-B2 intrabody was successful in preclinical animal models of ovarian, breast, and prostate cancers, and a phase I clinical trial was performed in patients with ovarian and extraovarian cancer (3). Delivery of intrabodies by gene therapy is anticipated to permit specific targeting of a large number of intracellular targets involved in cellular activities, such as cell division and cytokine production and infectious agents such as HIV and human papilloma virus (269). However, for intrabodies to reach their potential, highly efficient and targeted cell delivery will be required, a goal that has not yet been achieved for any form of gene therapy.

PRACTICAL CONSIDERATIONS FOR DEVELOPMENT OF ANTIBODY DRUGS

Delivery

Most therapeutic mAbs are now delivered either by intravenous infusion or subcutaneous injection. However, other routes of administration, such as oral or intranasal, that have been successfully used with some polyclonal antibodies for treatment of bacterial or viral infection (54,57,58,81,87,160,173,277), have not been extensively tested with mAbs. Intranasally administered anti–respiratory syncytial virus (RSV) antibodies are able to reduce pulmonary infection (88,277). A polymer device impregnated with an anti–herpes simplex virus (HSV) mAb placed in the vagina of mice allowed slow release of the antibody, resulting in reduced HSV infection (238). In a clinical trial to prevent oral *Streptococcus mutans* colonization, a monoclonal secretory IgA antibody produced in plants was applied to the teeth and prevented bacterial recolonization for at least 4 months (161). Aerosolized monoclonal anti-IgE antibody E25 was tested in one clinical trial in allergic asthma patients (72). Although the aerosolized E25 retained activity, the mAb concentrations in the tissues were too low to achieve clinical improvement. As novel delivery technologies improve the delivery of larger proteins, mAbs will increasingly be delivered topically, by inhalation, and possibly even orally (141,219).

Production Expense

Cost of production remains a critical issue in the commercialization of therapeutic antibodies. Currently, intact mAbs are manufactured in mammalian cells (218), whereas antibody fragments can be made in insect cells and yeast (21,30,68,111,114,139,208,224,225,239,283). To create manufacturing cell lines, murine myeloma cells or Chinese hamster ovary (CHO) cells are transfected with the antibody heavy- and light-chain genes (218). The transfected cells are grown in serum-free media in large stainless steel fermenters or hollow-fiber devices, and the mAbs purified from the culture supernatant fluid. This is an involved and costly process that results in a relatively high expense for antibody drugs. To reduce the cost of antibody manufacture, novel "bioreactors" are being explored. Both transgenic animals and plants are being tested for the production of antibodies (147,162,209). Transgenic mammals have been generated by fusing the antibody heavy- and light-chain genes to mammary gland–specific promoters to facilitate expression and secretion of large amounts of the antibodies in milk, from which the mAb is purified (157,209). Antibody genes also have been expressed in plants (103,162,250) and "Plantibodies" produced at agricultural scale are expected to be available at a fraction of the cost of currently available fermenter-produced antibodies.

THE FUTURE OF MONOCLONAL ANTIBODY–BASED THERAPEUTICS

According to a 1998 survey by the Pharmaceutical Research and Manufacturers of America, antibodies account for the largest group of investigational new drugs (INDs) filed among the different classes of biotechnologic drugs including cytokines, growth factors, enzymes, antisense, and gene therapy, and currently mAbs represent more than 20% of all biotechnology drugs in clinical development. An increasing amount of information about antibodies and related topics is readily available on the worldwide web (Table 91.5). Many problems encountered in the early history of murine mAbs have been solved, and human mAb-based drugs are fast becoming a major new therapeutic drug class. In addition, antibodies have proved important to identify and validate therapeutic concepts. In some cases, the antibodies themselves have been developed as the therapeutic agents. In other cases, the antibodies have been replaced by peptides or small organic drugs.

- http://www.ANTIBODIES-PROBES.com
- http://www.antibodyresource.com (Antibody resource page)
- http://www.biochem.ucl.ac.uk/~martin/antibodies.html (Antibody Structure and Sequence Information V1.2)
- http://www.biodesign.com (BIODESIGN International: THE ANTIBODY SOURCE)
- http://www.ocms.ox.ac.uk (Cytokines Web)
- http://www-chem.ucsd.edu/Faculty/goodman/antibody_____.html/onlinecomp.html (Finding an Antibody)
- http://bimas.dcrt.nih.gov/molbio/hla_bind (HLA Peptide Binding Predictions)
- http://www.antibodyresource.com/findantibody.html (How to Find an Antibody)
- http://www.path.cam.ac.uk/~mrc7/cig/immlinks.html (Immunology Web Links)
- http://immuno.bme.nwu.edu (Kabat Database)
- http://www.biochem.ucl.ac.uk/~martin/abs/simkab.html (KabatMan - Simple Interface V1.1)
- http://HSEQ.CRYST.BBK.AC.UK (MHC Home Page)
- http://wehih.wehi.edu.au/mhcpep (MHCPEP - a database of MHC binding peptides)
- http://www.path.cam.ac.uk/~mrc7/mikeimages.html (Mike Clark's Immunoglobulin Structure Home Page)
- http://www.atcc.org/hdb/hdb.html (Monoclonal Connection–Hybridoma Data Bank)
- http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html (V region database)

TABLE 91.5. Antibodies on the World Wide Web

Within a year, the entire sequence of the human genome will be completed. Characterization of each of the more than 100,000 gene products will require at least one and probably multiple mAbs. These reagents will find use in protein characterization and in biologic experiments. Some will be used directly or in modified form (i.e., as a fusion protein, bispecific, intrabody) as recombinant therapeutic antibody-based drugs. In addition, we predict that a significant subset of these mAbs will provide leads for drug development by demonstrating therapeutic concepts. Already mAbs are being used to model receptor/ligand binding, and mimics of antibody loops (231,280) may provide a rapid novel means to generate pharmaceutical leads. Novel display technologies (99,102) will speed the discovery process. Recently, so-called "psi-bodies," binding molecules having hypervariable loops similar in function to the antibody CDRs, have been selected to bind target antigens (193). These "designer proteins" may eventually represent a novel subgroup of antibody-like binding molecules. In the future, it is likely that only the imagination of antibody engineers will limit the application of these exquisitely designed and useful molecules!

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92 DRUGS THAT MODULATE THE IMMUNE RESPONSE

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Both the innate and adaptive immune systems evolved to prevent or eradicate invasion by microorganisms and to eliminate dead or injured tissue. These functions are essential for the maintenance of homeostasis, but overactive immune or inflammatory responses characterize the pathogenesis of a number of acute and chronic illnesses such as rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus (SLE), the “autoimmune” diseases in which an exaggerated or inappropriate immune or inflammatory response leads to significant disability. Although there are no cures for autoimmune diseases, great strides have been made in the last half century in the development of increasingly safe and effective therapies for these illnesses. Many of the drugs introduced were originally developed for other uses, and their mechanisms of action in the therapy of autoimmune diseases were not understood. In this chapter, the mechanisms of action and uses of common antiinflammatory and immunomodulatory drugs (Fig. 92.1) are discussed.

Figure 92.1. Drugs that affect the immune response.

CORTICOSTEROIDS

The doyen of immune-modulating drug therapy for half a century, corticosteroids have long been regarded as a mainstay in the treatment of inflammatory diseases. Hench's (1,2) benchmark and Nobel prize-winning discovery that corticosteroids have a profound antiinflammatory effect against rheumatoid arthritis brought attention to what was to become one of the most widely used and potent naturally occurring drugs in rheumatic and other inflammatory disease states. These four-ringed, 21-carbon steroid hormones are of adrenal medullary origin (cortisol/hydrocortisone) and are produced in response to stimulation by adrenocorticotropic hormone. Corticosteroids have received many pharmaceutical modifications and disguises over the years, which result in varying durations of action and relative glucocorticoid-to-mineralocorticoid potencies. Their widespread clinical use has been hampered by the recognition of their many side effects and toxicities, not least in relation to the suppression of the hypothalamic/pituitary/adrenal axis. Other major side effects involve multiple organ systems, as listed in Table 92.1.

Endocrine	Suppression of hypothalamus pituitary adrenal axis Diabetes
Skin	Hirsutism Skin thinning Striae Acne Purpura Alopecia
Gastrointestinal	Peptic ulcer disease Gastritis Pancreatitis
Eye	Glaucoma Posterior subcapsular cataract
Musculoskeletal	Osteoporosis Avascular necrosis Myopathy
Cardiovascular	Hypertension Dyslipidemia Dysrhythmia Premature coronary artery disease
Neuropsychiatric	Euphoria Depression Psychosis Insomnia Pseudotumor cerebri
Infectious disease	Increased risk of usual infections Opportunistic infections

TABLE 92.1. Major Adverse Effects of Corticosteroids

Antiinflammatory Effects

EFFECTS ON CELLS INVOLVED IN THE INFLAMMATORY RESPONSE

Neutrophils and Endothelial Cells

The short-lasting increase in the circulating pool of neutrophils, often persisting for less than 24 hours, is one of the best known of clinical effects after corticosteroid administration (3). A number of factors contribute to its occurrence, including an increase in the circulatory half-life of the neutrophils, perhaps due in part to a decrease in apoptosis of neutrophils; an increase in neutrophilic demargination from the vascular endothelium; the premature release of neutrophils from the bone marrow, partly in response to released granulocyte colony-stimulating factor; and a decrease in the egress of neutrophils into sites of inflammation (4,5,6,7,8,9,10,11,12 and 13). The exact mechanisms by which these phenomena occur are ill defined, but observations suggest various factors that might contribute to these effects. The change in electrophoretic mobility, reflecting cell-surface charge, was altered in neutrophils stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (*f*MLP) after corticosteroid administration (14). Neutrophil chemotaxis in response to *f*MLP and interleukin (IL)-8 is reduced by treatment with dexamethasone (15). The change in expression of adhesion molecules on both polymorphonuclear leukocytes and endothelial cells is another mechanism by which neutrophil attachment to blood vessels and subsequent recruitment to inflamed sites may be altered (16,17 and 18). Dexamethasone decreased L-selectin expression and b_2-integrin ligand-binding avidity on the surface of resting neutrophils, an effect mediated by the glucocorticoid receptor (19,20). Endothelial cell permeability and expression of major histocompatibility complex molecules may also be affected; and more importantly for inflammation, corticosteroids decrease endothelial expression of the adhesive molecules E-selectin and intercellular adhesion molecule (ICAM)-1 (21,22,23,24,25 and 26). Reduction in neutrophil binding to tumor cells may be responsible for the inhibition of neutrophil-mediated tumor cell cytostasis seen with corticosteroids (19). Neutrophil production of inflammatory mediators such as cytokines, prostaglandins,

leukotrienes, and reactive oxygen species may also be modulated by corticosteroids (15,27,28,29,30,31 and 32).

Monocytes and Macrophages

Corticosteroids affect the numbers, differentiation, and phenotypes of cells of the monocyte/macrophage lineage (23,33,34). Interaction between macrophages and other inflammatory cell types may also be modulated. The expression of adhesion molecules such as ICAM-1 may be suppressed by corticosteroids, an effect dependent on the preparation used and cell type studied (35,36,37 and 38). Through the production of cytokines, macrophages have a profound influence on the inflammatory behavior of multiple cell types, and corticosteroids have documented effects on the generation of cytokines such as IL-1 and tumor necrosis factor-a (TNF-a), contributing to the suppression of inflammation (39,40 and 41). As antigen-presenting cells, macrophages influence the production of T-helper (Th)2 cytokines by lymphocytes. This capacity is enhanced by treatment of macrophages with corticosteroids (42). Effective antigen presentation requires the participation of human leukocyte antigen (HLA)-DR antigens on the cell surface, and their expression may also be decreased by corticosteroid treatment, thus blunting any further macrophage-induced amplification of the inflammatory response. However, the evidence for this latter effect is contradictory, and inhibition of antigen presentation may occur regardless of the effect of corticosteroids on MHC expression (36,43,44).

Eosinophils

Corticosteroids reduce the survival of eosinophils, an effect that may be overcome by the influence of cytokines such as interferon (IFN)-g or tumor necrosis factor-a (TNF-a), thus leading to a decrease in circulating cell numbers (45,46 and 47). This may occur in part by induction of apoptosis in this cell population (7,48,49). Corticosteroids also induce the migration of peripheral blood eosinophils to several lymphoid organs, such as the spleen, lymph nodes, and thymus (50). Eosinophil chemotaxis, adherence, and degranulation may also be inhibited (23,51,52 and 53).

Lymphocytes

The circulating numbers of lymphocytes are reduced by corticosteroids (54). Redistribution to lymphoid tissues and the bone marrow may contribute to this decrease (55,56). The induction of apoptosis by corticosteroids seen in other cell types is also operative in lymphocytes, particularly thymocytes and immature lymphocytes (57,58 and 59). Lymphocyte proliferation, particularly that of the T-cell population, is also inhibited by corticosteroids (60,61). Corticosteroids also influence the expression of cytokines by T-cell subsets, generally shifting the balance in favor of a Th2-predominant profile (62,63). Downregulation of IL-2 production by T lymphocytes further attenuates an important stimulating signal for the proliferation and amplification of the lymphocyte population (64).

Phlogistic Effects

The powerful antiinflammatory actions of corticosteroids are manifested in their influence on almost every class of inflammatory mediators. An important effect is the stimulation of the production of lipocortins, which inhibit the activity of phospholipase A_2, which, in turn, attenuates arachidonic acid metabolism and eicosanoid production (65). Cyclooxygenase activity is reduced, and nitric oxide synthase expression is inhibited as well (66,67). The production of many inflammatory cytokines is suppressed, including IL-1, IL-2, IFN-g, and TNF-a; and the production of destructive enzymes such as collagenase and stromelysin is also downregulated (68,69).

Intracellular Signal Transduction

Glucocorticoids are potent regulators of the expression of a large number of gene products. On ligation of the glucocorticoid receptor, which normally resides in the cytosol complexed to heat shock protein (hsp)90 and immunophilin p56, homodimers of glucocorticoid receptors are formed and may enter the nucleus and positively affect gene transcription by binding to glucocorticoid response elements. Such regulatory processes contribute to some of the antiinflammatory properties of corticosteroids, particularly in relation to the effects of glucocorticoids on phlogiston production. However, since many corticosteroid-sensitive immunoregulatory genes do not have a recognizable glucocorticoid response element in their promotor regions, and corticosteroids inhibit the upregulation of many gene products stimulated by proinflammatory stimuli, alternative mechanisms must exist to explain how corticosteroids produce their antiinflammatory effects. The ligand-activated glucocorticoid receptor also possesses the ability to inhibit AP-1 and NF-kB–mediated transcription (70,71). Protein/protein interactions between NF-kB and glucocorticoid receptors may result in the repression of transcriptional activity (72). More strikingly, corticosteroids induce the production of an inhibitory protein, IkBa, which, by complexing with the heterodimeric NF-kB complex in the cytoplasm, effectively blocks the translocation of NF-kB into the nucleus, thus attenuating downstream transcriptional activity (see Chapter XX) (70). Glucocorticoids also promote the reassociation of free NF-kB with IkBa (73). Examples of NF-kB–regulated genes important in the inflammatory response that are repressed by corticosteroids include IL-2, IL2-Ra, IL-3, IL-6, IL-8, TNF-a, granulocyte-macrophage colony-stimulating hormone (GM-CSF), inducible nitric oxide synthase (iNOS), and adhesion molecules ICAM-1 and E-selectin (21,70,73,74). Interference with AP-1, the major enhancer for the collagenase promotor, may account for the downregulation of collagenase by corticosteroids, and a direct protein/protein interaction is known to exist between the glucocorticoid receptor and AP-1 (75). Whereas the glucocorticoid receptor is a potent inhibitor of AP-1 activity, c-Jun and c-Fos are potent repressors of glucocorticoid-receptor activity (76). The direct interaction between CREB-binding proteins and the ligand-binding domains of nuclear factors such as AP-1 may be responsible for the mediation of this activity without necessarily affecting the binding of AP-1 to DNA response elements (77).

METHOTREXATE

A disease-modifying drug that is now accepted as the gold standard for the treatment of rheumatoid arthritis, methotrexate was developed as one of the first chemotherapeutic agents for the treatment of malignant diseases. Methotrexate was developed as a folic acid analog that diminishes the cellular proliferation of malignancies by inhibiting the synthesis of purines and pyrimidines required to support cell growth (78). It was generally assumed that inhibition of the proliferation of cells involved in the immune and inflammatory response was responsible for the antiinflammatory action of methotrexate, as applied to inflammatory diseases such as rheumatoid arthritis. However, the doses of methotrexate used in the treatment of inflammatory diseases are from ten- to 1,000-fold lower than those used for its antimalignant effect. Furthermore, the addition of folic or folinic acid to the treatment regimen, a strategy that would nullify the antifolate action of methotrexate used for malignancies, has been widely adopted as an adjunct to counteract toxic side effects of methotrexate therapy, such as stomatitis and bone marrow suppression, processes that depend on cellular proliferation. In contrast to its effects on methotrexate-induced toxicity, folic acid supplementation does not result in any loss of antiinflammatory efficacy (79). It would therefore appear that the antiinflammatory actions of methotrexate must depend on mechanisms other than inhibition of dihydrofolate reductase.

The Pharmacologic Fate of Methotrexate

Methotrexate, whether administered orally or intramuscularly in inflammatory diseases, has a very short plasma half-life. Although a wide variation in interindividual levels exists, peak serum levels may be achieved in as little as 2 hours after oral administration (80). Methotrexate is hydroxylated to form 7-hydroxymethotrexate, which possesses many of the biologic properties of the parent compound (81). Both methotrexate and 7-hydroxymethotrexate are in turn polyglutamated, and as such remain intracellularly for extended periods while remaining biologically at least as active as methotrexate itself (82). Much of the excretion occurs through the renal route (83).

Molecular Actions

The molecular mechanisms by which methotrexate exerts its antiinflammatory actions have been well studied. There are currently three major hypotheses regarding the mechanisms of action of methotrexate, which may be complementary in explaining the molecular effects of the administered drug. The dihydrofolate reductase–inhibiting properties of methotrexate have long been known, and it has been suggested that low-dose methotrexate decreases purine and pyrimidine synthesis *in vivo* and thus diminishes lymphocyte proliferation. A single dose of methotrexate transiently decreases serum purine and pyrimidine levels (24 to 48 hours) (84). This decrease is associated with a transient reduction in antigen-stimulated proliferation of lymphocytes *ex vivo*. Methotrexate also induces apoptosis of activated peripheral T lymphocytes from patients with rheumatoid arthritis (85). Although these changes are quite consistent with the hypothesis, their transient nature does not correlate with the long duration of antiinflammatory action of a single dose of the drug.

The second proposed mechanism of methotrexate action posits that the immune pathways responsible for inflammatory diseases such as rheumatoid arthritis may be blunted by blocking transmethylation reactions intracellularly, an action accomplished by methotrexate. This hypothesis rests on the ability of methotrexate to inhibit the production of potentially toxic compounds such as the polyamines, spermine, and spermidine. Figure 92.2 illustrates the formation of *S*-adenosylmethionine, a methyl donor for transmethylation reactions within the cellular environment. The acquisition by homocysteine of a methyl group donated by tetrahydrofolate results in the formation of methionine, which is then converted to *S*-adenosylmethionine. The resultant *S*-adenosylmethionine is the primary methyl donor in intracellular reactions including the methylation of DNA and lipids, as well as the posttranslational modification of proteins, all essential for cellular survival. After the donation of the methyl group, *S*-adenosylhomocysteine is converted by *S*-adenosylhomocysteine hydrolase to adenosine and homocysteine, which can reenter the cycle. Blockage of cellular transmethylation reactions occurs with the accumulation of high concentrations of *S*-adenosylhomocysteine and consequent inhibition of the formation of *S*-adenosylmethionine, which occurs in the red cells and lymphocytes of children with inherited adenosine deaminase deficiency, a form of severe combined immunodeficiency syndrome, suggesting a critical role for transmethylation reactions in normal immune function (86,87,88 and 89). The inhibition of monocyte and

lymphocyte function that occurs with the blockade of transmethylation reactions lends further support to this hypothesis. Moreover, *S*-adenosylmethionine is required for the synthesis of polyamines such as spermine and spermidine, which may accumulate in the synovium of rheumatoid arthritis patients. Monocytes produce toxic metabolites from polyamines such as NH_3 and H_2O_2, which have been shown to inhibit lymphocytic IL-2 production (90,91,92 and 93). Polyamine formation also is associated with increased production of rheumatoid factor and altered monocyte function. Methotrexate, by inhibiting the formation of *S*-adenosylmethionine, can reverse these immunologic effects *in vitro* (Fig. 92.2). There are many other sources of toxic oxygen metabolites in the inflamed rheumatoid synovium, and the inhibition of polyamine formation alone is unlikely to account for the antiinflammatory effects of methotrexate. Moreover, the lack of clinical efficacy of 3-deaza-adenosine, an *S*-adenosylhomocysteine hydrolase inhibitor that diminishes transmethylation reactions and *S*-adenosylmethionine production, in the treatment of rheumatoid arthritis, suggests that inhibition of transmethylation pathways alone cannot account for the antiinflammatory actions of methotrexate (94,95,96 and 97; M. Weinblatt, personal communication).

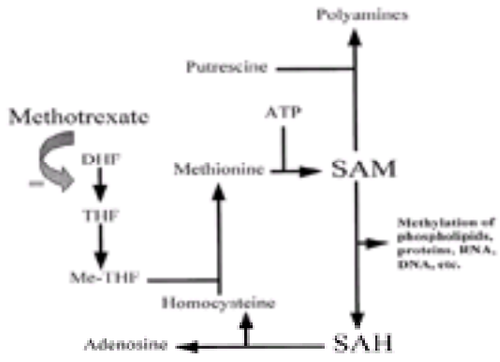

Figure 92.2. Methotrexate inhibits transmethylation reactions and polyamine formation. *DHF*, dihydrofolate; *THF*, tetrahydrofolate; *Me-THF,* methyl-tetrahydrofolate; *ATP*, adenosine triphosphate; *SAM, S*-adenosyl-methionine; *SAH, S*-adenosyl-homocysteine

Methotrexate polyglutamates are potent inhibitors of various intracellular folate-dependent enzymes, and one of those most effectively inhibited is 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, which catalyzes the conversion of AICAR into formyl-AICAR (Fig. 92.3). Inhibition of AICAR transformylase by methotrexate polyglutamates results in the accumulation of AICAR, and cancer patients treated with methotrexate have previously been shown to excrete increased amounts of aminoimidazole carboxamide in their urine (98). The accumulated AICAR has two important intracellular effects. First, AICAR is a direct inhibitor of the enzyme adenosine monophosphate (AMP) deaminase. The dephosphorylated metabolite of AICAR, AICARiboside, is an inhibitor of adenosine deaminase. AICAR accumulation can therefore lead to the release of adenosine, either directly through adenosine deaminase inhibition, or indirectly from AMP deaminase inhibition and hence the accumulation of AMP, which can be dephosphorylated to adenosine (99). In support of this hypothesis, Baggott et al. (100) recently observed an increase in urinary aminoimidazole carboxamide and adenosine in patients taking antiinflammatory doses of methotrexate.

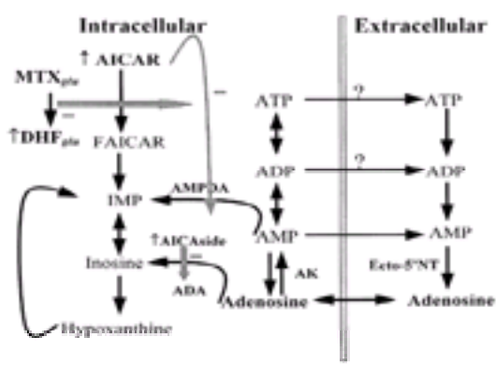

Figure 92.3. Pathways in adenosine formation. *AICAR*, 5-aminoimidazole-4-carboxamide ribonucleotide; *FAICAR*, formyl-AICAR; *IMP*, inosine monophosphate; *AMPDA*, adenosine monophosphate deaminase; *ADA*, adenosine deaminase; *AK,* adenosine kinase; *ecto-5'NT*, ecto-5'nucleotidase.

The released adenosine, acting through one of the four known types of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3, has a wide diversity of physiologic effects. Adenosine receptors are members of the seven-transmembrane-spanning receptor family related to adrenergic receptors (101). The A_1, A_{2A}, and A_{2B} receptors, unlike the A_3 receptor, are highly conserved during evolution. Many of the physiologic events mediated by adenosine are antiinflammatory in nature and involve a number of different cell types (102). With respect to the neutrophil, these include the diminution of their accumulation at the inflamed site, inhibition of stimulated neutrophil adhesion, and inhibition of the generation of toxic oxygen metabolites and neutrophil phagocytosis (103,104,105 and 106). Lymphocyte proliferation also is inhibited, and adenosine induces suppressor function and phenotype among lymphocytes (107,108 and 109). Secretion of cytokines by monocytes/macrophages also is modulated by adenosine, which enhances the production of antiinflammatory cytokines (e.g., IL-10) and suppresses the production of inflammatory cytokines (e.g., IL-6, IL-8, TNF-a), an effect mediated through adenosine A_{2A} and A_3 receptors (110,111,112,113 and 114). Ligation of adenosine A_1 receptors may decrease the production of collagenase, but not stromelysin or tissue inhibitor of metalloprotease by synoviocytes (115).

By releasing adenosine, methotrexate can therefore exert a variety of antiinflammatory effects (116). That adenosine mediates the antiinflammatory action of methotrexate is supported by the *in vivo* demonstration that adenosine-receptor antagonism can reverse or eliminate the antiinflammatory effects of administered methotrexate (117).

Aside from the desired effects that adenosine produces, it also may be responsible for some adverse effects encountered during methotrexate therapy. The mild, reversible renal dysfunction caused by methotrexate, thought to be vascular in origin, may be a function of the vasoconstrictive properties of adenosine acting through A_1 receptors in the kidney (118,119). The methotrexate-induced release of adenosine from macrophages promotes the formation of multinucleated giant cells, an effect again mediated through the adenosine A_1 receptor, and may account for the occurrence of subcutaneous nodulosis seen in patients with rheumatoid arthritis treated with methotrexate (120). Other adverse effects include gastrointestinal upset, cirrhosis, stomatitis, alopecia, interstitial lung disease, neurotoxicity, congenital abnormalities and abortion, lymphoproliferative malignancies, bone marrow suppression, and increased susceptibility to infections.

SULFASALAZINE

A compound originally introduced for the treatment of rheumatoid arthritis, sulfasalazine has gained clinical application in a wide range of inflammatory diseases, most notably in inflammatory bowel disease and the seronegative spondyloarthropathies. Composed of sulfapyridine and 5-aminosalicylic acid moieties, it possesses both antiinflammatory and antimicrobial properties. The active part of the drug may vary in different disease states, and whereas 5-aminosalicylate may mediate the therapeutic effects in inflammatory bowel disease, sulfapyridine is probably the responsible agent in the inflammatory arthritides (121,122 and 123).

Less than 20% of ingested sulfasalazine is absorbed intact, although some reenters the colon via biliary recirculation (124). The remaining sulfasalazine is metabolized by intestinal bacteria to sulfapyridine, which is well absorbed, and 5-aminosalicylate, which is only minimally absorbed, after which it is acetylated and excreted in the urine.

Sulfasalazine suppresses T-cell and B-cell proliferation *in vitro* and diminishes total immunoglobulin and rheumatoid factor levels *in vivo* (125,126,127 and 128). It may decrease the biologic effects of TNF-a by reducing its production and by inhibiting the binding of TNF to its receptor (125,129,130). Much as does methotrexate, sulfasalazine (but not sulfapyridine) inhibits AICAR transformylase, and the resultant AICAR accumulation inside the cell is associated with adenosine release, which in turn produces its salutary antiinflammatory effects, most likely mediated by adenosine A_2 receptors, because the sulfasalazine-induced inhibition of leukocyte accumulation in a murine air-pouch model of inflammation was reversed by an antagonist to the adenosine A_2 receptor (131,132 and 133). Inhibition of NF-kB may be the mechanism responsible for some of the physiologic actions of sulfasalazine, although the concentration of sulfasalazine required to inhibit translocation of NF-kB is

probably ten-fold higher than that achieved in patients (134,135).

Adverse effects of sulfasalazine therapy include gastrointestinal upset, urticaria and photosensitivity, Stevens-Johnson syndrome, aplastic anemia, interstitial nephritis, and oligospermia.

LEFLUNOMIDE

Leflunomide is an isoxazole derivative that is converted into the malononitrilamide compound A77 1726 (2-cyano-3-hydroxy-*N*-[4-trifluoromethylphenyl]-butenamide) before it becomes active. Because A77 1726 is highly protein bound in the plasma, it has a long half-life of more than 2 weeks. Excretion occurs through both feces and urine. It has been used in the prevention of solid organ transplant rejection and is approved for use in the treatment of rheumatoid arthritis, in which its clinical effects are evident when it is used either alone or in combination with methotrexate.

Leflunomide has been shown to be effective in diminishing lymphocyte proliferation (136,137). Humoral responses are also affected because B lymphocyte proliferation is suppressed during the progression through the S phase of the cell cycle with diminution of a cyclin-dependent kinase, Cdk2 protein (138). The antiproliferative action of leflunomide is believed to occur by inhibition of the *de novo* synthesis of pyrimidines in the G_1 phase of the cell cycle (139). The availability of pyrimidine nucleotides must be high enough to support the proliferation of cells such as activated lymphocytes, and salvage pathways alone do not fulfill the demands imposed by inhibition of *de novo* synthetic pathways (140). This notion is supported by the observation that administration of uridine reverses the antiproliferative effects of leflunomide *in vitro* (141,142). A77 1726 is an inhibitor of the enzyme dihydroorotate dehydrogenase, the activity of which is required for the *de novo* synthesis of uridine monophosphate from adenosine triphosphate (ATP) and glutamine, and this action may be important in the antiinflammatory effects of leflunomide in rheumatoid arthritis (143,144). The antiproliferative actions of very high concentrations of A77 1726 are, however, not reversed by the addition of uridine, suggesting that at these concentrations, leflunomide may exert its antiproliferative actions through a different mechanism, perhaps by inhibition of protein tyrosine kinases, as IL-2–induced tyrosine phosphorylation of Jak1 and Jak3, the protein tyrosine kinases initiating signaling by IL-2R, are inhibited by A77 1726 in high concentrations, and that tyrosine phosphorylation of the b chain of IL-2R, which is required for IL-2–driven proliferation, is also inhibited by A77 1726 (145). Leflunomide decreases stimulated NF-kB activation and also inhibits the adhesive process of peripheral blood and synovial fluid mononuclear cells (146,147).

Adverse effects of leflunomide therapy include gastrointestinal upset, respiratory and urinary tract infections, hypertension, peripheral edema, headache, alopecia and skin rash, hypokalemia, diabetes, dyslipidemia, anemia, leukopenia and thrombocytopenia, musculoskeletal pain, and elevated liver enzymes.

MYCOPHENOLATE MOFETIL

Mycophenolate mofetil is another immunomodulant used in the prevention of renal transplant rejection that is under study in clinical trials for the treatment of rheumatoid arthritis and SLE. It is well absorbed when administered orally and hydrolyzed to the active compound, mycophenolic acid. Mycophenolic acid is highly protein bound, and its metabolism takes place mainly by glucuronidation. Most of the excretion occurs through urine.

Mycophenolic acid is a potent inhibitor of the enzyme inosine monophosphate dehydrogenase and as such inhibits the *de novo* synthesis of purines (148). Lymphocytes are inherently dependent on the *de novo* rather than the hypoxanthine-guanine phosphoribosyl transferase salvage pathway for purine synthesis, and this imparts a relative selectivity for mycophenolic acid on cells involved in the immune response. The resultant decrease in the concentration of guanine nucleotides impairs DNA synthesis in lymphocytes and hence inhibits their proliferation. Mycophenolic acid suppresses antibody production by B cells and inhibits cytotoxic T-cell and NK-cell activity (149,150). It has also been shown to inhibit the production of cytokines by stimulated mononuclear cells after 48 hours of incubation, including IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IFN-g, TNF-a, TNF-b, and GM-CSF; and reduced the IFN-dependent induction of MHC in an *in vivo* model (151,152). In a murine model of SLE, mycophenolate mofetil suppressed the production of anti-DNA antibodies and reduced the number of $CD4^+$ T lymphocytes expressing very late antigen-4 and ICAM-1 without any effect on total immunoglobulins, CD4-, CD8-, or IgM-positive splenocytes. Survival of the treated mice was significantly greater than that of the control group after 60 days (100% vs. 10%) (153). Because mycophenolic acid reduces the guanine nucleotide pool in lymphocytes and monocytes, the synthesis of fucose- and mannose-containing saccharide components of membrane surface glycoproteins that are recognized by selectins is also inhibited. Thus mycophenolic acid may also limit neutrophil, lymphocyte, and monocyte recruitment (154). Side effects of mycophenolate therapy include fever; headache; abdominal, chest, and back pain; infection; hypertension; skin rash; gastrointestinal upset; insomnia; tremor; anemia; thrombocytopenia; leukopenia or leukocytosis; dyslipidemia; hyperglycemia; and electrolyte imbalance.

ANTIMALARIALS

The observation that patients with SLE improved while taking antimalarials led to the use of these agents in inflammatory diseases including rheumatoid arthritis. The variably absorbed drugs, chloroquine and hydroxychloroquine, which are both relatively weak antiinflammatory agents, have a predilection for pigmented tissues such as melanocytes and retinal cells. Excretion occurs via the urinary tract, but tissue deposition is associated with a prolonged half-life of more than 45 days (155). Retinal deposition of the drug causes retinopathy and blindness, although with the adoption of lower doses of hydroxychloroquine now commonly used in inflammatory diseases (6 to 7 mg/kg/day), retinal complications are rare (156). As they can easily traverse the plasma membrane, they gain ready access to acidic cytoplasmic vacuoles, where the acquisition of protons by these diprotic bases renders them acidic and unable to diffuse out of the vacuoles again. The concentrations of these antimalarial drugs are therefore higher within cytoplasmic vacuoles (157).

Antimalarials have varied antiinflammatory effects including inhibition of the production of multiple cytokines (TNF-a, IL-1 and IFN-g), phospholipases A and C activity, DNA and RNA polymerase reactions, and stimulated superoxide anion production by neutrophils (158,159,160,161,162 and 163). Their ability to buffer the acidic lysosomal pH has important ramifications in processes involved in the generation of the inflammatory response, most notably the interference of antigen presentation by macrophages (164). Side effects include skin rash and hyperpigmentation, gastrointestinal upset, headache, neuromyopathy, retinopathy, and corneal deposits.

CYTOTOXIC DRUGS

Various chemotherapeutic agents designed for the treatment of malignancies, including alkylating agents and antimetabolites, have been applied to the therapy of inflammatory diseases with the rationale that they target the most rapidly dividing cells, which in the case of inflammatory diseases are also the cells involved in the immune response. These drugs share many common features and toxicities including cytopenias, susceptibility to infection, gastrointestinal upset, and alopecia. We examine two examples of these agents used in the treatment of inflammatory diseases such as rheumatoid arthritis and SLE, azathioprine and cyclophosphamide.

Azathioprine

Azathioprine is an imidazolyl derivative of 6-mercaptopurine, which is a purine antimetabolite originally developed for the treatment of malignant diseases. It has been widely used in the management of solid organ transplants and in inflammatory diseases such as rheumatoid arthritis and SLE.

The ingested azathioprine is well absorbed and metabolized in the liver by xanthine oxidase to the active compound, 6-mercaptopurine. The subsequent conversion to 6-mercaptopurine nucleotides results in the inhibition of *de novo* purine synthesis. Alternatively, the conversion to thio-inosine monophosphate (thio-IMP) may interfere with the salvage pathway of purine synthesis, and the thio-IMP suppresses the conversion of IMP to AMP and guanine monophosphate, important in nucleic acid generation. The metabolism of thio-IMP yields thio-guanine monophosphate, which may be faultily interpolated into the DNA backbone.

Azathioprine induces the loss of NK-cell function without apparent change in cell numbers (165,166 and 167). It has the capacity to reduce antibody production and humoral response to challenges (168,169). Antibody-dependent cellular cytotoxicity is similarly suppressed (166). The circulating population of $CD8^+$ T lymphocytes also is reduced, with a resultant increase in the CD4/CD8 ratio (167). Production of cytokines such as IL-6 may also be diminished, and azathioprine reduces the expression of cytokine-induced neutrophil chemoattractant in an animal model (170,171). Chemotactic responses of neutrophils, monocytes, and lymphocytes are all reduced by azathioprine (172).

Cyclophosphamide

Cyclophosphamide is a prodrug that can be administered orally and intravenously. Absorption is complete when given orally. On metabolism by hepatic microsomal enzymes, aldophosphoramide is produced, which is cleaved into the phosphoramide mustard responsible for the alkylating action of cyclophosphamide, and acrolein, which is toxic to the bladder. Excretion occurs through the renal route.

The alkylation of guanine residues of DNA by cyclophosphamide renders the macromolecules cross-linkable and therefore interferes with DNA replication, transcription, and translation, overwhelming innate cellular DNA repair mechanisms (173). Cyclophosphamide has been shown to be markedly antiproliferative in mitogen-induced blastogenesis (174). B-lymphocyte function is altered, with suppression of immunoglobulin synthesis, although an increase in

immunoglobulin-secreting cells has been observed after cyclophosphamide therapy (175,176 and 177). Over time, cyclophosphamide decreases the number of circulating $CD4^+$ T cells with a lesser effect on $CD8^+$ T cells, thus reducing the CD4/CD8 ratio. T-cell proliferative responses were similarly suppressed with no apparent effect on NK cells or antibody-dependent cellular cytotoxicity (178).

CYCLOSPORINE

A powerful immunosuppressant used in organ transplantation and autoimmune diseases, cyclosporine is notable for targeting signal-transduction processes in immunocompetent cells, particularly T lymphocytes, and as such, shares many similarities with its more recently developed counterpart tacrolimus (FK506). This highly lipophilic compound has a high volume of distribution after oral or intravenous administration, particularly in cells high in cyclophilin content, such as leukocytes and erythrocytes. The many side chains arising from its complicated ring structure are subject to metabolism by the cytochrome P-450 system, and the principal route of elimination for these metabolites is through biliary excretion, although a small percentage undergoes urinary excretion. Adverse effects of therapy include nephrotoxicity and electrolyte imbalance, hypertension, tremor, hirsutism and acne, gastrointestinal upset, pancreatitis, hepatotoxicity, and infection.

The heterodimer formed from cyclosporine and cyclophilin is capable of inhibiting a calcium-dependent serine/threonine phosphatase activity by virtue of its ability to bind to and inactivate calcineurin (179,180). Thus regulatory proteins that include a subunit of the nuclear factor of activated T cells (NF-AT) in the cytoplasm, which require dephosphorylation before translocation into the nucleus, fail to be activated under the influence of cyclosporine. Because nuclear transcription of many inflammatory proteins requires the participation of these regulatory proteins in their activated form in the formation of transcription factors, the inhibitory process initiated by cyclosporine effectively attenuates the transduction signals that would otherwise result in the transcription of genes yielding products that amplify the inflammatory process. These products include cytokines, cytokine receptors, as well as protooncogenes. One such example is IL-2, important in mediating the mitogenesis of stimulated T lymphocytes (181). An alternative mechanism by which cyclosporine inhibits T-cell proliferation is the induction of a cell-cycle inhibitor, p21 (182). Stimulated gene transcription for IL-2 receptor is inhibited, and the synthesis of a number of other cytokines may also be affected, including IL-3, IL-4, IL-6, transforming growth factor-b, and IFN-g (183,184). NK-cell activity as well as B-cell function are also subject to inhibition by cyclosporine; and transcription of immunoglobulin genes may be inhibited (184,185 and 186). Production of cytokines by monocytes/macrophages such as IL-6 may be diminished by cyclosporine, and this diminution may occur at the posttranscriptional level because IL-6 synthesis but not IL-6 mRNA was inhibited by cyclosporine in a cell-culture model, and similar findings have been described in relation to the inhibition of TNF-a production. Thus cyclosporine may exert its antiinflammatory actions by mechanisms other than by regulating signal transduction via immunophilin binding, as described earlier (187,188).

CONCLUSION

The treatment of inflammatory diseases has enjoyed remarkable progress during the last half century after the introduction of immunomodulatory agents into clinical practice. It is worth noting that all the medications covered exert their antiinflammatory effects by influencing many different arms of the inflammatory network; and it would appear that such an approach is necessary to produce adequate and observable changes in outcome. Our understanding of the mechanisms of action of the commonly used immunomodulants complements the growing insights into the pathways of the immune network, and this knowledge will certainly find application in the design of more efficacious immunomodulants in the future.

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93 New Approaches to Vaccine Development

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The difficulties in generating effective vaccines for several highly prevalent infectious diseases, including acquired immunodeficiency syndrome (AIDS), malaria, and tuberculosis, have underscored the need to advance vaccine development with modern technologies. Typically, characterization of naturally occurring, protective immune responses have guided such efforts, but significant challenges are posed when the immune system does not confer protective immunity, for example, as in human immunodeficiency virus (HIV) infection. In such cases, several approaches may facilitate the development of highly effective vaccines. The identification of immunogens that elicit broadly neutralizing antibodies, an understanding of the molecular and cellular basis for immune responses to components of the infectious agent, identification of relevant forms of viral proteins for antigen presentation, stimulation of relevant T-cell types, and enhancement of antigen-presenting, dendritic cell function, may be required. A high priority for effective vaccine development is the quantitation of immune responses in animals and in humans, identification of surrogate markers of immune protection, streamlined vaccine production, and rapid evaluation of candidate vaccines for testing in clinical trials. Finally, the technologic advancements in genomics offer great potential to develop predictive algorithms that may facilitate vaccine design and allow identification of genetic determinants of immunogenicity for vaccine-induced responses.

Vaccines are among the most successful medical interventions for human disease. The smallpox vaccine served as the prototypic example, leading to the eventual eradication of one of the most devastating infectious diseases in history. This vaccine was developed more than 200 years ago and built on the observation that immunity to disease can be acquired naturally. For Edward Jenner, the knowledge that some persons became resistant to infection, specifically, milkmaids who had previously contracted cowpox, provided the critical lead that enabled the isolation of the virus that conferred immunity to disease. Nearly all successful vaccines since have built on examples of natural immunity to the offending pathogen. The definition of mechanisms of acquired immunity has formed the scientific basis for vaccine development. Immunologic protection can be mediated through antibodies that bind and neutralize virus infectivity. The role of cytotoxic T lymphocytes (CTLs) in the recognition of proteins processed by antigen-presenting cells (APCs) has also been established from the work of Zinkernagel, Doherty, and others (1), as has their importance in the elimination of cells infected with virus.

Modern immunology has provided important insights into disease pathogenesis and the identification of surrogate markers that may guide the development of vaccines. Some successful vaccines have been identified in this way; however, some medically important pathogens do not provide compelling examples of immunity to infection in nature. Such infections as HIV disease, malaria, and tuberculosis are of high medical impact but have thus far proven recalcitrant to immunization. In AIDS, for example, natural immunity to infection is rare: HIV-exposed seronegative sex workers have demonstrated a higher degree of resistance to infection, but they are not completely immune to viral infection (2). Although CTLs can be found in HIV-exposed persons who remain healthy after exposure to HIV-contaminated fluids (3), the role of these cells in protective immunity is not known. Except for these rare examples, the absence of known prototypic immunity for HIV and other infections requires that novel strategies be applied to these challenging problems. For malaria and tuberculosis, evidence indicates that immunization can contain or reduce the severity of disease, but complete resistance to infection is uncommon.

Historically, investigators have exploited different methods for vaccine delivery. These approaches include the use of live-attenuated or nonpathogenic viruses that can generate persistent viral gene expression, which can elicit cellular and humoral immune responses and can perhaps also interfere with virus replication. Second, inactivated viruses have been used to induce immunity as, for example, with the Salk polio or hepatitis A vaccines. Third, recombinant proteins can present viral antigens in the absence of infectious virions and have shown remarkable efficacy for viruses such as hepatitis B. Finally, inactive toxin or toxoids have been used to develop neutralizing antibodies for diseases such as diphtheria. The latter two approaches have often relied on adjuvants to enhance their effects.

GENE-BASED VECTORS

Live-attenuated viruses have provided compelling examples of protection against infection, perhaps best seen with the Sabin polio vaccine. The advantages of live-attenuated viruses are their sustained gene expression, their ability to induce protective immunity, and possible viral interference. The disadvantages are the potential for pathogenic infection, the possibility of enhanced replication during immune suppression, and the consequences of persistent immune stimulation and integration that may occur as a result of sustained viral replication. These vaccines stimulate cytolytic T cells to recognize multiple linear epitopes of viral gene products in infected cells and eliminate virus producer cells, possibly reducing the magnitude of the reservoir of infected cells. Conversely, it is not yet known that CTLs can persist for a sufficiently long period to provide long-lasting protection against infection. There may also be mechanisms to evade immune detection, including the inability to recognize virus in the absence of major histocompatibility complex (MHC) or specific viral mechanisms that reduce MHC expression, as the Nef protein of HIV or E3 gene products of adenovirus. Central to long-lasting protection against many viral infections is the ability to develop broadly neutralizing antibodies, which has proven critical to numerous successful vaccine efforts. Such antibodies are able to neutralize virus, to prevent new infection of cells, and to activate the inflammatory system, findings suggesting that they will be required for highly effective vaccines. Gene-based vaccines offer the potential to stimulate both types of immune responses.

Viral Vectors

Historically, viruses have been used to elicit immune responses that protect against infection. In many instances, these viruses are attenuated from the pathogenic agent, as in the case of the Sabin poliovirus or chickenpox. In other cases, viruses related to the pathogen may cross-react immunologically but do not cause disease, as was the case with modified poxvirus vectors that have built on the success of Jenner. The advantages of such vectors are the extensive experience and established safety in diverse populations. As new vaccines are needed, however, it is more challenging to adapt them to new pathogens because their widespread use may result in the development of antibodies to the vector in large segments of the population, as is the case for vaccinia virus vectors (4).

Over the past several years, new viral vectors have been defined, both from vaccine and gene therapy efforts, that may prove useful in vaccine development. These include additional poxvirus vectors, such as canarypox, fowlpox, and modified vaccinia Ankara (4,5 and 6). The use of uncommon strains of highly immunogenic viruses, such as adenovirus, may also prove useful, particularly when strains are identified that are not reactive with common antibodies (7), which could otherwise neutralize the virus and render it ineffective. Other vectors are also advancing in preclinical development, for example, the Sindbis (8,9) and Venezuela equine encephalitis virus alphaviruses (10,11). Replication-defective vectors derived from these viruses can synthesize high levels of recombinant gene products and, in some cases, can target delivery to dendritic cells. Finally, there is considerable interest in the identification of vectors that may allow for persistent transgene expression to provide continuous T-cell stimulation. Advances in lentiviral vectors have suggested that they, too, can be modified to address numerous safety concerns (12,13 and 14). In addition, adenoassociated virus has been useful for long-term gene expression and is the subject of investigation (15,16). Advances in molecular virology and vector development should allow rapid adaptation of newly discovered viruses for vaccine development.

Nonviral Vectors

Injection of plasmid DNA into muscle was shown to direct the synthesis of recombinant proteins (17), a finding suggesting that this gene transfer method would be useful for vaccination. Genetic immunization has been demonstrated in several model systems (18,19 and 20). More important, it has been shown to confer protective immunity to different infectious diseases in rodents, including influenza virus (21), malaria (22,23,24 and 25), tuberculosis (26), Ebola virus (27), rabies (28), lymphocytic choriomeningitis virus (29,30), and herpes simplex virus (31). It has also been applied to therapeutic immunization strategies for autoimmune and allergic diseases. This technology is now being refined in many ways.

Traditional vaccines composed of protein, peptides, or virus particles have been used to raise a specific immune response against the immunogen. DNA vaccines represent a more recent class of vectors that express protein immunogens *in vivo* (32,33). These plasmids allow eukaryotic cell gene expression and typically contain a prokaryotic origin of replication, a drug resistance marker for selection of the plasmid in bacteria, a strong eukaryotic promoter to drive expression of the immunogen gene, and a eukaryotic signal for polyadenylation following the immunogen gene. On injection, cells internalize the plasmid and transport the DNA to the nucleus, but they do not appear to integrate substantially into cellular chromosomes. The cellular transcription machinery transcribes the plasmid DNA in the nucleus to produce

mRNA encoding the immunogen, and different eukaryotic promoters, as well as autostimulatory polymerase regulatory elements, which are used to optimize the magnitude and duration of gene expression of the immunogen.

DNA vaccines have several potential advantages compared with traditional vaccines. In contrast to peptide vaccines, the entire protein is expressed from the DNA vaccine vector, and this allows processing and presentation of more epitopes to produce a better immune response in individuals with different MHC alleles. Compared with recombinant protein vaccines produced in bacteria or yeast cells, the proteins expressed from a DNA vaccine vector are more likely to fold into a native conformation. In addition, immunogens expressed from the DNA vectors undergo posttranslational modification. Appropriate posttranslational modification such as glycosylation or phosphorylation produces an immunogen more relevant to native protein for which immune protection is sought. Myocytes that express the DNA vaccine protein function poorly as APCs because they lack MHC class II antigens necessary to generate helper T cells (34). Although myocytes express low amounts of class I MHC, they lack other costimulatory molecules necessary to stimulate cellular immunity (33,35). Several studies, using bone marrow chimeras and reconstituted SCID (severe combined immunodeficiency) mice, have established that bone marrow–derived cells are required for antigen presentation after DNA injection into muscle (36,37), and peptide transporters are required in the APCs for cross priming *in vivo* (38). It appears that such APCs are recruited to the transfected myocytes through local inflammation (39), and these APCs present the antigens encoded by the DNA vectors (40). DNA vaccines can be delivered to the epidermis by either needle or gene gun delivery (18,41). Unlike intramuscular injection, intradermal injection causes little inflammation that would recruit APCs; however, professional APCs are abundant in the skin as tissue macrophages, Langerhans cells, and dendritic cells. Dendritic cells may be particularly important because of their ability to activate T cells (42,43). To generate mucosal immunity by DNA vaccination, it may be necessary to deliver the DNA at a mucosal surface using liposomes, microspheres, or other carriers.

Previous experiments suggested that a palindromic hexamer (AACGTT) found twice in the ampicillin resistance gene of many plasmid DNAs is an immune stimulatory sequence that favors a T-helper 1 (Th1)-type response (44). The coinjection of either cytokines or vectors expressing costimulatory molecules can also shift the type of immune response. The administration of granulocyte-macrophage colony-stimulating factor (45), B7 expression vectors (46,47), or other cytokine genes (48), together with a DNA vaccine vector, can increase antibody production. Thus, the dose of DNA vectors given, the route of injection, and the coinjection of immune system modulators can all have an effect on the type of immune response that is generated. Finally, the inclusion of inert carriers, such as liposomes (49), sustained release formulations, or *in vivo* electroporation (50), likely enhances the efficacy of this vaccine gene delivery strategy.

PROSPECTS FOR IMPROVED VACCINES AND CLINICAL TRIALS

Critical to the development of new vaccines is the ability to analyze vaccine candidates in humans. The ability of DNA vaccines to elicit potent cellular immune responses safely makes them strong candidates as a component of vaccines in humans; however, these responses have not been so strong that, on their own, they can be expected to be highly effective in clinical settings. In the absence of clear examples of natural immunity to an infectious agent, it is important to test the efficacy of different vaccine strategies and to compare candidates. The necessary safety studies are best done with rigorous immunologic analyses. Selected candidates can then be tested in relevant clinical populations. Phase III trials thus become the ultimate test of efficacy and form the basis of hypothesis-driven clinical research: the trial tests the hypotheses that a specific immune response elicited by a vaccine candidate can be successful in preventing infection in humans. The relatively complex nature of this effort requires multidisciplinary teams that cover a range of basic science and clinical disciplines.

New Technology and the Identification of Immunogens

To develop effective immunogens that elicit broadly neutralizing antibody responses, structural biology will increasingly contribute to rational approaches to this problem. In the case of HIV, x-ray crystallographic studies from the laboratories of Sodroski and Hendrickson (51) have yielded important structural data on gp160/gp120. The interactions of CD4 with gp120 and the recessed nature of the CD4 binding site provide better understanding required for the generation of broadly neutralizing antibodies. The molecular definition of the helical coiled-coil region (52) has defined highly conserved epitopes of the viral envelope critical to its function that are attractive targets for neutralizing antibodies. These considerations apply to challenging vaccine targets from other infectious agents. Other rational approaches to the identification of immunogens include rational mutagenesis using molecular genetic modification of target genes. With advances in recombinational polymerase chain reaction (53,54 and 55), it is also possible to generate large libraries of immunogens that may be screened for mutant versions of normal viral proteins that display enhanced immunogenicity.

Genomics, Informatics, and Vaccine Development

The use of genomics and bioinformatics can provide unprecedented opportunities for the clinical studies of vaccines. Genetic information within individual gene products determines alternative immune responses. Such is the case, for example, with gp160 and Nef. Identical immunization protocols for these two alternative gene products induce distinct immune responses for each, predominantly CTLs for env and antibodies for Nef. The control of this response is determined by the amino acid sequence of these proteins, and it should therefore be possible to define immune responses based on genetic sequence of the immunogen. By delineating such relationships, vaccine technology will be advanced.

Genomic analysis of the infectious agent has considerable potential to assist in vaccine design. The emerging genomic sequences of such pathogens as malaria (56) or *Haemophilus influenzae* (57,58) has allowed a focused analysis of target genes and provides a foundation that will allow greater understanding of their expression and role in disease pathogenesis. This information may assist in the identification of immunogens and the systematic analysis of combination gene vaccines that may prove helpful in vaccination.

Opportunities for the judicious use of genotyping in human trials will also facilitate the identification of genes that determine antigen responsiveness in human populations. This information must be collected in a way that maintains patient confidentiality, but it will provide opportunities to make vaccines more effective in specific persons and can be applied to larger populations. It will also provide insight into the genetic control of the immune response in humans.

Analysis of Human Immune Responses

Critical to the interpretation of human clinical studies is the analysis of human immune responses. Progress has been made in the development of assays that allow reproducible measurement of immune responses within and among persons. These evaluations are required for identification of vaccine candidates that induce strong immune responses that could potentially confer protection. Although CTL assays have been used for this purpose, this assay is relatively cumbersome, costly, and time-consuming. These analyses cannot be readily performed in large patient populations, thus making it impractical, particularly for phase III vaccine trials. Two assays have shown promise in their ability to quantify human immune responses. These approaches include the cytokine enzyme-linked immunosorbent assay, which provides a broad, sensitive, and consistent measure of immune function. More recently, another excellent analytic tool has become evident: analysis of intracellular cytokine induction by flow cytometry provides insight into the expression of immunologically activated genes and also into the analysis of T-cell subsets that are stimulated by different immunogens (59). It is likely that further refinements of immune assays will continue, although it may take time to understand their significance and relationship with conventional assays.

In performing large-scale clinical efficacy trials, it is important to anticipate the implications of such studies. Although sterilizing immunity, the ability to prevent infection entirely, may be observed, partial immunologic protection may nonetheless be useful. Partial protection could have several consequences. For example, complete protection may occur in a fixed percentage of vaccinees. Conversely, reduced disease pathogenicity or transmission could be seen with the same frequency as in unvaccinated control subjects. Yet another possibility is that vaccination could alter the disease course. For example, the infection could be converted from an aggressive to a more asymptomatic form, or the set point of viral load could be reduced. It will be critical to define these possible alteratives in the natural history of the disease that may be caused by vaccination. Animal models may serve this purpose well. For example, if a benign disease course is not accompanied by a reduction in transmissibility, a serious problem may ensue. Informed decisions about the appropriateness of vaccine candidates in large patient populations will be needed. Despite the urgent need to evaluate specific vaccines in humans, rigorous safety studies will be required.

Certain critical hurdles remain to be addressed in vaccine development. The field has progressed significantly and is poised to make significant advances. Immunogens have been identified that induce CTL responses, and it will become easier to assess their efficacy in human studies. Crystallographic structures have provided insight relevant to the development of broadly neutralizing antibodies. Immunologic methods to understand correlates of protection in humans and nonhuman primates have improved. Rigorous human trials, the need to prepare clinical-grade vaccine, and the need to improve methods to assess immune responses in humans will remain. The involvement of the biotechnology and pharmaceutical industries in vaccine production will be essential to these efforts, because it will be extremely difficult to develop, market, and distribute effective vaccines without their specialized infrastructure and expertise. The collective efforts of academia, industry, and government provide important synergy that will be needed to advance the field. This work on vaccines for infectious diseases has implications that extend beyond specific pathogens. This research not only will apply to other emerging viruses, parasites, and bacteria that threaten human populations, but also it has important potential for treatment and prevention of cancer, neurologic diseases, cardiovascular diseases, allergies, and autoimmunity. Expanded vaccine efforts may help to prevent outbreaks of infectious diseases and to set the stage for future treatments for a range of human diseases.

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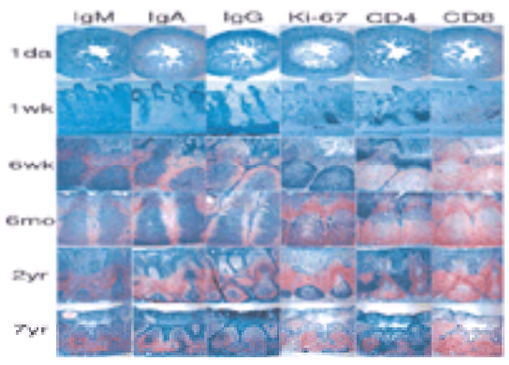
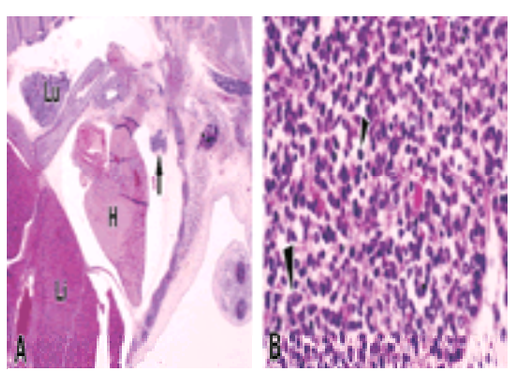
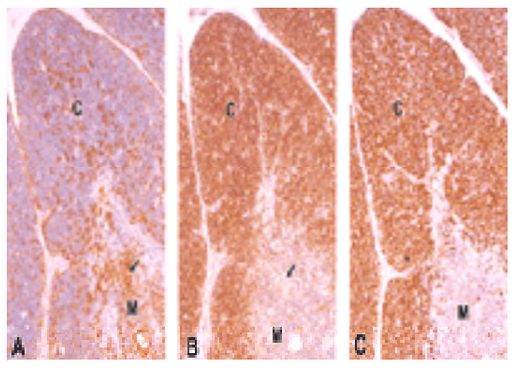
Volume 1 Color Figures



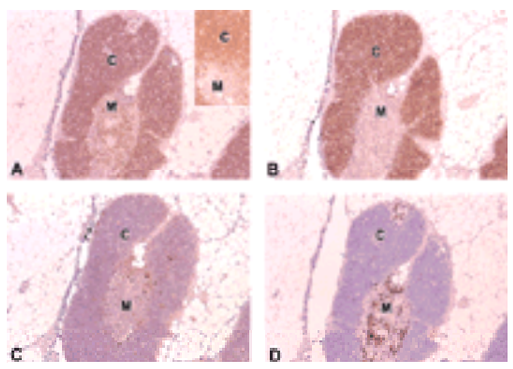
Color Figure 2.3. The rabbit appendix at various times after birth. The basic structure of the appendix is similar to that of the bursa of Fabricius (Fig. 2.2), with numerous ovoid or trapezoidal follicles containing B lymphocytes (B) in apposition to a supporting follicular epithelium. The development of follicular structure begins after birth and depends on the presence of a gut flora. With time, the follicles enlarge, reaching a maximum in young adult animals and then regressing with advancing age. The appendix contains well-described perifollicular T-cell zones, and $CD4^+$T lymphocytes (T) are scattered about in secondary follicles or germinal centers (GC). These germinal centers contain light and dark zones (LZ, DZ) (original magnification ×100). (From Dasso JF, Obiakor H, Bach H, et al. A morphological and immunohistochemical study of the human and rabbit appendix for comparison with the avian bursa. *Dev Comp Immunol* 2000;24:797–814, with permission.) (This figure is printed in black and white as Figure 2.3.).



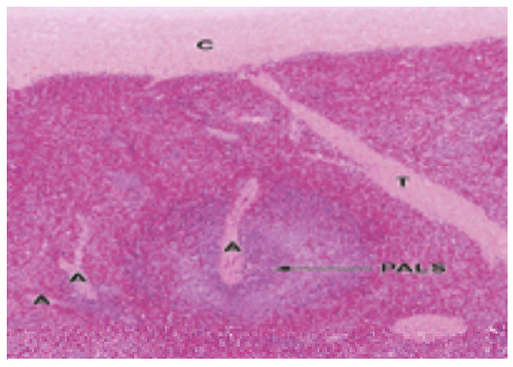
Color Figure 2.4. Human thymus at 8 weeks, gestation. **A:** The developing thymus is located in the anterior mediastinum overlying the heart (H). The position of the lung (Lu) and liver (Li) are also shown. **B:** At this stage of development, both lymphocyte precursors *(small arrowhead)* and epithelial cells *(large arrowhead)* are present; however, a distinction between cortex and medulla will not become apparent until week 14 of gestation (hematoxylin and eosin stain; original magnification ×3 in **A** and ×132 in **B**). (This figure is printed in black and white as Figure 2.4.).



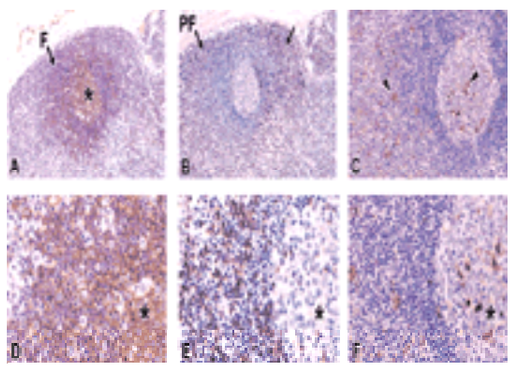
Color Figure 2.5. Architecture of the pediatric human thymus. Immunoperoxidase staining highlights cell types present in thymic cortex (C) and medulla (M). *Arrows* point to Hassall bodies. Cytokeratin antibodies highlight the meshwork of thymic epithelium **(A),** which is more attenuated in the cortex as compared with the medulla. The immature thymocytes present in the cortex can be uniquely identified as those cells reactive with antibodies specific for CD1a **(B)** and for the Ki-67 nuclear proliferation antigen **(C).** *Brown* indicates positive reaction (original magnification ×33). (This figure is printed in black and white as Figure 2.5.).



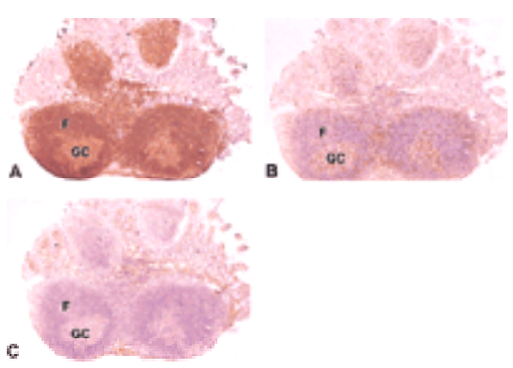
Color Figure 2.6. Architecture of the adult human thymus. Age-related thymic atrophy results in an increase in adipose tissue within the perivascular space (seen on the **left** and the **right** of each panel) concomitant with a decrease in the thymic epithelial space that is involved in production of new thymocytes. The architecture of the remaining cortex (C) and medulla (M) is relatively unaltered. Immunoperoxidase staining identifies the phenotype of cells present in the cortex and medulla. **A:** CD3 stain (T cells); the **inset** shows $CD8^+$ cells (immature cortical and a subset of medullary thymocytes). **B:** CD1a (immature cortical thymocytes and medullary dendritic cells).**C:** CD68 (macrophages). **D:** CD20 (B cells). *Brown* indicates positive reaction (original magnification ×16; ×40 for the **inset**). (This figure is printed in black and white as Figure 2.6.).



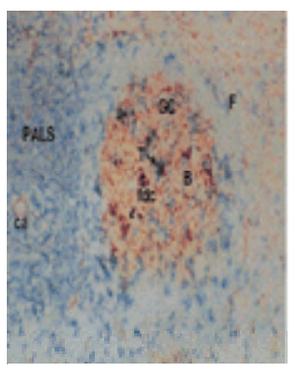
Color Figure 2.7. Architecture of the human spleen. The spleen is surrounded by a fibrous capsule (C) that invaginates to form trabeculae (T). The white pulp is arranged concentrically around arterioles (A) to form the periarteriolar lymphoid sheath (PALS) (hematoxylin and eosin stain; original magnification ×16). (This figure is printed in black and white as Figure 2.7.).



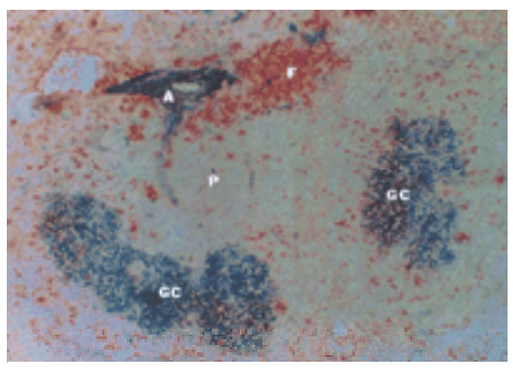
Color Figure 2.8. A–F: Architecture of the human lymph node. Lymphocytes within lymph nodes are organized into B-cell–rich follicles (F in **A**) and T-cell–rich perifollicular zones (PF in **B**). *Arrowheads* point out representative macrophages in **C.** Germinal centers are denoted by an *asterisk* in **A** and **D–F.** Immunoperoxidase stains identify specific cell types present in various lymph node regions. **A, D:** CD20 (B cells); **B, E:** CD3 (T cells); **C, F:** CD68 (macrophages). *Brown* indicates a positive reaction (original magnification ×33 for **A–B,** ×66 for **C,** and ×132 for **D–F.**) (This figure is printed in black and white as Figure 2.8.).



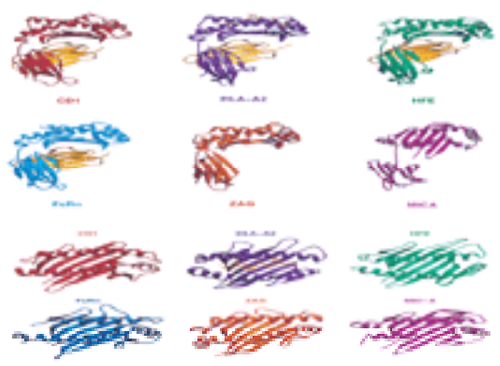
Color Figure 2.9. A–C: Architecture of Peyer patch. Peyer patches consist of organized aggregates of B-cell follicles (F) with germinal centers (GC) and intrafollicular zones of T cells that are present within the lamina propria of small bowel. Immunoperoxidase stains identify the locations of B cells (**A,** CD45RAB/B220 stain), helper-T cells (**B,** CD4/GK1.5 stain), and macrophages (**C,** F4/80 stain) (original magnification ×16). (This figure is printed in black and white as Figure 2.9.).



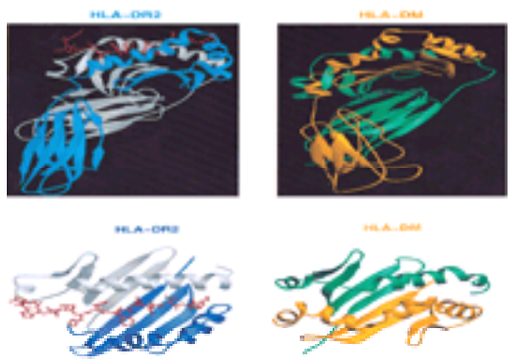
Color Figure 2.10. Germinal center reaction in murine spleen. Immunization with most thymus-dependent antigens initiates the germinal center (GC) reaction. GCs form within the B-cell follicle (F) by the expansion of antigen-specific T- and B-cell immigrants (T, B) from the periarteriolar lymphoid sheath (PALS). Both lymphocyte types proliferate in the reticulum of follicular dendritic cells (fdc) forming a distinct histologic structure containing light and dark zones (LZ, DZ). Original magnification ×200. Thus, the primary humoral immune response begins with antigen-dependent activation of lymphocytes [C, C']. Activated T- and B-lymphocytes then meet along the border of the PALS and follicle [D] where cognate interactions drive proliferation in both lymphocyte compartments. Some daughter B-lymphocytes remain adjacent to the T-cell area and differentiate into foci of antibody-secreting plasmacytes [E]. Other, clonally related, B-cells migrate along with specific T-cells back into the follicle and lodge in the processes of fdc where the GC reaction begins. Modified from ref. 71. (This figure is printed in black and white as Figure 2.10.).



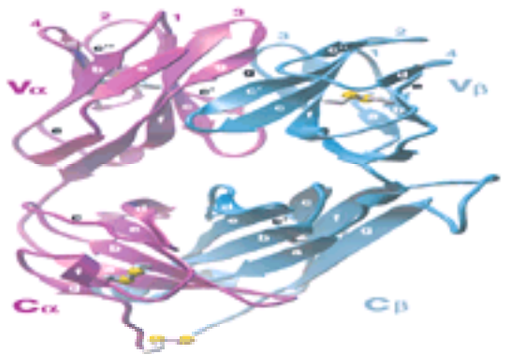
Color Figure 2.11. Plasmacytic foci and germinal centers in murine spleen. Following a period of proliferation near the periphery of the splenic T-cell zone, the periarteriolar lymphoid sheath (P), antigen-specific B lymphocytes differentiate into plasmacytes (F) and remain in close approximation to the T-cell zone. These large foci of AFC produce the earliest serum antibody and the plasmacytes within them may undergo IgM®IgG class switching but do not support V(D)J hypermutation. Original magnification ×200. From ref. 119. (This figure is printed in black and white as Figure 2.11.).



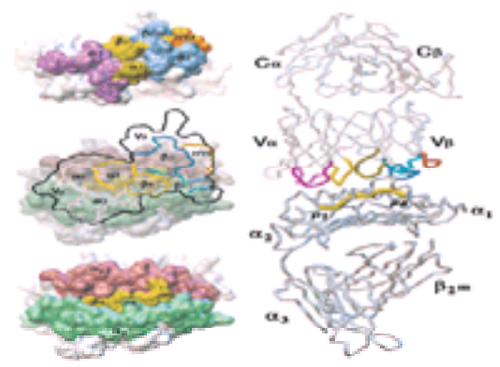
Color Figure 3.2. A: Structure of major histocompatibility complex (MHC) class I products and related structures. The HLA-A2 structure is representative of the so-called classic class I products, involved in presentation of peptide to T-cell receptors. The CD1 molecule presents lipid antigens. The HFE product interacts with and modifies internalization of the transferrin receptor. The neonatal Fc receptor (FcRn) is involved in transepithelial transport of immunoglobulins in the gut of the newborn. The ZAG protein is a zinc-binding serum protein. The MIC-A product is encoded by a gene linked to the MHC (Fig. 3.1) and is probably involved in antitumor immunity and eradication of cells subjected to stress. Note the presence of the common light chain, b_2-microglobulin, in CD1, HLA-A2, HFE, and FcRn. **B**: As in **A,** but structures viewed from above. Note the presence of the peptide in the cleft of the HLA-A2 molecule. (This figure is printed in black and white as Figure 3.2.).



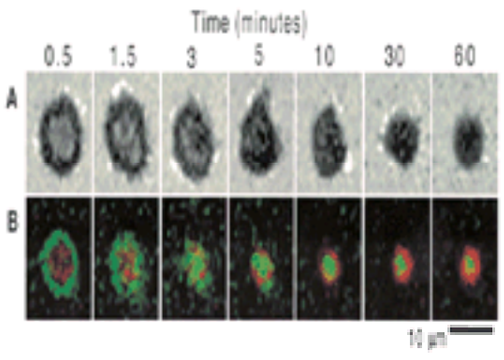
Color Figure 3.3. A: Comparison of the structure of the class II molecule HLA-DR2 and of the DM molecule, a catalyst involved in the peptide loading of major histocompatibility complex (MHC) class II molecules. **B**: As in **A,** viewed from above. (This figure is printed in black and white as Figure 3.3.).



Color Figure 4.2. T-cell receptor (TCR) structure. Ribbon diagram of the structure of an ab TCR heterodimer (extracellular portion). b Strands are indicated in *letters*, and complementarity-determining region (CDR) loops in *numbers*. The *yellow spheres* depict disulfide bonds within the V and C domains and joining the a and b chains. (From Garcia KC, Decagon M, Stanfield RL, et al. An ab T-cell receptor at 2.5Å and its orientation in the TCR-MHC complex. *Science* 1996;274:209–219, with permission). (This figure is printed in black and white as Figure 4.2.).

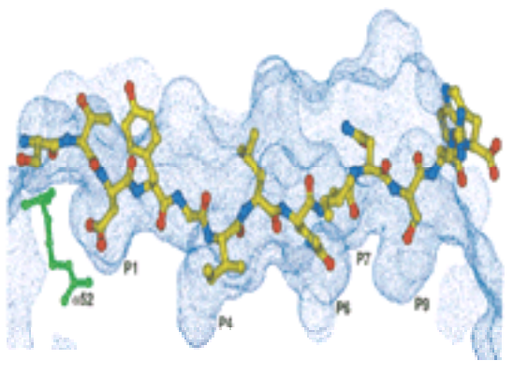


Color Figure 4.4. T-cell receptor (TCR)/peptide/major histocompatibility complex (MHC). The structure of an ab TCR binding to peptide/MHC. (From Garcia KC, Decagon M, Stanfield RL, et al. An ab T-cell receptor at 2.5Å and its orientation in the TCR-MHC complex. *Science* 1996;274:209–219, with permission.) (This figure is printed in black and white as Figure 4.4.).

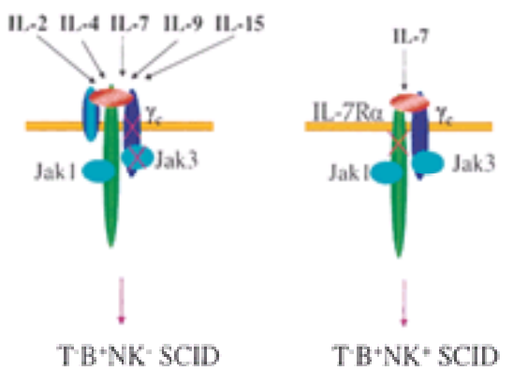


Color Figure 4.5. Formation of an immunologic synapse. An antigen-specific T cell **(A)** contacts an artificial lipid bilayer containing labeled major histocompatibility complex (MHC; *green*) and intercellular adhesion molecule (ICAM-1; *red*). Within minutes, it has recognized the antigen contained within the MHC and gathered the MHC and ICAM-1 into the distinctive “bulls-eye” pattern. (From Grakoui A, Bromley SK, Sumen C, et al. The immunological synapse: a molecular machine controlling

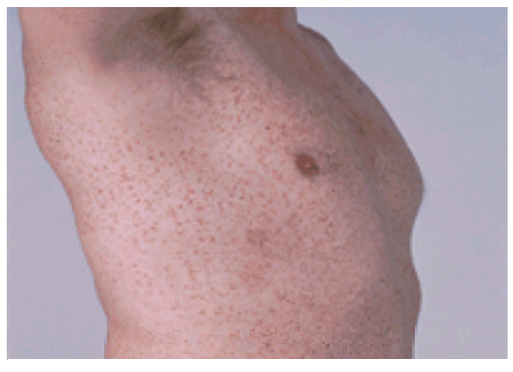
T-cell activation. *Science* 1999;285:221–227, with permission.) (This figure is printed in black and white as Figure 4.5.).



Color Figure 6.1. Peptide 52-61 of hen egg white lysozyme binds to I-A^k. **A:** Side view of the peptide. **B:** Ribbon diagram of the peptide as it is found on the I-A^k protein. The sequence of 52-61 is DYGILQINSR. The Asp52 residue is responsible for the selection of the peptide from HEL as well as in the binding affinity. Asp52 forms a salt-bridge with Arga52 situated at the base of the P1 pocket. Mutation of Asp52 to Ala52 reduces binding affinity severalfold. (Fremont DH, Monnaie D, Nelson CA, et al. Crystal structure of I-A^k in complex with a dominant epitope of lysozyme. *Immunity* 1998;8:305–317, with permission.) (This figure is printed in black and white as Figure 6.1.).

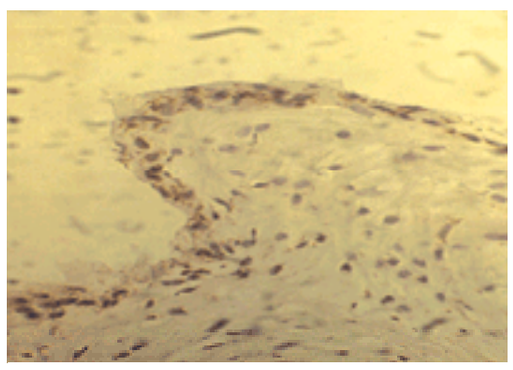


Color Figure 28.5. Cartoon showing on the left side that Janus kinase 3 (Jak3) is the major signal transducer for the common gamma chain (g_c) shared by multiple cytokine receptors. Mutations in the gene encoding g_c cause SCID-X1 and in the gene encoding Jak3 cause a form of autosomal recessive SCID that mimics SCID-X1 phenotypically. On the right side, it is seen that mutations in the gene encoding the IL-7 receptor a chain also result in another form of autosomal recessive SCID. The latter suggests that the reason for the severe T-cell defect in SCID-X1, and Jak3-deficient SCID is failure to signal through the IL-7 receptor. (Courtesy of Dr. Warren Leonard.) (This figure is printed in black and white as Figure 28.5.).

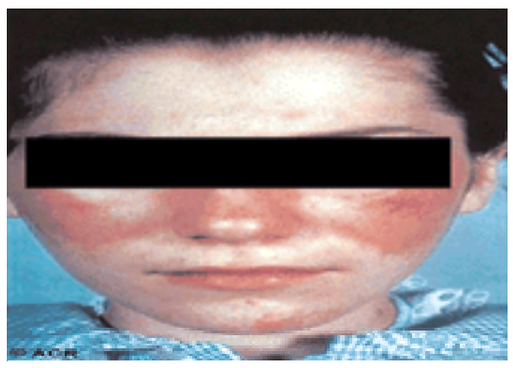


Color Figure 36.1. Urticaria pigmentosa in an adult patient with indolent disease. (This figure is printed in black and white as Figure 36.1.).

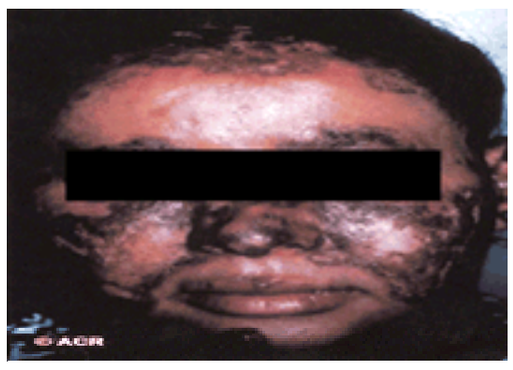
Volume 2 Color Figures



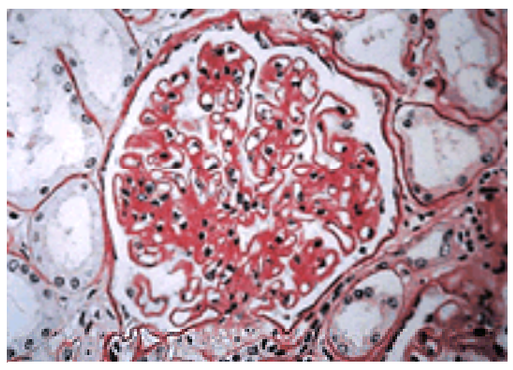
Color Figure 37.5. Noninflammatory osteoarthritis synovial membrane showing two or three layers of lining cells stained for stromal cell–derived factor-1 (SDF-1). (This figure is printed in black and white as Figure 37.5.).



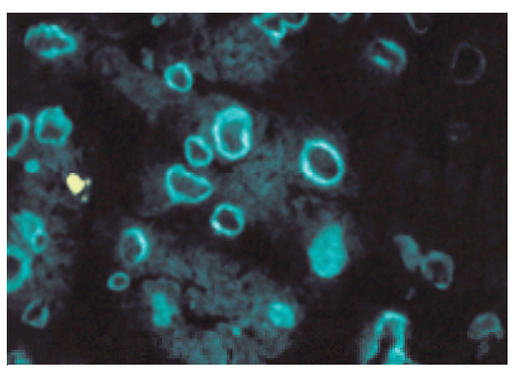
Color Figure 39.1. Malar rash. An erythematous facial eruption on the malar and chin areas. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (This figure is printed in black and white as Figure 39.1.).



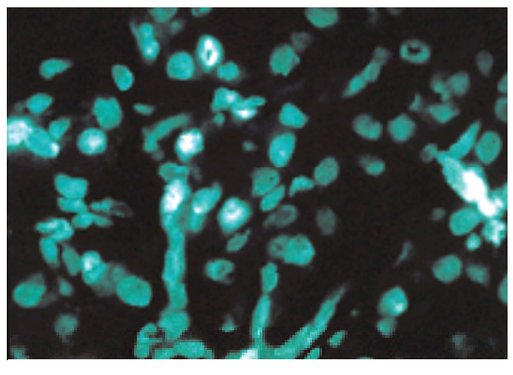
Color Figure 39.2. Discoid lupus. An erythematous and hyperpigmented rash on the face with central scarring and epidermal atrophy. Follicular plugging is also seen. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (This figure is printed in black and white as Figure 39.2.).



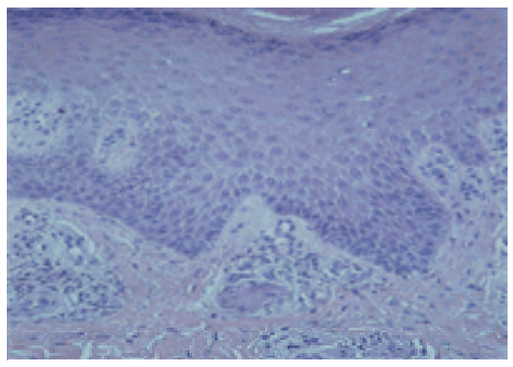
Color Figure 39.6. Membranous glomerulonephritis. Widespread, uniform thickening of capillary basement membranes of the glomerulus is seen on periodic acid-Schiff staining. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (This figure is printed in black and white as Figure 39.6.).



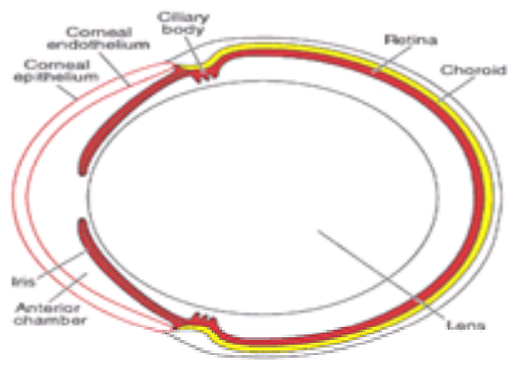
Color Figure 39.7. Antinuclear antibody: Rim (peripheral) pattern. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (This figure is printed in black and white as Figure 39.7.).



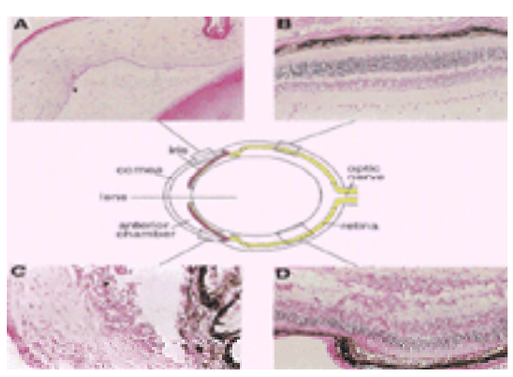
Color Figure 39.8. Antinuclear antibody: Homogeneous (diffuse) pattern. (Reprinted from the Clinical Slide Collection on the Rheumatic Diseases, American College of Rheumatology, with permission.) (This figure is printed in black and white as Figure 39.8.).



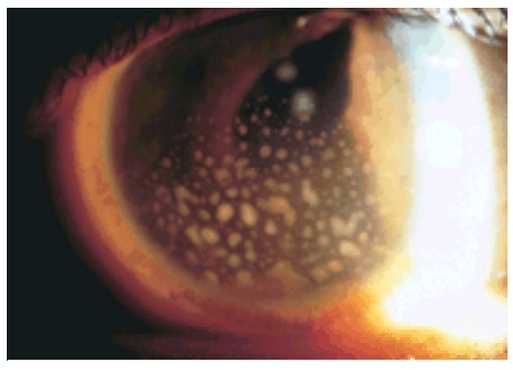
Color Figure 46.2. Cutaneous small vessel thrombosis in the antiphospholipid syndrome. Skin biopsy from the painful skin nodules of a 33-year-old woman with anticardiolipin, lupus anticoagulant, anti–b_2-glycoprotein I, deep venous thrombosis, and miscarriage. The hematoxylin and eosin–stained section of a skin biopsy shows a small dermal vessel in the center of the lower third of the frame that contains bland fibrin thrombus without inflammation. (Courtesy of Dr. K. Georgeous.) (This figure is printed in black and white as Figure 46.2.).



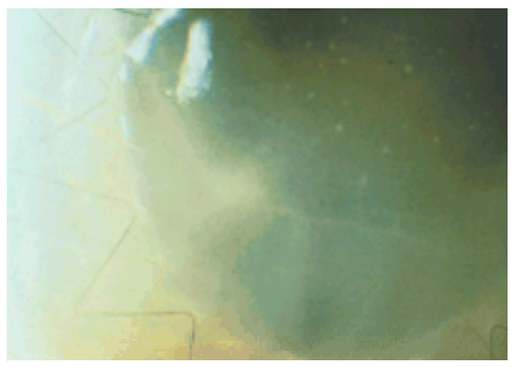
Color Figure 53.1. Fas ligand (FasL) expression. Schematic drawing of a mouse eye showing localization of FasL (FasL tissues in red). FasL is expressed on the corneal endothelium and epithelium, iris, ciliary body, and throughout the retina, forming a protective barrier (From Griffith TS, Brunner T, Fletcher SM, et al. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 1995;270:1189–1192, with permission.) (This figure is printed in black and white as Figure 53.1.).



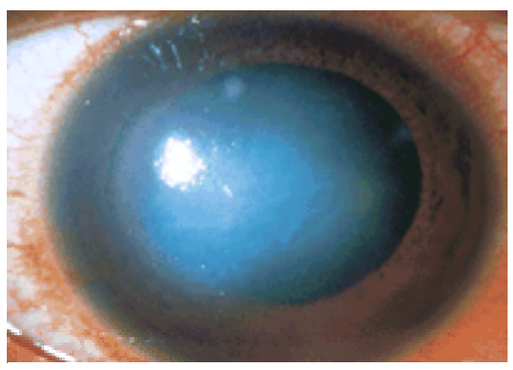
Color Figure 53.2. The consequences of not expressing functional FasL in the eye. B6 (**A, B**), B6-*gld* (**C, D**) mice were injected in the anterior chamber of the eye with 2.5×10^4 herpes simplex virus (HSV)-1 (KOS) as described (From Griffith TS, Brunner T, Fletcher SM, et al. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 1995;270:1189–1192, with permission.) Eyes were removed 10 days after infection, processed for paraffin sections, and stained with hematoxylin and eosin (H&E). *Boxes* on the schematic drawing of the eye (**center**) denote the area of detail for the H&E stain (× 200). (This figure is printed in black and white as Figure 53.2.).



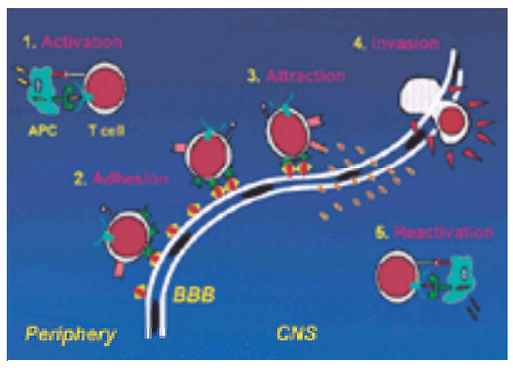
Color Figure 53.3. Typical appearance of granulomatous anterior uveitis on the cornea of a 46-year-old African-American woman with sarcoidosis. The corneal endothelium is covered in dense keratic precipitates, composed of epithelioid cells in small noncaseating granulomas. (This figure is printed in black and white as Figure 53.3.).



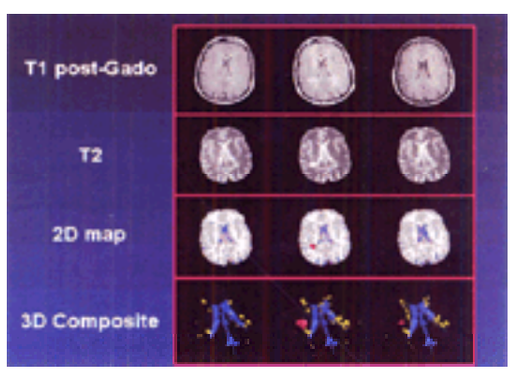
Color Figure 53.4. Allotypic corneal graft rejection. The *white line* in the graft represents an infiltrate of host lymphocytes. Very subtle rejection can be readily seen because of the transparency of the cornea. Despite routine use of non–human leukocyte antigen (HLA) matched donors and minimal immunosuppressive medication, corneal allografts are rejected in fewer than 10% of cases. (This figure is printed in black and white as Figure 53.4.).



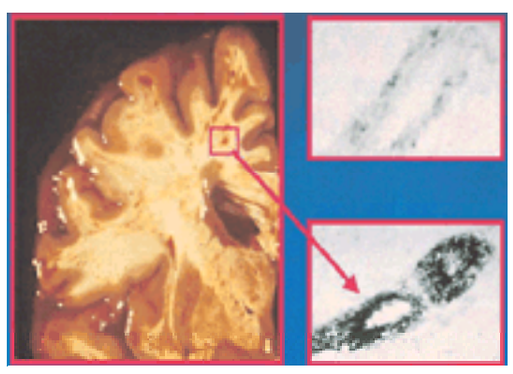
Color Figure 53.5. Herpetic stromal keratitis. After corneal infection with herpes simplex type 1 (HSV-1), a subset of patients subsequently develops a chronic, autoimmune keratitis. Herpesvirus is not recovered from these lesions. Murine models of this condition suggest that molecular mimicry between a corneal antigen and an HSV-1 coat protein causes the autoimmune keratitis. HSV-1 mutants lacking this antigen cannot trigger stromal keratitis, and mice made tolerant to this antigen are likewise protected from this disease. (This figure is printed in black and white as Figure 53.5.).



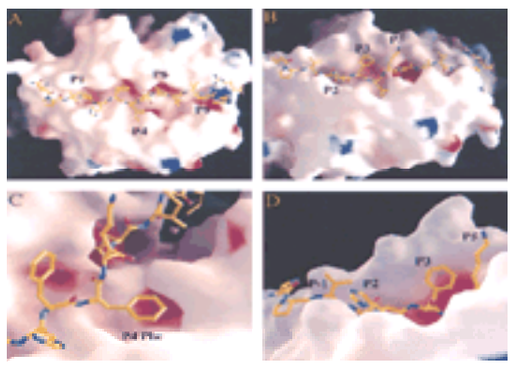
Color Figure 56.1. A model of multiple sclerosis (MS) immunopathogenesis. Myelin autoreactive T cells are found in an enhanced state of activation in the circulation of patients with MS. Upregulation of adhesion molecules *(1)*, chemoattraction *(2),* and elaboration of matrix proteinases *(3)* results in invasion of activated autoreactive T cells across the blood–brain barrier *(4)* (BBB). Inside the central nervous system (CNS), reactivation of T cells by local or infiltrating antigen-presenting cells (APCs) results in release of proinflammatory and cytotoxic mediators and leads to tissue injury. (This figure is printed in black and white as Figure 56.1.).



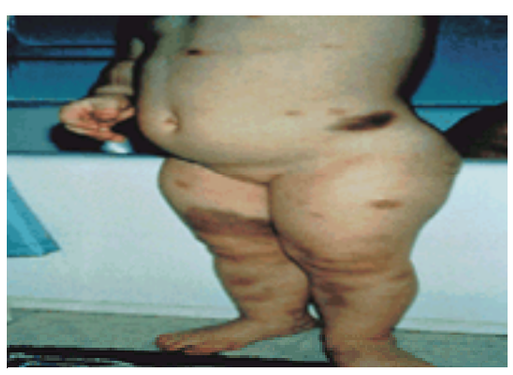
Color Figure 56.3. Serial magnetic resonance imaging (MRI) studies in multiple sclerosis (MS) highlight the dynamic disease pathophysiology. Serial brain MRI studies of a single patient with MS were taken at three different times **(left column:** study performed day 0; **middle column:** day 266; **right column:** day 362). **Top row:** Axial T1-weighted sequences obtained after gadolinium-contrast administration. Corresponding anatomic levels are compared at the three time points. A ring-enhancing lesion, absent in the first scan, is present on day 266 and disappears again by day 362. **Second row:** T2-weighted images at the same anatomic level demonstrate the appearance and subsequent shrinkage of this lesion, which remains visible on T2, but no longer enhances, at day 362. **Third row:** Computed algorithms enable the construction of accurate two-dimensional (2D) maps that distinguish and quantify white matter *(white),* gray matter *(gray),* ventricles *(blue),* and lesions *(yellow).* The ring-enhancing lesion is red. **Bottom row:** Three-dimensional (3D) composite model of the whole brain. The gray matter and white matter are stripped away to reveal the dynamic changes in lesional activity and burden over time (see text for further discussion). (From Weiner HL, Guttmann CR, Khoury SJ, et al. Serial magnetic resonance imaging in multiple sclerosis: correlation with attacks, disability, and disease stage. *J Neuroimmunol* 2000;104:164–173, with permission from Elsevier Science.) (This figure is printed in black and white as Figure 56.3.).



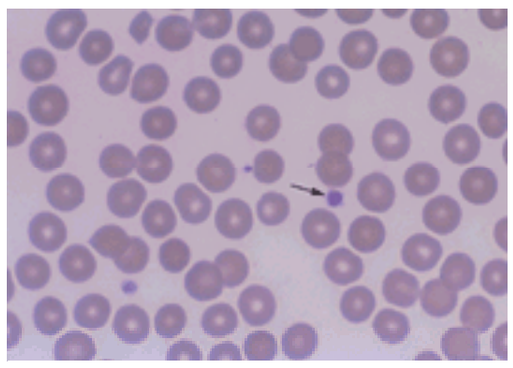
Color Figure 56.4. The pathology of multiple sclerosis (MS). **Left**: Coronal section of a cerebral hemisphere of a patient with MS. Multiple areas of discoloration, such as the one outlined in the **box,** are seen in the periventricular and subcortical regions and represent the classic MS plaques. **Lower right:** Microscopic view of a cryostat section from outlined subcortical plaque, stained with anti-CD4 antibody (magnification ×98). The hallmark perivascular inflammatory infiltrate is demonstrated. **Upper right:** Antikeratin control stain. See the text for a discussion of the pathologic heterogeneity of MS lesions. (From Windhagen A, Newcombe J, Dangond F, et al. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J Exp Med* 1995;182:1985–1996, with permission of the Rockefeller University Press.) (This figure is printed in black and white as Figure 56.4.).



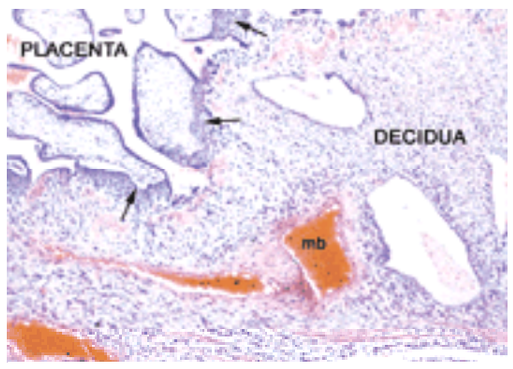
Color Figure 56.5. Structure of the HLA-DR2–myelin basic protein (MBP) peptide complex. **A** and **B**: Top and side views of the complex; 14 residues are included for the MBP peptide. P1 and P4 are hydrophobic pockets in the DR molecule and are occupied by the P1 Val and the P4 Phe of the MBP peptide, respectively. These interactions anchor the peptide in the groove. Peptide atoms are shown as ball-and-stick structures. **C**: Close-up view of the P4 pocket. The Gln b70 of the DR2 molecule (not labeled) is positioned over the P4 Phe of the MBP peptide. **D**: The T-cell receptor contact residues of the MBP peptide. P2 His, P3 Phe, and P5 Lys (previously shown to be important for T-cell recognition of the MBP peptide) are prominent, solvent exposed residues. (From Smith KJ, Pyrdol J, Gauthier L, et al. Crystal structure of HLA-DR2 (DRA*0101, DRB1*1501) complexed with a peptide from human myelin basic protein. *J Exp Med* 1998;188:1511–1520, with permission of the Rockefeller University Press.) (This figure is printed in black and white as Figure 56.5.).



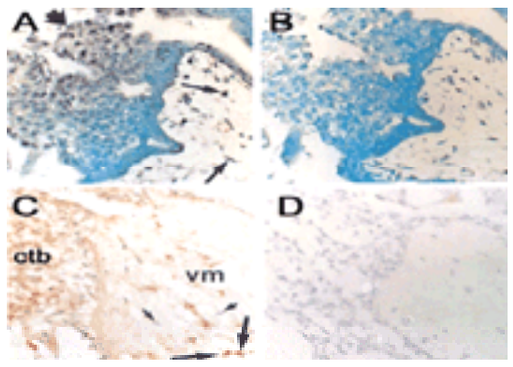
Color Figure 58.1. Large bruises (purpura) on the trunk and extremities in a young child with immune thrombocytopenia. (This figure is printed in black and white as Figure 58.1.).



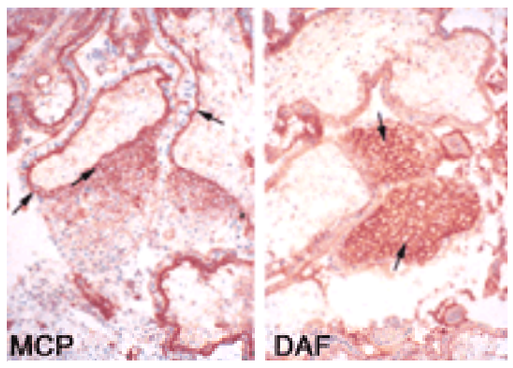
Color Figure 58.2. Peripheral blood smear of a patient with immune thrombocytopenia. Few platelets are present, but those that are newly released into the circulation (*arrow*) are larger than the normal diameter of 1 to 2 µ. (This figure is printed in black and white as Figure 58.2.).



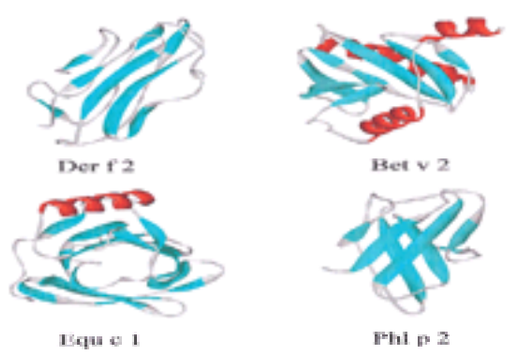
Color Figure 60.5. Histologic section of a gestation week 8 human placenta showing attachment and invasion of the decidua by extravillous trophoblast cells emerging from villi (*arrows*). Modified maternal arteries surrounded by fibrinoid maternal and containing pools of maternal blood (mb) mark the implantation site in the decidua. Stained with hematoxylin and eosin. Original magnification, ×100. (From the Boyd Collection, Department of Anatomy, Cambridge University, courtesy of G. Burton.) (This figure is printed in black and white as Figure 60.5.).



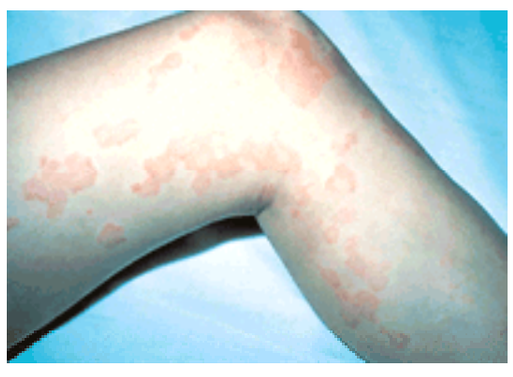
Color Figure 60.9. *In situ* hybridization and immunohistochemical staining demonstrate that human leukocyte antigen-G (HLA-G) mRNA and protein are present in discrete populations of cells in first-trimester placentas. **A**: HLA-G mRNA is evident in migrating trophoblast cells (*large arrow*) as well as macrophage-like villous stromal cells (*smaller arrows*). **B**: A sense version of the HLA-G probe does not hybridize to placental tissues. **C**: HLA-G protein is identified using the mouse monoclonal antibody, 87G, specific for HLA-G1. Signal is present in migrating cytotrophoblast cells (ctb) and in some villous mesenchymal cells (vm). **D**: The immunostaining control is negative. Paraformaldehyde-fixed tissue was used for (A,B), and flash frozen, acetone-fixed tissue was used for (C,D). Original magnifications: ×250. (This figure is printed in black and white as Figure 60.9.).



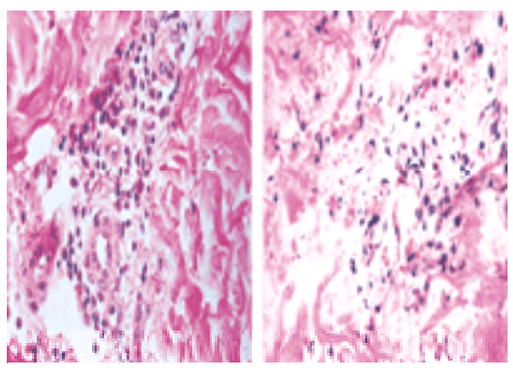
Color Figure 60.12. Immunohistochemical staining of human first-trimester placentas for membrane cofactor protein (MCP, CD46) and decay accelerating factor (DAF, CD55). Note that both these complement regulatory proteins are expressed but are differentially located, with MCP being prominent on villous cytotrophoblast (left panel, *arrows*) and DAF being prominent on migrating cytotrophoblast cells (right panel, *arrows*). Original magnifications: ×250. (This figure is printed in black and white as Figure 60.12.).



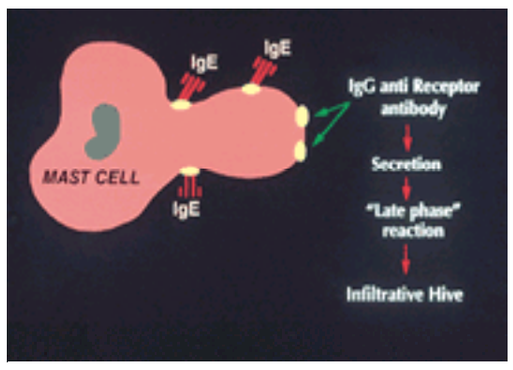
Color Figure 61.5. Three-dimensional structures of the mite allergen, Der f 2 [Protein Data Bank file 1AHK.pdb (103)], the birch pollen profilin, Bet v 2 [1CQA.pdb (69)], the horse allergen, Equ c 1 (126), and the major timothy grass pollen allergen, Phl p 2 [1WHO.pdb, (70)]. Figure kindly prepared by Dr. Anna Poms. (This figure is printed in black and white as Figure 61.5.).



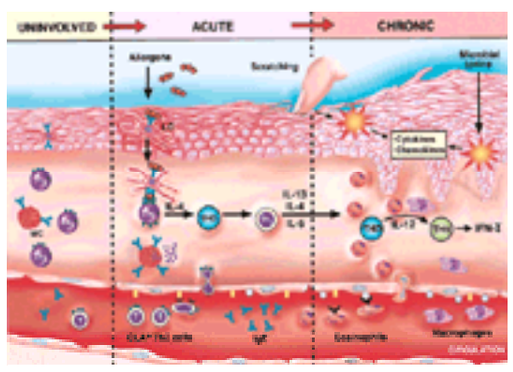
Color Figure 66.1. Typical lesions of chronic urticaria with serpiginous, elevated lesions borders variable degrees of central clearing. (This figure is printed in black and white as Figure 66.1.).



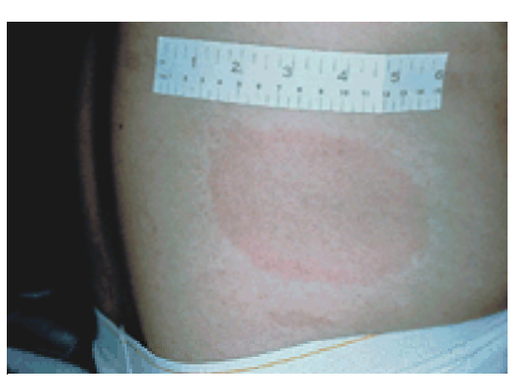
Color Figure 66.2. Comparison of biopsy specimens from a patient with chronic urticaria and a patient with cutaneous vasculitis. **A:** The vasculitis biopsy has a small venule just to the right of center that is destroyed. There is a predominantly neutrophilic infiltrate with fragmented cells (leucocytoclasis) throughout. **B:** In chronic urticaria, the integrity of the blood vessel wall is maintained, and a nonnecrotizing, predominantly mononuclear cell infiltrate is seen. (This figure is printed in black and white as Figure 66.2.).



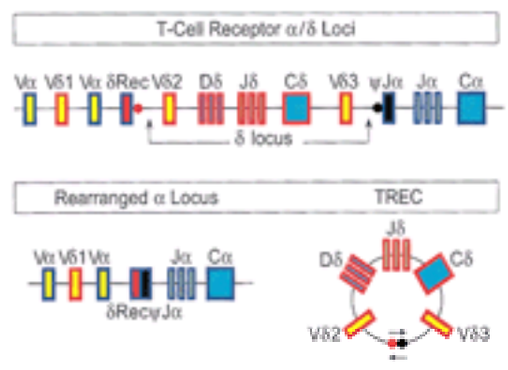
Color Figure 66.5. Diagrammatic representation of cutaneous mast cell activation by immunoglobulin G (IgG) antibody to the IgE receptor. (This figure is printed in black and white as Figure 66.5.).



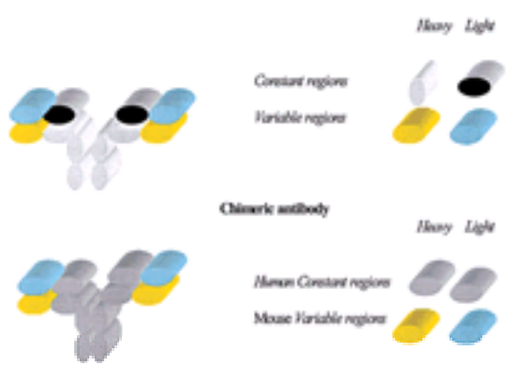
Color Figure 67.1. Cellular interactions in atopic dermatitis. The acute skin lesion is associated with overexpression of Th2 cytokines, whereas the chronic skin lesions show a shift to Th1 cytokine expression. Two potential reasons for the shift to Th1 cytokine expression include interleukin (IL)-4–induced eosinophil secretion of IL-12 or superantigen-induced stimulation of macrophage, or dendritic cell secretion of IL-12. *MC*, mast cell. (This figure is printed in black and white as Figure 67.3.).



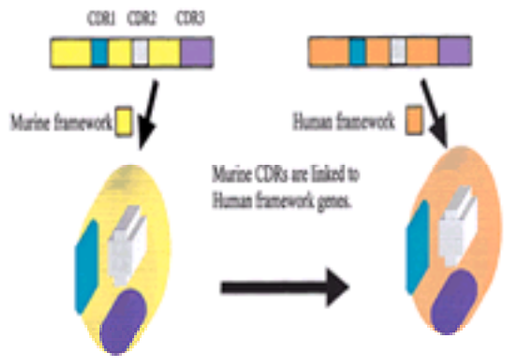
Color Figure 79.1. A characteristic erythema migrans skin lesion is shown. A 10-day-old lesion has gradually expanded to 10 cm and is beginning to have a brighter red border. (Reprinted from Steere AC, Bartenhagen NH, Craft JE, et al. The early clinical manifestations of Lyme disease. *Ann Intern Med* 1983;99:76, with permission.) (This figure is printed in black and white as Figure 79.1.).



Color Figure 81.2. Derivation of T-cell receptor excision circles (TRECs) during rearrangement of the T-cell receptor a gene. (This figure is printed in black and white as Figure 81.2.).



Color Figure 91.1. Chimeric murine/human antibody created by linking murine antigen-binding variable regions to human heavy- and light-chain constant regions. (This figure is printed in black and white as Figure 91.1.).



Color Figure 91.2. Humanization of antibodies using complementarity-determining region (CDR) grafting. CDR loops transfer antigen binding to variable region constructed with human framework regions. (This figure is printed in black and white as Figure 91.2.).

Appendix A METHODS

Thomas A. Fleisher, M.D.

Interest in evaluating the immune response began with the need to assess the efficacy of vaccines. This early technology opened the way for experimental models and studies that dramatically extended our understanding of immune responses and the immune system. These data have evolved and allow a clearer understanding of the immunopathologic mechanisms underlying many human diseases. This understanding, in turn, has increased the need for more sophisticated and specific means to assess the immune response. Applications of immunologic studies in clinical medicine include evaluation of patients for primary or secondary immune deficiency, as well as the investigation of diseases involving autoimmune, allergic, infectious, and malignant processes. The human immunodeficiency virus (HIV) pandemic has further increased the clinical application of immunologic testing because these results provide prognostic information that is directly linked to the development of new therapies. The future is likely to herald an increasing emphasis on assays of not only general but also specific immune function.

This discussion is directed at general concepts and standard applications of current laboratory methods used to characterize and quantitate immunoglobulins (Igs), assay-specific antibody levels, immunophenotype lymphocytes, evaluate lymphocyte function, study neutrophil function, test monocyte function, assess complement, and examine immediate hypersensitivity. In view of space limitations, the presentation focuses on an overview of the various topics; more complete discussions of these various subjects are available in texts devoted to laboratory immunology (1,2).

CHARACTERIZATION AND QUANTITATION OF IMMUNOGLOBULINS

Igs are glycoproteins secreted by terminally differentiated B lymphocytes or plasma cells after encounter with specific antigen. Serum Igs are polyclonal because their production involves multiple B-cell clones, each synthesizing a unique antibody molecule that binds with varying strength to the stimulating antigen. The interaction between antibody and antigen is influenced by the strength of the reversible attraction between antigen and antibody (affinity), as well as by the stability of the complex formed between antigen and antibody in solution (avidity). Antibody production after initial antigen exposure is referred to as the *primary response,* whereas subsequent exposure induces a *secondary, or anamnestic, antibody response.* The latter occurs more rapidly and generates primarily IgG as well as higher specific antibody levels than a primary response.

There are five different classes (isotypes) of Ig produced by human B cells. The basic molecular structure of each of these classes consists of two identical polypeptides, referred to as heavy chains (a, d, e, g, or μ), that bind two identical light chains (k or l) forming a Y-shaped monomeric protein. Each monomer (two identical heavy and two identical light chains) contains two antigen combining sites referred to as the *Fab portion* (antigen binding fragment). The sites of other biologic functions are found in the constant region of this molecule, referred to as the *Fc part* (crystallizable fragment). In some isotypes such as IgM, multiple monomers are bound together to form a polymeric structure. The various classes differ with regard to isoelectric point, molecular weight, carbohydrate content, and susceptibility to enzyme digestion. In addition, each class has certain significant functional differences (Chapter 13). This chapter reviews selected qualitative and quantitative assays applied for assessment of Igs that include techniques based on electrophoresis, immunoprecipitation, and nephelometry.

Zone Electrophoresis

The principle that charged particles migrate at different rates in an electrical field forms the underlying principle of *zone electrophoresis.* The application of this technique in the evaluation of serum proteins was first described by Tiselius in 1937 (3). In this report, serum proteins subjected to an electrical field were demonstrated to be resolved into five zones (bands), identified as albumen and a-1, a-2, b, and g globulin fractions (Fig. A.1). Igs fall primarily in the g globulin band, although IgA and IgM also migrate into the b and a-2 globulin regions (4). In this semiquantitative method, the solution of interest is applied to a support medium (e.g., agarose, methyl

cellulose) that is saturated with buffer; an electric potential is then introduced to separate the proteins; the final step requires the addition of a stain to visualize the separated protein bands. The rate of migration depends on the charge of the different proteins, the strength of the electrical field, and the resistance of the matrix. The relative quantity of each separated band is based on the total protein level and can be evaluated with a densitometer. This approach is useful in assessing overall plasma protein status and also can be used to screen for monoclonality, although low-level monoclonal and oligoclonal proteins may be missed (5). The initial description of a patient with hypogammaglobulinemia was based on the observation that the g globulin band, as determined by zone electrophoresis, was absent in a boy with recurrent infections (6). Zone electrophoresis can be applied to other fluids including cerebrospinal fluid (CSF) and urine. This test, however, is only a semiquantitative method, and quantitative methods (e.g., radial immunodiffusion, nephelometry) should be used to determine the actual concentration of Igs.

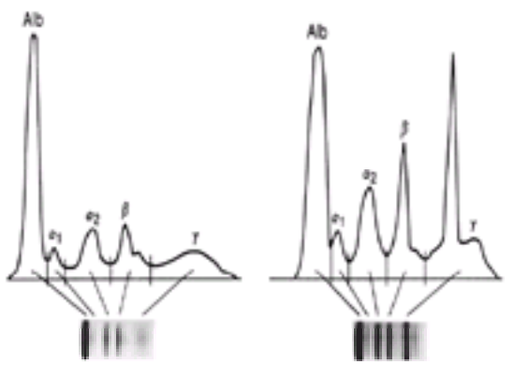


Figure A.1. Zone electrophoresis. The electrophoretic pattern and densitometric tracing demonstrate the five major bands (albumen plus a-1, a-2, b, and g globulins) from a normal **(left side)** and from a patient with a clonal immunoglobulin.

Immunoelectrophoresis

The combination of zone electrophoresis (described earlier) and immunodiffusion underlies the technique of *immunoelectrophoresis* (7,8). This two-step method applies electrophoretic separation of proteins in a gel, followed by the addition of specific antisera into troughs cut parallel to the direction of the electrophoretic migration. The antisera diffuse through the support medium at right angles to the separated proteins. Interaction between antibody and the separated, specific proteins (antigens) produces antigen-antibody complexes in the gel. These complexes immunoprecipitate in the region where the antigen-antibody concentrations are at or near equivalence (Fig. A.2). The degree of precipitation decreases in areas of antibody excess (prozone) and antigen excess (prezone or postzone) because of the increasing solubility of the immune complexes. The site and pattern of the resulting precipitin arcs depend on the specificity of the antiserum used; in standard immunoelectrophoresis, the antibodies typically are directed against each of the three major heavy-chain (a, g, and µ) proteins and both light-chain (k or l) proteins. Abnormal (clonal) proteins alter the appearance of the precipitin and often produce a spur or thickening of the arc (Fig. A.3).

Figure A.2. Immune complex immunoprecipitation curve. The insolubility of complexes when antigen and antibody concentrations are near equivalence is demonstrated. The concentration dependence of immune complex precipitation is central to immunoprecipitation assays.

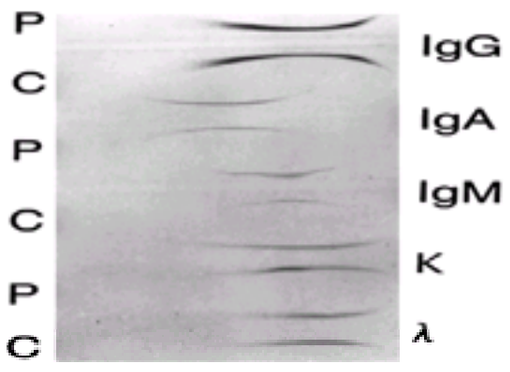


Figure A.3. Immunoelectrophoresis. Gel demonstrates a normal pattern with the control (C) serum and µ heavy-chain and k light-chain spurs with the patient (P) compatible with immunoglobulin M-k monoclonal gammopathy.

Immunoelectrophoresis is commonly used for identification of monoclonal Igs, particularly myeloma and Bence Jones proteins, because it is relatively insensitive to antigen excess (8). It also is the preferred method for identifying protein components with similar electrophoretic mobility present in complex mixtures (8). This is a nonquantitative technique and should not be used as a primary means of evaluation for presence or absence of Igs. In addition, the interpretation of the varied immunoelectrophoretic patterns requires considerable experience to master (9).

Immunofixation Electrophoresis

This technique is similar to immunoelectrophoresis in that it combines zone electrophoresis with the unique specificity of an antigen-antibody reaction (8,10). Proteins are electrophoretically separated in a support medium, and then monospecific antibodies are overlaid directly onto the surface of the gel, in contrast to the diffusion from troughs used in immunoelectrophoresis. The overlaying of specific antisera results in immune complex formation and immunoprecipitation in the region of antigen-antibody equivalence. The gel can be stained for visualization, and normal serum (polyclonal) Igs demonstrate a diffuse band after staining (Fig. A.4). This pattern contrasts with the intense, narrow band formed by a monoclonal Ig or the multiple intense bands resulting from oligoclonal Igs. Automated immunofixation assay systems are now available that provide consistent results for clinical laboratories. This approach is used as the primary technique for identifying clonal Igs by many clinical laboratories, whereas some still use either immunoelectrophoresis or a combination of these two techniques.

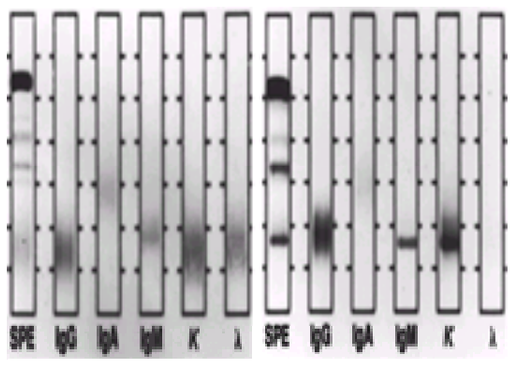


Figure A.4. Immunofixation electrophoresis. The **left panel** lanes demonstrate a normal pattern, whereas the **right panel** shows an immunoglobulin M (IgM)-k monoclonal gammopathy. The clonal protein is also seen on the serum protein electrophoresis (SPE) in the **left lane of the right panel.** The location of this clonal immunoglobulin is consistent in all three positive lanes (SPE, IgM, k), the *darker staining* in the IgG lane is an application artifact.

The advantages of this method over immunoelectrophoresis include more rapid diffusion, easier interpretation, direct comparison with zone electrophoresis results, and higher sensitivity for clonal Igs (8). The last point makes this technique particularly useful in identifying oligoclonal Igs in serum, CSF, and other body fluids. Immunofixation yields more specimens with low-level monoclonal and oligoclonal Igs. The impact of identifying monoclonal gammopathy in otherwise asymptomatic patients has been evaluated in longitudinal studies, and it appears that as many as one-fourth of patients with this finding ultimately develop a malignant B-cell process (11). These observations have led to the term *monoclonal gammopathy of unknown significance,* rather than benign monoclonal gammopathy, when describing this finding (11). The disadvantages of immunofixation electrophoresis include its detection insensitivity in the presence of antigen excess and the difficulty in resolving monoclonal proteins with similar electrophoretic mobility.

Single Radial Immunodiffusion

Single radial immunodiffusion is technically a noncomplex method that allows quantitative assessment of a protein in solution (8). The approach consists of adding a sample (serum or other biologic fluid) to a well that is cut into agarose containing specific antiserum. The sample proteins (including the antigen of interest) diffuse into the agarose radially, and the specific antigen forms immune complexes on interaction with antibody contained in the support medium. An immunoprecipitate forms in the region of antigen-antibody equivalence; thus, the diameter of the immunoprecipitin ring varies according to the concentration of the diffusing protein, because the antibody level in the agarose is fixed (Fig. A.5). Controls, with known antigen concentrations, can be used to generate a standard curve from which the protein level in an unknown sample can be determined. Two different testing approaches are used for radial immunodiffusion: one involves reading the immunoprecipitin ring once it has reached the end point, often referred to as the Mancini method (12). The other technique evaluates the immunoprecipitate at a fixed time, before end-point equivalence has been reached, and is referred to as the Fahey and McKelvey method (13). A linear relationship is found between the antigen concentration and the square of the ring diameter when using the end-point method and between the log of the antigen concentration and the ring diameter in the fixed-time diffusion approach. Radial immunodiffusion is a simple and reliable method to quantify Igs, IgG subclasses, complement components (e.g., C3, C4, factor B, C1 inhibitor), and other proteins. Low-level kits are commercially available that extend the lower limits of detection to approximately 0.03 mg/L; these can be used in the determination of CSF IgG levels. However, this technique is cumbersome for high-volume protein quantitation as well as requiring 1 to 3 days before the immunoprecipitin rings can be read and results generated.

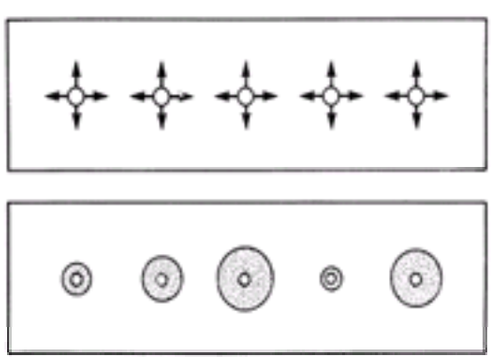


Figure A.5. Radial immunodiffusion. The size of the immunoprecipitate ring correlates with the concentration of the protein being measured because the concentration of the antibody (in the gel) is constant. The three rings on the **left** are controls, the fourth ring represents a patient with decreased levels of the protein being measured, whereas the fifth represents a person with a normal result.

At least three situations may result in erroneous results using conventional radial immunodiffusion testing. The presence of monomeric (low molecular weight) IgM, which is seen in Waldenström macroglobulinemia and ataxia telangiectasia, may result in the reporting of incorrectly high IgM levels. This is caused by the low-molecular-weight IgMs diffusing more rapidly than the pentameric IgM standards. Second, the presence of IgG rheumatoid factor in high concentrations can result in rheumatoid factor-IgG complexes that diffuse more slowly than the IgG standard, thus causing a spurious decrease in the total IgG level to be reported. Finally, antibovid species antibodies are found in certain patients with IgA deficiency. These can bind to Bovidae (e.g., goat) produced antisera used in the support medium and can produce a precipitin ring despite a deficiency of IgA.

One-Dimensional Electroimmunodiffusion

One-dimensional electroimmunodiffusion, also known as rocket electrophoresis, is an active immunodiffusion technique developed by Laurell (14). This technique is a variation of radial immunodiffusion in which multiple dilutions of a sample are placed in a series of wells cut in an agarose gel containing specific antibody. The sample is introduced into the well and then is subjected to an electrical field that causes the protein to migrate into the antibody-containing agarose. Antigen-antibody complexes are produced as the antigen moves into the antibody-containing support medium, and an immunoprecipitate is produced in the region of antigen-antibody equivalence. The immunoprecipitate forms in the shape of a spur or “rocket,” and the antigen concentration is directly proportional to the length of the “rocket.” Controls of known concentration are run to generate a standard curve from which the protein level of unknown samples can be generated. This method has a sensitivity similar to that of radial immunodiffusion but requires less time for completion, because the electrical field–induced immunodiffusion step is complete in hours rather than days. However, it is used relatively infrequently in most clinical laboratories even though commercial systems for assaying Igs and complement components are available.

Nephelometry

The basic principle underlying *nephelometry* is based on measuring the amount of incident light scattered by immune complexes in solution (15). The soluble immune complexes are generated by the addition of specific antibody to the protein (antigen) of interest, by allowing time for the immune complexes to form, and then by measuring the amount of scattered light. Nephelometry is performed in the antibody excess region of the precipitin curve where the complexes remain in solution, in contrast to precipitin reactions that require antigen-antibody equivalence to produce an immunoprecipitate. The concentration of the antibody added to each sample is constant, so changes in light scatter reflect differences in the antigen concentration. Because the method is designed to operate in dilute solutions, polyethylene glycol is often added to enhance the formation of immune complexes under the operational conditions. Currently, two different technical approaches are used: rate nephelometry, which measures light scatter associated with the peak rate of immune complex formation; and fixed time nephelometry, which evaluates light scatter at the same time point for all reaction mixtures. Both methods enable accurate measurement of IgG, IgA, IgM, C3, C4, factor B, C-reactive protein, IgG subclasses and certain other serum proteins (16). In addition, they are adaptable for the determination of proteins found in extremely low levels, including CSF IgG and albumen. Nephelometry is currently the standard method used in most busy clinical laboratories to quantify protein analytes because of the high throughput capabilities, together with the excellent precision provided by the commercially available automated nephelometers.

DETECTION AND QUANTITATION OF SPECIFIC ANTIBODIES

The *detection of specific autoantibodies* has become a standard approach used in the diagnostic evaluation of a variety of autoimmune diseases. In addition, testing for microorganism-specific antibodies provides serologic evidence for present or past infection. Substantiating the diagnosis of primary infection may be done by evaluating for the presence of organism-specific IgM, such as IgM antiviral capsid antibody in Epstein-Barr virus (EBV) infection. This approach is based on the finding that IgM is generated initially during the primary antibody response, whereas IgG appears slightly later and persists during the convalescent phase. This approach is most reliable if acute and convalescent samples are obtained to assay for both IgG and IgM antibody determinations (preferably tested in the same assay). Additional evidence of an acute or semiacute infection is a rising titer of antibody specific for the organism.

The evaluation of specific antibodies can be performed semiquantitatively using various methods including immunodiffusion, immunodiffusion combined with electrophoresis, agglutination, indirect immunofluorescence, and Western blotting. Quantitative assessment for specific antibodies is performed most commonly using an immunoassay such as radioimmunoassay or enzyme-linked immunosorbent assay (ELISA).

Double-Gel Immunodiffusion

Two-dimensional, double-gel (Ouchterlony) immunodiffusion is a test that facilitates the qualitative identification and characterization of specific antibodies (or antigens) in solution (7,17). In this method, a reference material (control antigen) and an unknown sample are placed in separate wells, and a characterized antibody is put into a central well (Fig. A.6). The antigens diffuse radially from their site of application into the support medium, where they encounter the antibody preparation diffusing radially from the central well. Immune complexes are formed when specific antibody interacts with the antigen to form an immunoprecipitate at the point of antibody and antigen equivalence. The test is designed to compare the reference material with the neighboring unknown samples based on three qualitative reaction descriptions: identity, nonidentity, or partial identity. This evaluation is derived from the pattern formed at the intersection of the reference antigen-antibody immunoprecipitate and the unknown sample-antibody immunoprecipitate (Fig. A.6). Fusion of the two different immunoprecipitin bands at the point of intersection represents the reaction of identity, and crossing of both bands constitutes nonidentity, whereas crossing of only one band signifies partial identity. Serial dilutions of a sample can be used for a semiquantitative titer of a specific reactant. The reactants can be reversed by using characterized antigen in the central well to detect and compare for the presence of specific antibodies contained in samples placed into the surrounding peripheral wells. Clinical application of this method includes the evaluation of autoimmune disorders for the presence or absence of autoantibodies to extractable nuclear antigens, including Sm, RNP, SS-A, SS-B, as well as other antigens such as Scl-70 and Jo-1. It lacks the detection sensitivity of many quantitative methods and requires time for the immunodiffusion to occur. However, it is technically easy, can be performed with antigen preparations that are only partially purified, is highly specific, and serves as an excellent screening test for the presence of a specific antibody or antigen in solution.

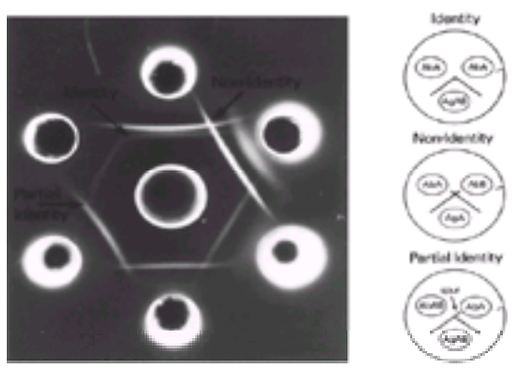


Figure A.6. Double-gel (Ouchterlony) immunodiffusion. Immunoprecipitation patterns at the intersection points demonstrate reactions of identity (fusion), nonidentity (both crossing), and partial identity (spur).

One-Dimensional Double Electroimmunodiffusion

This method, also known as *counter immunoelectrophoresis,* consists of placing an antigen-containing sample into a cathodal well and antibody into an anodal well within a specific type of gel (18). The reactants are then exposed to an electrical field, and antigen, with a relative negative charge, will move in the direction of the electrical current, whereas the relatively neutral antibody will move in the opposite direction as a result of endosmotic flow. Specific, high electroendosmosis grade agarose is required for this technique to ensure that Igs migrate cathodically. The reactants migrate toward each other and produce immune complexes that immunoprecipitate in the region of antigen-antibody equivalence. Because this represents active diffusion, the test can be performed far more rapidly than the passive method of double-gel immunodiffusion (Ouchterlony). This semiquantitative technique has been used primarily for identifying the presence of antigens or antibodies specific for certain infectious diseases in patient samples.

Agglutination Assays

Agglutination is a technique in which antibody reacts with antigen to produce multivalent binding (19). When antibodies combine with more than one particulate antigen, the result is the bridging of multiple antigens and antibodies with visible clumping. Direct agglutination assays use particles that naturally express the antigens of interest (e.g., red blood cells), whereas indirect agglutination assays use inert particles (e.g., latex spheres) that have been coated with the antigen of interest. This technique represents a simple and rapid method for determining the presence of specific antibody. Various serum dilutions are added to the antigen-coated (or antigen expressing) test particles, followed by incubation and observation for the presence of visible clumping. In agglutination assays, the evaluation of specific antibody that demonstrates wide variation in levels can result in a false-negative value when testing a single dilution as a result of the prozone effect (antibody excess with no immunoprecipitate). Avoiding this effect requires testing a range of sample dilutions rather then simply screening a single dilution of the sample.

Examples of agglutination tests that are routinely used clinically include red blood cell cross-matching (direct hemagglutination), rheumatoid factor (indirect, latex agglutination), and anticytomegalovirus antibody (indirect, latex agglutination). Agglutination assays are technically easy to perform, require no sophisticated instrumentation, and generate relatively quantitative results rapidly (20). They are less sensitive than immunoassays and have a bias in detecting IgM antibodies over IgG antibodies (21). Modification of the agglutination assay by the addition of an anti-Ig reagent, referred to as the Coombs test, improves detection of IgG antibodies.

Indirect Immunofluorescence Assays

The detection of an antibody to antigens that are expressed in some solid phase can be performed using *indirect immunofluorescence.* The clinical application of this technique typically uses cultured cells or a tissue section fixed on a microscope slide as the antigen substrate (22). The slide is overlaid with a sample (e.g., serum) that allows specific antibody, if present, to bind to the antigen of interest on the cells. After a wash step, fluorochrome-labeled antibody to human Ig is added, free antibody is washed away, and any bound antibody is detected by examination using fluorescence microscopy. Indirect immunofluorescence is used to test for antinuclear antibodies and specific antiviral antibodies (23). The availability of a cultured human cell substrate (such as Hep2 cells) for antinuclear antibody testing facilitates the identification of certain antibody specificities (e.g., anticentromere antibody and definition of cells in mitosis) (24). Other tissue substrates used for autoantibody testing include neutrophils (antineutrophil cytoplasmic antibody), stomach (anti–smooth muscle antibody), Purkinje cells (anti-Yo antibody), and hepatocytes and kidney tubules (antiliver kidney microsomal antibody). The substrate for antiviral antibody testing is usually infected cells or cell lines that express specific viral antigens (25). Indirect immunofluorescence tests can distinguish between IgG and IgM using fluorochrome-conjugated, class-specific antibodies. Although this sensitive test is relatively easy to perform, it requires technical knowledge and the availability of fluorescence microscopy.

Western Blot

The *Western blot* technique, a type of immunoblotting, is a semiquantitative method to identify specific antibodies (or antigens) in a solution (26). This technique combines the selectivity of gel electrophoresis with the specificity of an antibody-antigen interaction. The method involves electrophoretic separation (usually by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of a mixture of proteins (antigens), followed by transfer from the separation gel onto a synthetic membrane. Protein binding to nitrocellulose, the most commonly used synthetic membrane, is essentially irreversible over a wide range of protein concentrations. The actual detection phase of the test is initiated by overlaying the sample of interest onto the membrane containing the separated antigens. Specific antibody in the sample binds to its appropriate antigen, and after a wash step, labeled (enzyme, radionuclide) antihuman Ig is added to identify the presence of antigen-specific antibody (Fig. A.7). The general location and molecular weight of the separated protein antigens are known, and a control membrane is stained with either colloidal gold or amido black to confirm the presence and location of the specific proteins (antigens). Although this method is only semiquantitative, it is extremely sensitive, with a lower limit of detection in the range of 10 to 100 pg protein. It also has the added advantage of characterizing the pattern of antibody reactivity directed at certain distinct protein

antigens. This feature has enabled more specific assessment of the antibody response in HIV infection and Lyme disease as well as other infectious diseases. The availability of commercial Western blot test kits with prepared methylcellulose strips has significantly simplified the performance of this assay and has allowed the widespread use of the Western blot as a confirmatory test in assessing HIV serologic status (27). This approach also may have utility in the characterization of autoantibody reactivity in certain autoimmune disorders. Western blotting has wide applicability in the research laboratory to characterize antibody responses to complex antigens.

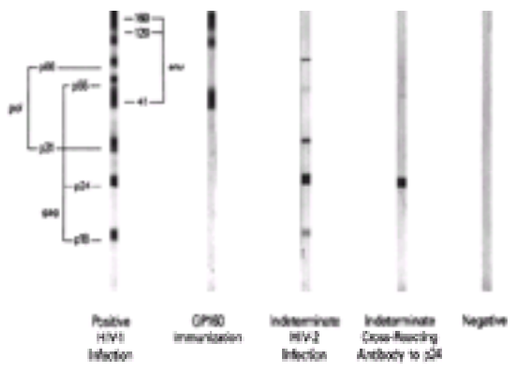


Figure A.7. Western blot. Various patterns of anti–human immunodeficiency virus antibody reactivity including a positive with antibody to all the major antigens, a postimmunization serum demonstrating the limited reactivity to the GP160 (envelope) proteins, and indeterminate and negative sera.

Radioimmunoassay

Until Yalow and Berson described a method to measure human insulin levels in the 1950s, the evaluation of circulating proteins present in relatively low concentrations had been a major technical obstacle (28). This initial report detailed the competitive binding immunoassay in which a constant amount of antigen-specific antibody is placed together with a small, predetermined amount of the antigen that has been previously radiolabeled. Next, standards, consisting of known quantities of unlabeled antigen, as well as unknown samples, are added to individual tubes containing the antibody and labeled antigen. After incubation, antigen-antibody immune complexes are precipitated, and their radioactivity is measured. Because the displacement of labeled antigen depends on the concentration of the antigen in the standard (or unknown sample), a concentration curve can be generated using the data from the standards. The radioactivity of the unknown samples then can be plotted on the standard curve to determine the concentration of the antigen in each sample tested.

This assay can be applied to determine specific antibody concentration by reversing the reagents, starting with a fixed concentration of purified antigen mixed with a small amount of labeled antibody. The second step involves the addition of either an antibody standard or an unknown sample that contains antibody. As noted earlier, displacement of the labeled protein (antibody) depends on the concentration of specific (unlabeled) protein (antibody) in each sample. The radioactivity observed in each of the precipitated immune complexes is inversely related to the antibody concentration.

A modification of the initial *radioimmunoassay* method uses a solid phase to immobilize one of the reactants (either antigen or antibody) (29). Binding of the unknown to the reagent attached on the solid phase can be detected with a second reagent that is radiolabeled or by using a sequence of antiantibody reagents with the final antibody being radiolabeled. The use of more than one reagent such as an antiantibody in the detection mode can increase the low-end sensitivity of the test, but it also can produce higher levels of nonspecific reactivity. The radioimmunoassay is a sensitive test for the quantitation of specific antigens or antibodies. The major disadvantages of this type of test are the requirements for expensive equipment to determine the level of radioactivity (i.e., g counters), radiolabeled reagents with short shelf lives, and special means for disposal of the radioactive waste.

Enzyme-Linked Immunosorbent Assay

A nonradioactive modification of the solid-phase immunoassay method, *ELISA,* was developed to address the disadvantages previously noted for the radioimmunoassay (30,31). This method depends on detection using an enzyme labeled reagent plus an appropriate, chromogenic substrate to produce a color. The intensity of the color generated is directly related to the concentration of the analyte tested. The indirect method has been used extensively for the detection of antibody, typically using polystyrene microtiter plates, tubes, or beads as a solid phase to which a purified antigen is bound. Serum (or other fluid) is added to the antigen-coated surface to enable any specific antibody to bind the immobilized antigen. The bound Ig then can be detected using an enzyme-labeled antihuman Ig followed by addition of the chromogenic substrate (Fig. A.8A). Washing is required between each individual step to remove unbound reactants. The optical density of the color reaction is measured and is proportional to the antibody concentration present in the sample. A simple modification of this method again involves an indirect detection method that uses a primary unlabeled antibody (antihuman Ig), followed by a second, labeled antibody that is reactive with the primary Ig. This may enable detection of lower concentrations of antibody, but it also may result in higher nonspecific reactivity.

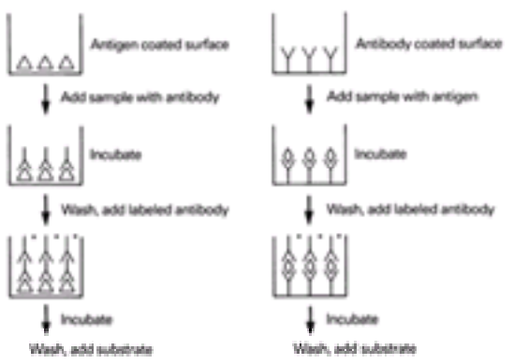


Figure A.8. Solid-phase immunoassay: indirect assay **(A),** sandwich assay **(B).** The label on the antibody can be an enzyme plus substrate (as shown), a radionuclide, or a fluorochrome.

An alternative approach for the detection of specific antigen is referred to as a *sandwich assay.* This method uses antigen-specific antibody bound to the solid phase that “captures” specific antigen. This is followed by the addition of a second, enzyme-labeled antibody specific for the antigen; after a wash step, substrate is added, and the color intensity is measured (Fig. A.8B). When only impure antigen is available, a specific antibody bound to the solid phase can be used to capture the antigen of interest from the heterologous mixture. The detection method also can use an indirect procedure with antigen-specific antibody and then antiantibody after the antigen capture step. The sandwich assay can be modified to detect antibody by initially binding specific antigen onto the solid phase and using enzyme-labeled antigen plus chromogen for the detection step. ELISA testing also can be set up in a competitive binding assay similar to the method discussed in the radioimmunoassay section. In each of these various approaches, control samples of predetermined concentration are used to construct a standard curve from which the specific levels of the various unknowns can be derived. Numerous test kits are available commercially for determining the levels of antibodies or other proteins. These usually include coated microtiter wells and standards as well as software to generate the concentrations of unknown samples. Laboratory-developed ELISA tests take advantage of the readily available reagents, automated ELISA readers, and various data reduction programs.

The sensitivity of current ELISA assays is comparable to that of radioimmunoassays. This method can be used for quantitating a host of specific antibodies or antigens, and it has several advantages, including performance simplicity, excellent reproducibility, and no requirement for isotopes. ELISA testing is currently the standard assay for various antiviral antibody tests, including the primary HIV antibody test. It is also a popular test method for detecting certain other immunologic proteins including IgG subclasses and IgE. The major disadvantage of ELISA is nonspecific binding to the solid-phase surface (microtiter plate, tube, or bead). This problem is common to all solid-phase assays, and it can be diminished by using specific blocking steps (saturate the nonspecific binding with unrelated protein) and washing thoroughly between steps.

The newer-generation immunoassays have incorporated certain changes, including improved solid phase (e.g., beads) to increase the amount of antigen available, the

use of recombinant or well-characterized proteins as targets, better reporter systems using either fluorescence or chemiluminescence, and automated platforms. These changes have resulted in assays with greater sensitivity and expanded dynamic range that are also less labor intensive with a lower test unit cost. As with all laboratory tests, immunoassays require careful test design, with appropriate controls to evaluate and monitor test performance, including the level of nonspecific reactivity.

FLOW CYTOMETRY

Flow cytometry is a method that uses an instrument to measure light emission generated by fluorochrome-labeled cells. The illumination source is typically a monochromatic beam generated by a laser and chosen to provide appropriate excitation energy for the fluorochrome reporters. Most instruments can provide data on at least five different measurements or parameters per cell. This approach has the distinct advantage over bulk techniques of retaining the collected data as discrete events specific to each cell are evaluated. It affords the opportunity to examine a variety of cell characteristics including surface antigen expression, intracellular protein production, and cell-cycle or viability status.

Testing for the presence of specific antigens (receptors) on the surface of lymphocytes allows for the identification of specific cell types or subtypes. This approach, referred to as *immunophenotyping,* can provide qualitative and quantitative data regarding cell lineage, stage of differentiation, functional potential, and state of activation. The clinical application of immunophenotyping has been shown to be important for assessing disease status in HIV infection as well as for characterizing leukemia and lymphoma cells. This technique also is being used as an adjunct in the evaluation of immune deficiencies and immune-mediated diseases, as well as in monitoring patients undergoing immunomodulatory therapy or organ transplantation. The most common method for analysis uses fluorochrome-conjugated monoclonal antibodies that are specific for lymphocyte-surface antigens.

Monoclonal Antibodies and Fluorochromes

The capacity to produce a *monoclonal antibody reactive with a specific antigen* was first described by Kohler and Milstein (32). The approach described in this seminal report consists of fusing an “immortal” murine myeloma cell line with normal murine plasma cells obtained from the spleens of mice that had been specifically immunized. The myeloma cell and the plasma cell are physically fused using polyethylene glycol to yield a hybridoma. This somatic hybrid combines the advantage of immortality with survival in selective medium. After a period in culture, the cell-free supernatants are examined for the presence of specific antibody. Cultured cells identified as producing specific antibody are cloned using a limiting dilution technique based on a Poisson distribution. The cells producing antibody are expanded to provide additional antibody for further characterization. Finally, specific antibody-producing clones can be expanded either by *in vitro* culturing techniques or by *in vivo* growth in the peritoneal cavity of a mouse. This antibody “factory” can produce a long-term supply of a specific antibody. The ever-increasing numbers of monoclonal antibodies to different lymphocyte cell-surface antigens that are available are categorized according to a CD (cluster of differentiation) numeric convention (Table A.1) (33). The CD assignments are made at international workshops that are held periodically to characterize and assign CD specificities to more recently developed monoclonal antibodies. This designation system is also used to describe monoclonal antibodies directed at surface antigens on other hematopoietic cells, including monocytes, platelets, and granulocytes, as well as other cells (e.g., endothelial cells).

TABLE A.1. Selected Lymphocyte-Surface Antigens

The availability of a large number of monoclonal reagents directed at lymphocyte-surface antigens has allowed for significant progress in the understanding of lymphocyte differentiation and maturation. In addition, other monoclonal antibodies have been produced that yield information about mechanisms of cell adhesion, activation, and function. The immunophenotyping of lymphocytes can provide information regarding the presence or absence of particular cells or specific cell characteristics, and the potential for certain functions. However, identification of cells using this technique is not the equivalent of evaluating actual cell function.

The detection of antibody binding to the cell surface requires a reporter, which typically is a fluorochrome, a compound that absorbs light (excitation) of a defined wavelength and converts this energy into light (emission) of a longer wavelength (lower energy). Cells are exposed to a monochromatic source of light designed to excite fluorochrome conjugated antibody bound to the cell surface. The light emitted by the fluorochrome is detectable with the human eye using fluorescence microscopy or with a flow cytometer.

The simplest technique for immunophenotyping consists of using monoclonal (or occasionally polyclonal) antibodies that have been directly conjugated with a fluorochrome. This approach is referred to as *direct staining* because the reaction is accomplished in one step. The alternative approach, referred to as *indirect staining,* uses a nonconjugated primary reagent (antibody) that is detected in a second step with a fluorochrome conjugated secondary reagent (e.g., fluorochrome labeled goat antimurine IgG).

The fluorochromes that are routinely used in clinical immunophenotyping include fluorescein isothiocyanate (FITC) and phycoerythrin (PE). Both these compounds are excited by blue light (488 nM), with FITC producing green light (525 nM) and PE emitting orange light (575 nM). A newer fluorochrome, peridinin chlorophyll protein (PerCP), is also excited by blue light generating a red signal (675 nM). An alternative for the third color uses two fluorochromes linked together, with one serving as the source of excitation energy for the second, a process referred to as *energy transfer.* The most common example of this approach is using blue light to excite phycoerythrin, which emits orange light that serves as the excitation light source for a cyanin dye such as Cy5. The combination of either PerCP or the energy transfer pair with FITC and PE enables direct staining with three different monoclonal antibodies using a single excitation (blue) beam to perform a three-color analysis (34). This would provide eight potential cell populations (i.e., positive or negative with each of the three reagents yielding 2^3 combinations). Other fluorochromes, including Texas red, rhodamine, and allophycocyanin, are used primarily in research settings, because alternative wavelength light (a second light source) is required to excite these compounds.

Research-based flow cytometers with multiple excitation sources enable workers to perform studies with up to six or seven different fluorochromes (“colors”) simultaneously; this situation could generate 64 (2^6) or 128 (2^7) phenotypically distinct cell subpopulations from one combination of 6 or 7 antibodies. Significant effort is being expended to develop additional fluorochromes to enable even greater numbers of reagent combinations. However, the identification of large numbers of cell subpopulations poses a significant challenge with regard to data management and interpretation.

Flow Cytometer

A *flow cytometer* consists of a light source, focusing optics, fluidics, photodetectors, and a computer system (Fig. A.9) (35). An air-cooled, argon laser is the standard source for the monochromatic blue light beam. A system of fluidics using hydrodynamic focusing regulates the flow and location of the cells passing through the focused beam of light. Photodetectors consisting of photomultiplier tubes and photodiodes detect and amplify the specific light signals derived from the cells, and the computer allows for data acquisition and analysis. This system is capable of collecting the multiple pieces of data (parameters) obtained from each individual cell at rates that range from 500 to 2,000 events per second or greater. Many specific software packages are available that allow a correlated analysis of these multiparameter data to identify qualitative and quantitative characteristics about various cell subpopulations. The quantitative, multiparameter analysis of large numbers of cells provides a significant advantage over conventional fluorescence microscopy, particularly when dealing with small populations or subpopulations of cells. In addition, the various reagents can be detected independently on each cell without the interference of the other bound reagents (fluorochromes).

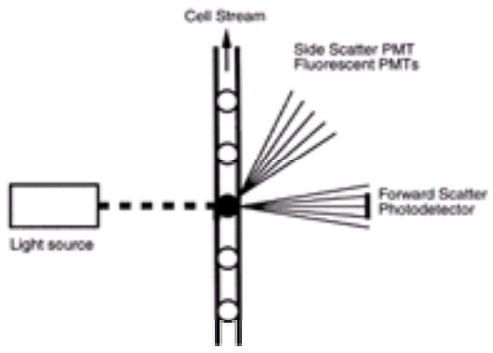


Figure A.9. Basic design of a flow cytometer. This instrument has a monochromatic light, an optical system to focus the light onto a specific area, fluidics to move the cells in suspension through the path of the light, and photodetectors to collect nonfluorescent and fluorescent light signals.

Most flow cytometers can evaluate two nonfluorescent parameters: forward-angle (low-angle) light scatter, measured in the plane of the excitation beam, and side (orthogonal, 90-degree) light scatter, measured at a right angle to the incident beam and the cell flow. The former is a reflection of cell size, whereas the latter depends on cell granularity and topography. A standard flow cytometer also can collect at least two (usually three or four) fluorescent parameters using photomultiplier tubes arranged in the same plane as the side scatter detector. Each detector is "tuned" to a specific wavelength ("color") through the use of optical filters that are chosen to match the emission characteristics of the fluorochromes used.

Lymphocyte Phenotyping

As noted earlier, fluorochrome-conjugated monoclonal antibodies directed at specific cell-surface antigens in combination with flow cytometry are used to differentiate *lymphocyte* subpopulations. The technical approach employed in many clinical laboratories is a whole-blood lysis method that consists of adding one or more directly (fluoro-chrome-) conjugated monoclonal antibodies to a small volume of whole blood (36). The mixture is incubated to allow the antibody to bind the appropriate cell-surface antigen; next the red blood cells are lysed, and the sample is then ready for evaluation with a flow cytometer. The major advantage of the whole-blood technique is decreased sample handling with diminished likelihood of selective cell depletion. An alternative approach to the whole-blood technique uses lymphocytes that have been separated by means of a density gradient. The staining procedure is similar, except lysis of red cells is not necessary. This technique provides relatively pure population of mononuclear cells (lymphocytes and monocytes), although the separation method may deplete certain cell subpopulations.

The computer software typically displays single-parameter immunophenotypic data graphically as a histogram (Fig. A.10). This is a distribution plot with cell frequency (number) on the Y-axis and fluorescent intensity on the X-axis. Integration of the area under a curve or part of a curve yields information regarding cell distribution. Two color studies are usually displayed by means of a contour (Fig. A.9), a dot, or an isometric plot. This system allows the simultaneous display of two parameters versus frequency, and it defines four separate populations: cells that express both antigens, cells that express neither antigen, and those that express exclusively one or the other antigen (37). Resolution of antigen coexpression is best appreciated with two-parameter displays. The graphic display of three-color studies requires sequential analysis examining two-color data on cells identified as positive or negative for the third marker.

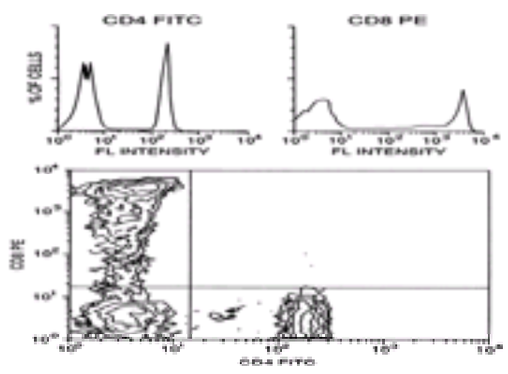


Figure A.10. Flow cytometry–derived histograms and contour plot. CD4 and CD8 histograms from a sample stained simultaneously with CD4-fluorescein isothiocyanate (FITC) and CD8-phycoerythrin (PE) showing the fluorescent ntensity (X-axis) versus cell frequency (number) on the Y-axis. The dual-parameter study is also displayed as a contour plot in which four basic populations can be distinguished: CD4-FITC positive **(lower right quadrant),** CD8-PE positive **(upper left quadrant),** CD4-FITC positive/CD8-PE positive (double positive, **upper right quadrant),** and CD4-FITC negative/CD8PE negative (double negative, **lower left quadrant).**

The proper characterization of lymphocytes requires differentiating the cells of interest from the other leukocytes (monocytes, granulocytes), nonleukocytes (red blood cells, platelets), and debris. A directed analysis confined specifically to lymphoid cells in a mixed cell population depends on a process referred to as *lymphocyte gating.* This is usually accomplished by evaluating the two nonfluorescent parameters, forward angle and side scatter (38). As previously noted, the former is a reflection of cell size, whereas the latter is a measure of cell granularity and topography. These two parameters enable the separation of leukocytes into three relatively distinct populations: granulocytes (greatest amount of side scatter), monocytes (greatest amount of forward scatter and intermediate side scatter), and lymphocytes (combination of low forward scatter and side scatter) (Fig. A.11). The inclusiveness of this three-part differential can be confirmed using monoclonal gating reagents consisting of the panleukocyte specific monoclonal antibody (CD45) with a monocyte-specific reagent (CD14) (39). This approach identifies lymphocytes (CD45 brightly positive, $CD14^-$), monocytes ($CD45^+$ and $CD14^+$), and granulocytes (CD45 dimly positive, $CD14^-$) as well as nonleukocyte contaminants ($CD45^-$ and $CD14^-$). The confirmation of the lymphocyte gate using the combination of CD45 and CD14 is especially critical when one uses the whole blood lysis method, because nonlymphocytes represent the majority of leukocytes in the peripheral blood of adults. Proper gating ensures that the percentages obtained for the various lymphocyte immunophenotypes are correct. Thus, exclusion of nonlymphocytes and inclusion of most lymphocytes by the primary gating technique are the first steps in providing valid immunophenotypic data. Incorrect gating, resulting from either including significant numbers of nonlymphocytes in the lymphocyte gate or excluding significant numbers of lymphocytes from the lymphocyte gate, can invalidate a study.

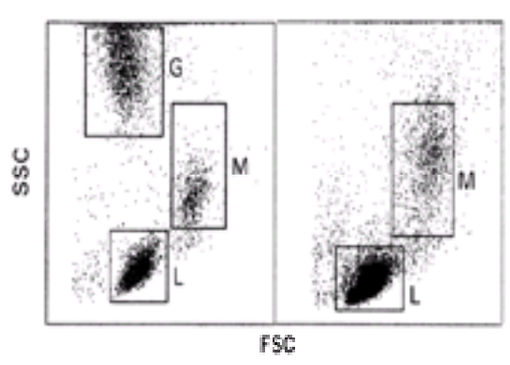


Figure A.11. Lymphocyte gating. The nonfluorescent parameters, forward scatter (FSC) and side scatter (SSC), identify three populations in lysed whole blood: lymphocytes (L), monocytes (M), and granulocytes (G). These parameters reveal the presence of lymphocytes (L) and monocytes (M) after density gradient separation (same sample). Most granulocytes have been depleted by this method of cell preparation.

Normal immunophenotypes vary with age, race, and sex; thus, patient data must be interpreted in the context of appropriate control values (40). Representative immunophenotyping data for immunologically normal adults derived in our laboratory are presented in Table A.2. Complete immunophenotypic data must include both the percentage and the absolute number (based on the total lymphocyte number) of the various subpopulations studied.

Surface Antigen	95% Confidence Interval	
	Percentage (%)	Cell Number (per mm³)
T cell		
CD2	74–89	990–2080
CD5	60–82	830–1990
CD3	61–84	830–2030
CD4	32.5–60	480–1340
CD8	18–46.5	350–910
B cell		
CD19	5.5–16	95–380
CD20	5–16	90–330
NK cell		
CD3-/CD16 and CD56	6.5–29.5	120–490

NK, natural killer.

TABLE A.2. Normal Adult Immunophenotype Data

Within a specific study, certain internal relationships can be used to validate the results. T cells + B cells + natural killer (NK) cells should equal 100% (plus or minus 5%) in any phenotyping study that reports all three major lymphocyte subpopulations. At least three different situations could alter the sum of the parts: improper lymphocyte gating with the inclusion of nonlymphocytes, the presence of an abnormal lymphoid population that is not stained by the reagents chosen, and technical problems. Additional internal controls include the finding that in immunologically normal adults, approximately 75% of circulating lymphocytes are T cells; most T cells express either CD4 or CD8, usually in a ratio of 2:1; the remaining non-T lymphocytes are generally equally divided between B cells and NK cells. There also should be internal consistency when specific reagents are used more than once as well as consistency between two different reagents that identify essentially the same population or subpopulation (e.g., CD19 and CD20 on B cells).

The most commonly requested flow cytometry test is a CD4 count because it is used as a diagnostic criteria for the acquired immunodeficiency syndrome, to develop prognostic data regarding HIV infection, and to make decisions regarding therapy. The evaluation of clonal excess, cell lineage, and state of differentiation by flow cytometry has become a standard approach in the assessment of leukemia and lymphoma cells. Additional applications of this method include evaluation for increases or decreases in specific cell subpopulations, as well as the status of activation markers in a variety of diseases. Generally, flow cytometry provides data used to support a diagnosis, as well as prognostic information. In many other settings, it is currently used for investigative types of studies.

Intracellular Flow Cytometry

The evaluation of *intracellular characteristics using flow cytometry* has been applied in different settings. In these applications, the common methodologic approach is to open the interior of the cell to probes using membrane fixation and permeabilization. An example of this approach is the evaluation for the presence of intracytoplasmic cytokines after mitogen or antigen stimulation (41). This has proven to be a useful tool in exploring the T-helper cell Th1 and Th2 paradigm (Chapter 10 and Chapter 11), and it has been applied in the setting of studies of allergic and autoimmune disease as well as investigation of allergen immunotherapy. An additional application of intracellular testing is the evaluation of phosphorylated proteins after cytokine activation (42). A flow cytometer also can be used to study cell cycle by evaluating total cellular DNA or RNA with certain specific fluorescent probes including those that intercalate into DNA or RNA (43). These studies can be combined with the evaluation of specific cell-surface antigens. This combination approach is used to evaluate cell-cycle or ploidy status of tumor cells. It also can be applied to a cell mixture containing malignant cells by using a tumor-specific monoclonal antibody to identify the malignant cells for cell-cycle assessment.

Finally, flow cytometry can be used to assess other intracellular characteristics associated with cell activation. These include evaluation of intracellular parameters with fluorescent probes that are sensitive to changes in calcium, pH, or transmembrane potential (44). An additional activation directed assessment is to evaluate oxidative burst in granulocytes and other leukocytes. This method is diagnostic for patients with chronic granulomatous disease, as well as the X-linked carriers of this disorder (45). It depends on loading granulocytes with a specific dye, dihydrorhodamine-123 (DHR), activating the cells, and then evaluating cell fluorescence using flow cytometry. The activation step induces hydrogen peroxide production through reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase pathway converting DHR to an active fluorochrome that can be detected by the flow cytometer. Patients with chronic granulomatous disease have a defect in this enzyme pathway, and their granulocytes show only background fluorescence, whereas normal cells show bright fluorescence and cells from X-linked carriers demonstrate two cell populations, normal and abnormal.

Tetramer Technology

A more recent application of flow cytometry allows for the enumeration of antigen-specific T cells (46). The reporter in this assay is a soluble *tetrameric antigenic peptide-major histocompatibility complex (MHC) complex* that serves as a ligand for T cells specific for the antigenic peptide and MHC. Flow cytometric detection is achieved by binding an avidin-linked fluorochrome to streptavidin contained within the tetrameric complex. Application of this technology requires that the composition of the antigenic peptide and its human leukocyte antigen (HLA) restriction are known. To date, most applications have focused on MHC class I–mediated (CD8) T-cell responses; however, detection of antigen-specific CD4 T cells with tetramers generated from soluble MHC class II and peptide has also been reported (47). These approaches should help to clarify the kinetics of primary and secondary T-cell responses. This method can also be combined with surface phenotype and intracellular functional markers. Tetramer-based flow cytometry is proving useful for the study of the immune response to infectious agents and vaccines. It is likely that this new technology will find many more applications.

EVALUATION OF B-CELL FUNCTION

Quantitative Immunoglobulin

Obtaining IgG, IgA, and IgM levels represents the initial screening test in evaluating B-cell function. These results must be interpreted with age-matched controls, because of significant changes with age, as well as more minor differences based on gender and race (48,49). Most normal ranges report a 95% confidence interval, meaning that 2.5% of normal values fall below and 2.5% fall above this range. The most common laboratory methods used to determine Ig levels are automated nephelometry or radial immunodiffusion.

IgG subclass levels are also available to investigate more subtle abnormalities in B-cell function. This approach is based on the findings of low normal total IgG accompanied by a selective depression of one or more IgG subclasses in certain patients with recurrent sinopulmonary infection (50). These results must be balanced with the observation that most persons with abnormal IgG subclass levels are clinically normal (51,52). Thus, the assessment of IgG subclass levels does not represent a screening test for immune deficiency. Furthermore, it is a moderately expensive test and should be reserved for evaluating patients with a history of recurrent bacterial infections in the setting of low normal to minimally depressed IgG levels. This test is most useful in the setting of IgA deficiency associated with recurrent sinopulmonary infections. Identifying an IgG subclass deficiency generally requires demonstration of a functional antibody abnormality *in vivo* to justify initiating replacement therapy.

Functional Antibody Testing

Assessment of specific antibody levels in vivo may be useful in screening for B-cell immune competence (53). This approach includes evaluating isohemagglutinins, as well as testing for antibody levels to documented prior immunizations or natural infections (e.g., tetanus toxoid, polio, rubella, rubeola). An important approach for evaluating B-cell immunity is the testing of *in vivo* antibody production in response to recall antigens. This consists of measuring preimmunization titers and 2- to 3-week postimmunization titers to both protein antigens (e.g., tetanus toxoid, diphtheria toxoid) and polysaccharide antigens (e.g., pneumococcal polysaccharides) (54,55). Evaluation of specific antibody levels is available at many medical centers, as well as from selected commercial laboratories and state department of health laboratories. There are also neoantigens, including bacteriophage fX174, that are studied under research protocols to evaluate the primary antibody response (56). No patient with suspected immune deficiency should receive an immunization with a live virus vaccine. Overall, this type of testing provides definitive evidence for the presence or absence of normal B-cell function. In the absence of a major depression in the Ig levels, information regarding *in vivo* antibody production is absolutely

essential before one considers intravenous Ig therapy.

In Vitro Antibody Production

Testing B cells for the capacity to undergo terminal differentiation and to secrete Igs after *in vitro* stimulation with either nonspecific polyclonal activators (mitogens) or specific antigens is used in many research laboratories (53,57). These studies generally involve culturing purified B cells in combination with T cells or T-cell products together with an activator for a prescribed time, followed by evaluation of the cell-free culture supernatant for secreted antibody or *in situ* using the ELISPOT immunofixation method (58). This can provide data regarding B-cell responsiveness as well as the function of regulatory T cells involved in the control of Ig production. The application of this testing may enable investigation into basic questions, including those directed at evaluating abnormalities in isotype switching and possible cytokine alteration in B-cell responsiveness or T-cell immunoregulation. This approach is generally reserved for more basic investigations directed at studying underlying mechanisms of disease or identifying disease variations and does not represent testing used for diagnostic purposes.

EVALUATION OF T-CELL FUNCTION

Delayed-Type Hypersensitivity Testing

Assessment of the capacity of T cells to respond to recall antigen *in vivo* is performed using the method of *delayed-type hypersensitivity skin testing.* Intradermal injection of a recall antigen induces an inflammatory process that requires the proliferation of antigen-specific memory T cells, cytokine release, and infiltration of monocytes (59). This process produces maximal local inflammation 48 hours after antigen exposure, as evidenced by erythema and induration at the injection site. The test site should be examined at 24, 48, and 72 hours, with the early assessment time used to rule out the alternative inflammatory response mediated by IgG (Arthus response), which peaks at 12 to 24 hours. Failure to mount a delayed-type hypersensitivity response can result from either immune dysfunction or lack of prior exposure to the test antigen. To minimize the latter possibility, a panel of antigens is useful based on the observation that virtually all immunologically normal persons respond to at least one antigen when they are tested with a combination of tetanus toxoid, *Candida,* and mumps antigens (60). The absence of reactivity to a panel of recall antigens strongly suggests cutaneous anergy with an underlying T-cell deficiency. Establishing that normally functioning T cells were present in the past is provided by obtaining a history of contact sensitivity including common responses to *Rhus* (poison ivy) or nickel. An absolute lymphocyte count should be obtained when initiating an *in vivo* evaluation of cell-mediated immunity, because T cells normally comprise approximately 75% of the circulating peripheral lymphocytes. A significant decrease in T cells would likely result in a decrease in the overall lymphocyte count, and as such, the count represents a simple screening test for the presence of circulating T lymphocytes.

Proliferation Assays

The *in vitro* correlate of delayed-type hypersensitivity testing is *T-cell proliferation* in response to a recall antigen. This test evaluates a complex process involving ligand-receptor interaction, signal transduction, generation of specific receptors, production of cytokines, and, ultimately, cell proliferation. In addition to recall antigens, alloantigens (mixed lymphocyte culture) mitogens (e.g., phytohemagglutinin, concanavalin A, pokeweed mitogen), and specific monoclonal antibodies (e.g., OKT3) can also be used to induce T-cell proliferation (53,61,62 and 63). The conventional approach is to evaluate cell proliferation by separating mononuclear cells from the peripheral blood on a density gradient and then culturing the cells in the presence or absence of an activator. At the end of the culture period, tritiated thymidine is added for a predetermined interval, to allow cellular incorporation of this radioactive DNA precursor to measure DNA synthesis. Tritiated thymidine incorporation is quantitated using a scintillation counter, and the results are expressed either as counts per minute (cpm) or disintegrations per minute (dpm). In addition, a stimulation index for each culture condition can be generated using the following equation:

$$\text{Stimulation Index} = \frac{\text{cpm of simulated cells}}{\text{cpm of unstimulated cells}}$$

Mitogen stimulation activates a significant proportion of normal T cells and is generally assessed after 3 days of culture. Antigen or alloantigen stimulation initially activates only those T cells with the appropriate antigen receptor, followed by recruitment and activation of additional cells such that a longer culture period of 6 to 7 days is generally used. Assessment of proliferation to recall antigens should include more than one antigen because, as with delayed-type hypersensitivity testing, the response to a recall antigen is predicated on previous exposure. In evaluating possible immune deficiencies, it may be useful to evaluate multiple doses of each activator and to examine more than one culture interval.

Longitudinal investigation in patients with HIV infection has demonstrated that unresponsiveness to recall antigen can be present early in the course of the infection, a finding suggesting the presence of a relatively subtle T-cell disorder already at this stage in the infection (64). As the disease progresses and the degree of T-cell deficiency increases, the T-cell response to alloantigens becomes depressed. Finally, in the later stages of disease, as the immune deficiency becomes more extensive, there is loss of the response to the mitogen, phytohemagglutinin. Thus, the degree of T-cell deficiency appears to be reflected by a progression in the level of the *in vitro* proliferative defect. These data suggest that the *in vitro* response to recall antigen is the most sensitive test for T-cell abnormalities, whereas mitogen (phytohemagglutinin) unresponsiveness is associated with profound T-cell deficiency.

Alternative techniques to evaluate T-cell activation involve flow cytometry. One such method is directed at quantitation of receptors that are upregulated after activation. Included among these is evaluation of CD69 expression that occurs within hours of the activation signal (65). Alternatively, the transferrin receptor (CD71) and the high-affinity interleukin-2 (IL-2) receptor (CD25) can be quantitated later after activation. This approach does not represent a standard method for evaluating T-cell activation, although some laboratories use it for screening. The expression of these activation antigens is not the equivalent of cell proliferation and thus may not fully reflect the findings with culture studies outlined earlier. A flow cytometric alternative for evaluating lymphocyte proliferation depends on evaluation of cell cycle after lymphocyte stimulation. This can be performed using DNA intercalating dyes to determine the distribution of cells in each phase of the cell cycle. Dividing cells can also be identified flow cytometrically by evaluating the incorporation of bromodexosyuridine into newly synthesized DNA using a labeled antibody directed at this substance. Finally, fluorescence intracellular, tracking dyes have been used with flow cytometry to evaluate cell proliferation based on the 50% decrease in total cell fluorescence associated with each round of cell division (66).

Cytotoxicity

The critical role of MHC (HLA) restriction, together with antigen specificity in T-cell recognition, was first established using an *in vitro* test for *T-cell–mediated cytotoxicity* (67). The assay described T-cell killing of virally modified self-targets, which is now known to involve $CD8^+$ T cells recognizing endogenously processed viral antigen in the context of class I MHC molecules (HLA-A and B molecules) (68). It has also been established that $CD4^+$ T cells can act as cytotoxic T cells, and these effectors recognize antigens, after endosomal digestion of the antigen, in the context of class II MHC molecules. Class I and class II MHC-restricted cytotoxicity is mediated by T cells with T-cell antigen receptors that express the ab chains. The underlying requirement for cytolytic activity is that the effector T cell has been previously sensitized (exposed) to the target antigen. Alternatively, some non–MHC-restricted cytotoxic T-cell activity does not require prior sensitization for cytolytic activity. These cells constitute a small percentage of the circulating T cells, most of which have T-cell receptors that express the gd chains.

In a conventional T-cell cytotoxicity assay, the target cells are labeled with a radioactive intracellular marker (chromium-51 [^{51}Cr]), and T-cell lysis of the target cell is assayed by measuring radioactivity released into the cell-free supernatant (69). Standard target cells include virally infected (modified) cells, allogeneic cells, mitogen-stimulated blasts, and tumor cell lines. In MHC-restricted assays of virally modified cell killing, previously sensitized T cells are initially cultured for 4 to 5 days, with the cells expressing the specific viral antigen to generate active cytotoxic cells. ^{51}Cr-labeled target cells are then cocultured with the activated effector cell preparation at a variety of prescribed effector cell–to–target cell ratios; the cell-free supernatants then are collected and are assayed for released ^{51}Cr. The degree of lysis is compared between normal and patient effector cells, with results expressed as the percentage of cytotoxicity, calculated as follows:

$$\%\ \text{cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}}$$

Maximal cpm (lysis) is determined by assaying the supernatant from detergent lysed target cells, whereas spontaneous cpm (lysis) is the amount of radioactivity in the supernatant obtained from target cells incubated without effector cells. Additionally, the results can be expressed as lytic units, where one lytic unit is defined as the number of control cells required to lyse a designated percentage of the target cells. An alternative method using cell tracking dyes and flow cytometry to evaluate the level of target cell lysis has also been described.

Analysis of T-cell–mediated cytotoxicity is not routinely available in most laboratories because the assays are difficult to set up, and the clinical utility of the results remains to be clearly defined. T-cell–mediated cytotoxicity directed at virally infected cells probably is a major protective mechanism in controlling or eradicating certain viral and other intracellular microbial infections as well as playing a role in tumor immunity (70). This theory has resulted in studies that monitored the evolution of

HIV-specific cytotoxic T cells and the production of specific antibody levels after immunization with experimental HIV vaccines.

Cytokines

Lymphocyte production of *cytokines* as a result of cell activation can be assessed by evaluating the supernatant obtained from cells cultured in the presence of mitogen, antigen, or other activator (Chapter 10 and Chapter 11) (71). Assay methods for quantitating these proteins include commercially available immunoassays most commonly based on an ELISA assay. Functional assays, technically more complicated, test for specific cytokine activity using appropriate *in vitro* culture systems. The evaluation of cytokine levels *in vivo* is complicated by the short half-life of most cytokines, together with the high-affinity binding of these proteins to their respective cell-surface receptors. At this time, testing for *in vivo* cytokine levels has provided useful information regarding immunologic mechanisms underlying certain chronic inflammatory conditions, particularly at the local level. Additional approaches include the evaluation of local cytokine production, the identification of cytokine antagonists, and the assessment of cytokine-specific receptors on cells or in a soluble form.

EVALUATION OF NATURAL IMMUNITY

Natural Killer Cell Function

NK cells are a population of circulating lymphocytes distinct from T cells and B cells that are characterized morphologically as large granular lymphocytes. Phenotypically, most of these cells are $CD16^+$ and $CD56^+$, but $CD3^-$ (72). In addition, approximately two-thirds to three-fourths of NK cells are $CD2^+$, whereas one-third to one-half are dimly positive for CD8. NK cell–mediated cytotoxicity does not require prior sensitization for the effector cell to lyse a susceptible target cell (69). *In vitro* assays typically use a cultured cell line (e.g., the K562 erythroleukemia cell line) as a target. The target cells are radiolabeled with ^{51}Cr and then are cocultured with density gradient, separated peripheral blood mononuclear cells, or a preparation of enriched NK cells. There is no requirement for a sensitization step, and cytolytic activity is tested immediately after cell processing. The assay method is similar to the assay described for T-cell cytotoxicity using various ratios of effector cells to target cells. The results are expressed either as a percentage of cytotoxicity or in lytic units.

Proliferation and cytokine production can also be assessed in NK cells using nonlabeled target cells as stimulants. The clinical utility of this approach has not been established.

The NK cell appears to be important in dealing with specific viral infections, allograft rejection, and tumor rejection. Absence of NK cell activity appears to be rare, with patients reported to have undetectable NK cell function in association with recurrent herpes infection (73). In addition, diminished NK cell function has been noted in certain situations, including in patients with advanced cancer.

Antibody-Dependent Cellular Cytotoxicity

The NK cell is one of several different cell types that mediate *antibody-dependent cellular cytotoxicity* (ADCC) (69). In this type of cytotoxicity assay, the cytotoxic effector cells attach by their IgG Fc receptors to IgG bound to the target cells. The actual role of ADCC in host defenses is still unknown, although it is theorized to play a part in the eradication of IgG-coated tumor or infected cells. ADCC is tested with a standard cytotoxicity assay system using ^{51}Cr-radiolabeled target cells that have been preincubated with anti–target cell antibody (IgG). Various effector cell–to–target cell ratios are used, release of the radioactive label into the supernatant is measured as a reflection of target cell lysis, and the results are expressed either as a percentage of cytotoxicity or in lytic units. The clinical application of ADCC testing is primarily of research interest at this time.

Cytokine-Enhanced Cytotoxicity

Preincubation of NK cells with specific cytokines, including interferons and IL-2, has been noted to *enhance in vitro* cytotoxicity. Interferon addition directly to the *in vitro* cytotoxicity assay enhances the level of target cell lysis (74). Alternatively, preincubation of the effector cells with IL-2 for 24 hours or longer increases not only the level of cytotoxicity, but also the range of susceptible target cells (75). Thus, *in vitro* preincubation of effector cells with IL-2 results in the lysis of target cells that would not be killed in a standard NK cell assay (e.g., autologous tumor cells). These IL-2–stimulated cytotoxic cells have the phenotypic characteristics of NK cells and are referred to as lymphokine-activated killer (LAK) cells. The observation that IL-2 enhances the effectiveness of cytotoxic cell lysis of tumor cells has led to cellular immunotherapy protocols for selected cancers. In these studies, mononuclear cells are obtained from patients, are cultured *in vitro* with IL-2 to generate LAK cells, and then are infused into the patient for possible cellular antitumor effect.

EVALUATION OF NEUTROPHIL FUNCTION

The *evaluation of neutrophils* can be separated into specific features of normal cell function that include chemotaxis, phagocytosis, generation of the respiratory burst, and microbial killing (Chapter 20) (76,77). In addition to evaluating each of these processes, assessment for the presence of the specific leukocyte adhesion molecules, CD11/CD18, also may be indicated in selected patients who have a history of recurrent bacterial infections with a predilection for the skin, periodontal tissue, and lungs. More than 15 congenital disorders affect neutrophil function, including congenital neutropenia as well as disorders of motility, adhesion, chemotaxis, granular unction, and oxidative metabolism. In addition, there are age-related changes in neutrophil function, as well as a various acquired phagocytic defects secondary to disease, pharmacologic agents, and other influences.

Neutrophil Chemotaxis

Directed cell migration can be studied using isolated peripheral blood neutrophils in an apparatus called a Boyden chamber (78,79). This device is designed with a millipore filter membrane that allows separation of the cells from a solution containing a chemoattractant (e.g., C5a, formyl-methionyl-leucyl-phenylalanine [FMLF]) or control material. The neutrophils are incubated in the chamber for a predetermined period to allow for directed cell movement in response to the chemoattractant. The filter is removed and stained to allow enumeration of neutrophils that migrated toward the chemoattractant. These results are compared with migration in response to a control stimulus as well as the response of normal neutrophil in the same assay system. An alternative approach involves evaluating chemotaxis under agarose in response to standard chemoattractants (79,80). Neutrophils are placed in a central well cut in the agarose; chemoattractant and control solution are placed in two different wells, equidistant from the site of the cells. The agarose plate is incubated for 1 hour, to allow directed and random cell migration. The cells are fixed, and the migration pattern is visualized under a microscope. The distance from the origin to the leading edge of cells in response to the chemoattractant is measured and is compared with the random movement of cells in the direction of the control well. Chemotaxis assays must be set up with normal controls, and results also must be compared with age-matched data. In addition, *in vivo* chemotaxis can be studied with the Rebuck skin window method by making a superficial skin abrasion and covering it with a sterile coverslip. The numbers of cells that migrate onto the coverslip are counted periodically over 1 day.

Abnormalities of chemotaxis have been found in the leukocyte adhesion deficiency, the Chédiak-Higashi syndrome, the Pelger-Huët anomaly, juvenile periodontitis, and some patients with the hyper-IgE syndrome. Certain drugs may interfere with chemotaxis, and monocytes can secrete substances that inhibit chemotaxis. Demonstration of a significant decrease in neutrophil response to chemoattractants *in vitro* is usually associated with a diminished inflammatory response *in vivo.*

Oxidative Burst

The oxidative response that follows neutrophil activation and phagocytosis increases hexose monophosphate shunt activity, oxygen consumption, hydrogen peroxide production, and superoxide radical formation. Functional screening of this process can be performed using qualitative assays such as the nitroblue tetrazolium (NBT) test or using a flow cytometric assay (45,81,82 and 83). In the NBT assay, stimulation of neutrophils with a potent activating agent such as phorbol myristate acetate (PMA) induces *oxidative burst* (81). This reduces the NBT dye within the cell and generates blue insoluble crystals of formazan, which can easily be detected by microscopic examination. Absence of NBT dye reduction is a classic finding in patients with chronic granulomatous disease. Similar information can be obtained using a flow cytometric method in which the neutrophils are loaded with DHR (discussed in the section on intracellular flow cytometry); after PMA activation, the increase in intracellular hydrogen peroxide produces a marked increase in fluorescence (45). This method has proven to be an extremely reliable approach to the diagnosis of chronic granulomatous disease and X-linked carriers of this disorder (83).

Quantitative assessment of the oxidative burst can be performed with the chemiluminescence assay or the ferricytochrome C reduction test (79). The chemiluminescence assay measures oxidative burst–dependent generation of light (energy) by activated neutrophils. Addition of ingestible particles (e.g., zymosan) or a soluble stimulator (e.g., PMA), activates neutrophils and induces phagocytosis; this results in the release of light (energy) which, in the presence of luminol, can be measured with a scintillation counter. Alternatively, the reduction of ferricytochrome C, manifested by increased light absorbance at 550 nM, is an accurate measure of superoxide release. A patient response in either the chemiluminescence or cytochrome C reduction assays should be compared with normal responses, and the results usually parallel the findings with the NBT or the flow cytometry tests.

Microbial Killing

Assessment of the capacity of neutrophils to kill microorganisms involves mixing neutrophils with opsonins and bacteria *in vitro* (79). The cell-free supernatant and the lysed leukocyte pellet are cultured for residual bacterial growth at prescribed time intervals. Patients' cells are compared with normal cells for both the uptake of organisms from the culture supernatant and the effectiveness of intracellular killing. Different types of microorganisms may yield varied results in this type of neutrophil assay; for example, cells from patients with chronic granulomatous disease demonstrate markedly diminished killing of *Staphylococcus aureus* but relatively normal killing of *Escherichia coli.* Accuracy of this assay requires establishing optimal bacteria-neutrophil ratios and well-defined normal controls.

Adhesion Molecule Testing

The evaluation of three related *adhesion molecules,* leukocyte factor antigen-1, Mac-1, p150,95, has evolved from the description of patients with a defect in the expression of these cell-surface receptors (77,84). These patients were noted to have recurrent infections with minimal inflammatory response, delayed wound healing, and persistent neutrophilia. All three of these molecules are heterodimers, each consisting of a unique a-chain protein (CD11a, CD11b, CD11c) linked to a common b-chain protein (CD18). They can be identified flow cytometrically with monoclonal antibodies to the a chains as well as to the common b chain. Patients with leukocyte adhesion deficiency type 1 have depressed or absent expression of these surface antigens in both resting and activated neutrophils related to a defect in the b-chain gene. Additional features in this disorder include decreased cell adherence, chemotaxis, and phagocytosis. This abnormality in expression of b integrin expression is found on other leukocytes and results in abnormalities in selected *in vitro* lymphocyte assays.

EVALUATION OF MONOCYTE-MACROPHAGE FUNCTION

The *evaluation of monocyte function* can be directed at the assessment of antigen presentation for T-cell proliferation, ADCC, tumor cell cytotoxicity, chemotaxis, microbial killing, and cytokine receptor expression. The availability of these tests has been limited primarily to research settings because the clinical indications for testing monocyte function are not well defined (85,86).

Antigen Presentation

Antigen presentation can be evaluated by adding soluble antigen to a preparation of monocyte-enriched cells. These cells can be obtained from peripheral blood mononuclear cells either by harvesting plastic adherent cells or by using a discontinuous density gradient (Percoll) centrifugation. After an incubation period, remaining soluble antigen is removed by washing, and the monocytes are then cocultured with autologous (or allogeneic MHC class II–matched) T cells. Memory CD4 T cells with the appropriate antigen receptor are activated through interaction with the processed antigen displayed on the monocytes' class II MHC antigens (86). The T cells are then cultured for a defined time, and cell proliferation is assayed using standard methods. This evaluates the capacity of monocytes to process and present antigen to T cells. However, an abnormal result would require additional studies because this can be the result of either a monocyte defect in antigen processing and presentation or a deficiency in T-cell function.

Monocyte-Mediated Killing

Evaluation of monocyte-mediated ADCC uses a standard assay system. The choice of ^{51}Cr-labeled target cell determines the primary effector cell being assayed: IgG-coated chicken red blood cells reflect predominantly monocyte-mediated killing, whereas IgG-coated Chang cells (a cultured tumor cell) are primarily NK cell targets. Assays of monocyte-mediated tumor cell killing are also available. Assessments of monocyte chemotaxis and microbial killing represent specialized testing performed infrequently. Testing requires using monocyte-enriched cells and assays analogous to those described in the section of this chapter on neutrophils.

Interferon Receptor Deficiency

Patients with recurrent *Mycobacterium avium-intracellulare* infections were identified who had a deficiency in the expression of the *interferon-g receptor* (87). This appears to define a subgroup of patients with more limited recurrent opportunistic infections, and the defect can be detected using flow cytometry, to evaluate for expression of the interferon-g receptor on monocytes. The method requires identification of monocytes based on standard gating procedures and comparing the binding of a labeled anti–interferon-g antibody between normal cells and patients' cells. The receptor defect has been associated with abnormalities in the genes coding for either of the protein chains in the interferon-g receptor (87,88).

ASSESSMENT OF COMPLEMENT

The *complement system* is made up of multiple protein components, some with subcomponents, which can be activated in a cascadelike fashion (89,90). In addition, plasma proteins and membrane proteins act to regulate the complement cascade. Activation of complement has a significant role in host defenses through its opsonic and lytic activity, as well as by providing inflammatory mediators, promoting the catabolism of circulating immune complexes, and playing a role in regulation of the immune response. The three pathways of complement activation are the classical, lectin, and alternative pathways (Chapter 26). The first is initiated by immune complexes formed from the interaction of IgG (IgG1, IgG2, IgG3) or IgM with specific antigen; the second is formed by the interaction of protein lectins such as mannan-binding lectin to surfaces rich in the appropriate sugar, and the third can be activated by the presence of repeating polysaccharides (e.g., lipopolysaccharide) and certain other polymeric structures. The alternative pathway is greatly accelerated by bound antibody of any class, and it also amplifies classical pathway activation. The complement proteins are labile, and complement activity is lost if the serum sample is not handled properly. To prevent loss of complement activity, samples must be stored at –70°C as well as diluted in special buffers and placed on ice during test procedures.

Complement Activity

Activity of the classical pathway of complement can be evaluated using the total hemolytic complement (CH50) assay (91). In this test, sheep red blood cells are sensitized by preincubating the cells with an anti–red cell antibody (hemolysin). Addition of various dilutions of patient or control sera serves as the source of complement for an aliquot of sensitized sheep red blood cells. The tubes are incubated at 37°C, and the degree of red cell lysis is measured by assaying the supernatant spectrophotometrically for hemoglobin released from lysed sheep red cells. Standard lysis curves are generated, and the CH50 result is reported as the inverse of the serum dilution that produces 50% lysis of the sensitized red cells. Currently, modifications of the traditional CH50 tube test use microtiter plates to simplify the procedure. There is also a commercial hemolytic complement test (CH100) in which test serum is added to wells cut in agar containing sensitized sheep red blood cells; after incubation, the diameter of red cell hemolysis around each well is determined and plotted on a standard curve. The lability of complement components means that improper sample handling or storage can result in diminished or absent total hemolytic complement activity with otherwise normal samples.

The most common abnormality of the CH50 assay is increased complement activity resulting from an acute-phase response (91,92). This results from a generalized increase in protein synthesis, including the complement components, and can be seen in a variety of inflammatory diseases. The opposite situation, depression in CH50 activity, can result from either hyposynthesis or hypercatabolism of the complement components. An extreme case of the former is seen with congenital deficiency of a component, in which the CH50 is zero because of the absence of any individual classical pathway component (93). This test has proven to be an effective means for identifying congenital component deficiency, and it is particularly useful in diagnosing late component defects that are observed in certain patients with recurrent *Neisseria* infections. The alternative explanation for depressed complement activity, hypercatabolism of complement components, is most frequently associated with complement activation resulting from immune complex generation. This would be expected in a systemic autoimmune disease, such as systemic lupus erythematosus, in which the generation of immune complexes is significant. The CH50 assay has been found to be abnormal at some period in most patients with systemic lupus erythematosus, but the clinical utility of this finding remains controversial (94). In most patients, alterations in CH50 activity has not proven to be a reliable predictor of impending disease flare or a satisfactory monitor of therapeutic efficacy.

Assessment of the alternative complement pathway is performed only in research laboratories, because these data are required in few clinical situations. The assay, referred to as the APH50 test, consists of adding various dilutions of test serum to (nonsensitized) rabbit red blood cells and assaying for hemolysis. The rabbit red cell expresses surface molecules that spontaneously activate the alternative complement pathway in the absence of specific antibody. The data generated in this test are handled in the same fashion as those from a CH50 test, with the results expressed as the inverse of the serum dilution producing 50% red blood cell lysis. The presence of serum antibody reactive with rabbit red blood cells would increase the hemolytic activity in the APH50.

Component Testing

Evaluation of specific complement components may be included in the laboratory evaluation of suspected congenital deficiency of a component, as well as immune-mediated inflammatory diseases. Component testing has limitations similar to those of the CH50 assay when used as a monitor of inflammatory disease. Immunoassays for complement components are technically the simplest means of assessing for component concentration, and they usually involve either radial

immunodiffusion or nephelometric testing. Most clinical laboratories use one of these methods to quantitate selected complement components, including C3, C4, factor B, and C1 inhibitor. The immunologic assessment of these proteins is satisfactory, except in the unusual situation of a functionally deficient component that is antigenically indistinguishable from its normal counterpart. An example of this is observed in some patients with hereditary angioedema, a condition that results from a deficiency of the complement regulatory protein, C1 inhibitor. This disease is associated with recurrent, nonpruritic swelling of the skin, respiratory tract, and gastrointestinal tract. A few patients with hereditary angioedema have normal levels of C1 inhibitor by immunoassay because of a functionally abnormal protein that is antigenically indistinguishable from normal C1 inhibitor. Thus, in these uncommon patients, only a functional assay (but not the immunoassay) for C1 inhibitor will establish the diagnosis.

Functional assays evaluate the actual activity of a specific complement component. The indicator system is usually a CH50 assay constructed to contain all components except the one tested. The unknown sample is added to provide this missing component and is compared for degree of red cell hemolysis with the results generated using a normal serum sample. These assays are labor intensive and generally are performed only in specialized laboratories that focus on complement testing. The main clinical application for component testing is in a patient with clinical findings suggesting a complement defect with laboratory findings of depressed or absent CH50 activity. Additionally, functional testing may be indicated in disorders of the various complement regulatory proteins, particularly in the clinical setting suggestive of C1 inhibitor deficiency (see earlier).

Assessment for evidence of complement activation as a result of immunologically mediated disease theoretically could predict flares or therapeutic efficacy of interventional therapy and could be useful in individual patients with systemic lupus erythematosus (95). However, the overall prognostic or predictive utility with either CH50 or component assays remains limited (96). The reason may be that the level of any component reflects the net balance of production and consumption. Studies suggest that assaying complement cleavage products could be a more reliable indicator of complement activation despite their short half-life and the high binding affinity of their specific receptors (97,98). These reports have used tests directed at one or more of the following cleavage products: C4d, C3a, iC3b, Bb, SC5b-9 (99). The ultimate clinical utility of this approach for the assessment of complement activation in clinical settings awaits additional studies.

Immune Complex Assays

Assessment of circulating immune complexes can be performed using various different techniques (100,101). Standard approaches currently used include solid-phase immunoassays to detect immune complexes through the binding of a complement component attached to the antigen-antibody complex. Included are three methods: the anti-C3 assay using antibodies to immune complex–bound C3 products (iC3b, C3d, C3dg); the C1q binding test that uses immobilized C1q to bind immune complexes containing IgG and IgM; and the conglutinin assay, which uses an immobilized bovine protein (conglutinin) that attaches to immune complex fixed iC3b. The immune complex detection step in all these systems involves addition of a labeled antibody that binds human Ig. The results are expressed as micrograms of immune complex based on a standard that is derived from a heat-aggregated IgG. Additional methods include the Raji cell assay, which uses an EBV cell line expressing receptors for C3b, iC3b, and C3d to capture immune complexes and the radiolabeled C1q precipitation assay. The clinical utility of testing for circulating immune complexes has not been clearly established, and detection remains primarily of research interest.

IN VITRO ALLERGY TESTING

Immunoglobulin E Quantitation

IgE is present in concentrations that are four to five orders of magnitude less than concentration of IgG, IgA, and IgM. This means that the determination of IgE concentrations requires alternative testing techniques. The most common method for IgE detection involves a sandwich immunoassay in which an nti-IgE antibody is immobilized on a solid phase (102,103 and 104). The sample is added to the antibody-coated solid phase (tube, microtiter plate, beads), which allows the binding of IgE. This is followed by a washing step and the addition of a labeled (radioactive, enzyme) second antibody to IgE. The amount of labeled antibody attached correlates directly with the quantity of IgE in the sample. A World Health Organization standard for IgE was developed to allow laboratories to standardize test results (103).

The measurement of total IgE is not a useful screen for allergy because of significant overlap between allergic and nonallergic subjects (103,104). It does appear that infants with elevated IgE levels have increased risk of allergies, and adults with extremely low IgE levels (less than 50 ng/mL) are relatively unlikely to have allergies. Marked elevation in IgE levels is seen commonly in patients diagnosed with the Wiskott-Aldrich syndrome and hyper-IgE syndrome, as well as with systemic parasitism. Serial IgE levels are useful in assessing the effectiveness of corticosteroids in the treatment of patients with allergic bronchopulmonary aspergillosis.

Allergen-Specific Immunoglobulin E

Testing for allergen-specific IgE is usually performed with epicutaneous skin tests. *In vitro* testing for allergen-specific IgE is usually reserved for situations that include extraordinary sensitivity to allergen, abnormal skin conditions, and required medication that interferes with skin test results (104,105). The *standard in vitro* test uses specific allergen attached to a solid-phase support (allergosorbent) as the test substrate. Serum is added to the allergosorbent to allow any specific antibody to bind, unbound antibody is washed away, labeled anti-IgE is added, and the amount of bound label is measured as a reflection of the allergen-specific IgE concentration. This test is called a *radioallergosorbent test* when the anti-IgE antibody is labeled with a radionuclide. It is also possible to use alternative reporters, including enzyme- or fluorochrome-labeled anti-IgE, in the allergosorbent test and to measure color change or fluorescence. The most recent assays use a World Health Organization IgE reference standard and express the results as the level of allergen-specific IgE per unit volume rather than using a numeric grading system. Studies using this new allergosorbent system suggest that the level of allergen-specific IgE may predict a systemic reaction in children with certain food allergies (106). The major obstacle in interpreting these data relates to the difference in results generated with assays from different manufacturers. In light of this situation, standardization of the various reagents and assays represents a desirable goal for *in vitro* allergen-specific testing.

The basophil histamine release assay is an alternative *in vitro* approach for assessing allergen-specific IgE that theoretically is more representative of an allergic reaction (107,108). The indicator system involves the addition of an allergen preparation to the patient's whole blood or separated leukocytes as a source of basophils. The allergen cross-links specific IgE bound to the basophil surface, which induces mediator (e.g., histamine) release similar to an *in vivo* allergic response. Histamine is measured in the supernatant and is compared with the total basophil histamine content. This test also can be used to examine the effect of drugs and other substances on the allergic response. It generally offers no advantage over skin testing and is primarily a research assay.

Mast Cell Products

Levels of plasma tryptase, plasma histamine, and urinary metabolites of histamine can be assayed as a measure of systemic *mast cell degranulation* (109,110 and 111). Testing for tryptase and histamine is performed by immunoassay, whereas evaluating histamine metabolites is done by high-performance liquid chromatography. An example of a recognized use of these techniques is the evaluation of plasma tryptase levels after a systemic shocklike reaction and for patients with systemic mastocytosis (111).

TEST ASSESSMENT

Sensitivity, specificity, and predictive value are used in evaluating the effectiveness of individual tests applied to specific clinical situations. With increasing concerns over health care costs, it will become even more important to choose the most appropriate laboratory test based on these criteria.

Sensitivity

Sensitivity is the frequency of positive test results in patients with a specific disease.

$$\text{Sensitivity (\%)} = \frac{\text{Number of true positives}}{\text{number of true positives + false negatives}} \times 100$$

Specificity

Specificity is the frequency of negative test results in persons without a specific disease.

$$\text{Specificity (\%)} = \frac{\text{Number of true negatives}}{\text{number of true negatives + false positives}} \times 100$$

Predictive Value

Predictive value is calculated both for positive and for negative test results and is influenced by disease prevalence in the population tested.

Predictive value of a positive test =

$$\text{Predictive value of a positive test} = \frac{\text{Number of true positives}}{\text{number of true positives} + \text{false positives}} \times 100$$

Predictive value of a negative test =

$$\text{Predictive value of a negative test} = \frac{\text{Number of true negatives}}{\text{number of true negatives} + \text{false negatives}} \times 100$$

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Appendix B GLOSSARY

Accommodation Xenograft resistance to acute vascular rejection.

Adaptive immunity The ability to recognize a foreign antigen and develop a specific immune response to antigens.

Adjuvant Substance able to potentiate an immune response.

Adoptive transfer Transfer of the immune state from one subject to another via lymphoid cells or antibody (the latter condition is called passive immunity).

Affinity chromatography Technique by which a material with a selective affinity for a substance is coupled to an inert matrix. The substance to be purified interacts with over the material-matrix complex and adheres to it.

Agammaglobulinemia Absence of immunoglobulin in plasma. Properly speaking, this term is a misnomer because people and animals, even if immunodeficient, always have *some* immunoglobulins. *See also Hypogammaglobulinemia.*

Agglutination Reaction in which particles of antigen or particles coated with antigen are clumped by specific antibody.

Allele One of a pair of genes at a particular locus.

Allelic exclusion Expression of just one gene in a cell containing two allelic genes at that locus.

Allergen Antigenic substance capable of inducing sensitization by virtue of its ability to stimulate production of IgE.

Allergy Altered state of immunologic reactivity that can be cellular or humoral.

Allogeneic Immunologic relation between genetically different members of one species.

Allograft Graft of a tissue or organ placed in a genetically dissimilar recipient (previously *Homograft*).

Allotype Structurally different set of proteins present in one species.

Alpha-fetoprotein (AFP) A specific b-globulin, similar to albumin, that is present in serum and prominent during fetal life.

Alternative complement pathway System of complement proteins, that can be activated without antigen-antibody complexes, including properdin and factors D, B, C3b, finally activating C3 through C9.

Amyloidosis Disorder of protein folding in which normally soluble proteins misfold and aggregate as insoluble amyloid fibrils, with cross-b structure, and these accumulate in the tissues causing disease.

Anamnesis Positive immunologic memory: a second or later exposure to specific antigen causes a heightened immunologic response.

Anaphylactoid reaction Clinical picture similar to that of anaphylaxis.

Anaphylatoxins Group of complement protein derived peptides (C3a and C5a) that cause mast cell activation and histamine release.

Anaphylaxis Clinical syndrome caused by specific antigen reacting with IgE bound to mast cells and basophils, leading to one or more of the following: rhinitis, wheezing, angioedema, urticaria, cardiac dysrhythmia, hypotension, and rarely death.

Anchor residues Positions accommodating only specific amino acids in MHC peptide binding clefts.

Anergy Clinically, the inability to express delayed-type hypersensitivity via skin test challenge. Also, a state of unresponsiveness to antigen induced in T or B cells. *See also Tolerance.*

Angiocentric Growing around and into blood vessels.

Angiogenesis The formation of new blood vessels.

Antibody (Ab) Immunoglobulin produced by B cells that is capable of specifically combining with antigen.

Antibody combining site Cavity formed by the variable segments of the heavy (H) and light (L) chains of an antibody molecule, into which the specific antigenic epitope fits.

Antibody-dependent cell-mediated cytotoxicity (ADCC) Form of cytotoxicity in which an antibody-coated target cell is killed by an effector cell interacting with the Fc portion of the bound antibody.

Anticardiolipin (aCL) antibodies Autoantibodies detected in a cardiolipin ELISA; in the antiphospholipid syndrome the actual antigen detected is b_2-glycoprotein I, which itself binds the cardiolipin.

Antigen Substance capable of inducing a specific immunologic response.

Antigenic competition Interference of the immune response to an antigen produced by immunization with another, unrelated antigen.

Antigenic determinant Portion of an antigen that determines the specificity of the immune response. *See also Epitope.*

Antigenicity The ability of a molecule to be recognized by the product of a preexisting immune response.

Antigenic modulation The covering or alteration of a cell surface antigen following its interaction with specific antibody.

Antigen presentation Antigen processing and display of resultant MHC-peptide combination at cell surface.

Antigen-presenting cells (APC) Cells capable of presenting antigen to T and B cells. They include monocytes and macrophages, B cells, dendritic cells, and endothelial cells.

Antigen processing Conversion of an intact protein into an entity that can be recognized by T cells.

Antiglobulin (Coombs) test Method of detecting antibody bound to cells (direct test) or antibody in serum capable of binding to cells, usually red blood cells (indirect test).

Antilymphocyte serum Serum (usually heterologous) raised by immunization with lymphocytes and designed for immune suppression.

Antinuclear antibodies (ANA) Antibodies directed against nuclear antigens, such as DNA or histones, and associated with a number of autoimmune syndromes.

Antitoxins Antibodies capable of combining with and inactivating toxins, particularly those made by bacteria.

Apheresis Process of selectively removing blood elements or plasma. *See also Plasmapheresis.*

Apoptosis Programmed cell death through fragmentation of a cell into membrane-bound particles that are then eliminated by phagocytosis.

Arthus reaction Local inflammatory reaction, usually mediated by antibodies, one form occurs when antigen is introduced into a previously immunized subject.

Association constant (K value) Mathematic representation of the binding affinity between two molecules, usually an antibody and an antigen.

Atopy States of immediate hypersensitivity mediated by IgE, genetically influenced and including one or more of the following: allergic rhinitis, allergic asthma, urticaria, eczema (atopic dermatitis).

Autoantibody Antibody directed to self antigens.

Autoantigen Self antigen.

Autograft Tissue or organ graft transferred from one place to another in the same recipient.

Autoimmunity Immunologic reactivity to self antigens.

Autoradiography Method to detect radioisotopically labeled materials in tissue sections or cell suspensions by overlaying x-ray or photographic film.

Bacille Calmette-Guérin (BCG) Attenuated strain of *Mycobacterium bovis* used for immunization against M. *tuberculosis.*

Backcross Mating of a parental animal to a related hybrid.

B cells (B lymphocytes) Lymphocytes derived from bone marrow or yolk sac (or bursa of Fabricius in birds) capable of making antibodies, or in the developmental line of such cells.

Bence-Jones proteins Monoclonal light chains present in the urine of some patients with paraproteinemias.

Blast cell Large immature lymphoid or other cell with a large nucleus and active biosynthesis.

Blocking antibody Immunoglobulins capable of blocking cell-mediated lympholysis or promoting growth of tumors. Also, IgG antibodies capable of binding antigen and preventing IgE mediated mast cell degranulation.

Bradykinin Proinflammatory nonapeptide derived enzymatically from a serum b_2-globulin precursor.

Bursa of Fabricius Lymphoid organ near the cloaca of birds that controls the development of B cells.

Cadherins Calcium-dependent adhesion molecules.

Carcinoembryonic antigen (CEA) Antigen expressed on fetal endodermal cells and on some gastrointestinal malignant cells, particularly carcinoma of the colon.

Cardiolipin Material primarily composed of phospholipids; derived from beef heart; used as a substrate in antitreponemal serologic tests.

Carrier Immunogenic material which, when coupled to a hapten, renders the hapten immunogenic.

C3b receptor (CRI) Molecular complex on cells able to bind the C3b fragment of complement.

CD (cluster of differentiation) Denotes a molecule recognized by a monoclonal antibody. Usually the molecule is a cell-surface marker.

Cell-mediated cytotoxicity The strategy used by effector T cells to destroy, by direct contact, cells expressing the peptide/MHC complex to which their antigen receptors are directed.

Cell-mediated immunity (CMI) Immunity mediated by T cells without a requirement for B cells or antibody.

Cell-mediated lympholysis (CML) Specific destruction of lymphoid, tumor, or other target cells by specific T cells, usually of the CD8 class.

Centimorgan Unit of distance on a chromosome, equal to a 1 percent frequency of recombination between nearby genes.

Central lymphoid organs Organs essential for the ontogeny of lymphocytes, including the thymus and bone marrow (and in birds the bursa of Fabricius).

Chemokines Chemoattractant molecules that direct movement of hematopoietic cells.

Chemotaxis Process by which mobile cells are attracted to a source of cytokines.

CH_{502} unit Measure of lytic ability of serum in a standard hemolytic complement test.

Classical complement pathway Cascade of enzyme-substrate and protein-protein interactions of serum proteins beginning with C1 and ending with C9.

Class I MHC antigens Histocompatibility antigens encoded by A, B, and C MHC loci in humans and by other loci in animals.

Class II MHC antigens Histocompatibility antigens encoded by HLA-DR, HLA-DP, and HLA-DQ antigens in humans and by other antigens in other species.

Class III MHC antigens C2, C4, and complement factor B encoded by genes within the MHC.

Clonal anergy Antigen-induced unresponsiveness in a clone of B cells.

Clonal deletion Removal of clones of lymphocytes, primarily during ontogeny-a mechanism thought to be largely responsible for the absence of self-reactive lymphocytes.

Clonal selection theory Concept developed independently by Burnet and Talmage in which clones of lymphocytes with different antigen receptors develop randomly prior to antigen exposure, and where self-reactive clones are generally deleted.

c-*myc* gene Oncogene.

Cognate recognition MHC restricted interaction with a specific antigen.

Cohn fractionation Method of separating serum globulins on a large scale. Cohn fraction II contains most of the serum immunoglobulins.

Cold agglutinins Antibodies that agglutinate bacteria or cells more efficiently in the cold than at 37°C.

Colony-stimulating factors (CSFs) Group of hematopoietic cytokines able to promote the growth of hematopoietic stem cells.

Complement Complex series of serum proteins activated by antigen-antibody complexes and other substances; responsible for modulation and amplification of immunologic and inflammatory reactions.

Complement fixation Standard serologic method to detect the combination of antigen and antibody by depletion of complement via the antigen-antibody complex.

Concanavalin A (Con A) Glycoprotein plant lectin derived from the jackbean; used for polyclonal stimulation of T cells *in vitro.*

Congenic Denotes a strain of animal (usually mice) in which a portion of the genome, usually part of the histocompatibility complex, has been substituted from a different strain by appropriate backcrossing. (Previously *Congenic resistant.*)

Constant region Carboxy-terminal region of H or L chains that is identical in amino acid sequence with other cognate regions in molecules of the same class, subclass, or allotype.

Contact pathway (intrinsic pathway of coagulation) The portion of the coagulation pathway activated by the plasma being in contact with an activating surface such as collagen in vivo or glass *in vitro.*

Contact sensitivity Form of delayed hypersensitivity to antigens expressed on the skin or mucous membranes.

Contrasuppression Inhibition of suppressor activity.

Copolymer Polymer, either random or ordered, of at least two chemical substances.

Coproantibody Antibody present in the feces.

Counterimmunoelectrophoresis (CIE) Form of immunodiffusion in agar in which antigen and antibody diffuse toward each other in an electrical field.

C-reactive protein (CRP) The classical acute phase protein, a member of the pentraxin family, that increases in concentration in the plasma in response to most forms of tissue injury, infection, and inflammation. It binds to sugar moieties on microbes.

CREST Acronym for five manifestations of a rare limited form of scleroderma: *c*alcinosis, *R*aynaud's phenomenon, *e*sophageal dysmotility, *s*clerodactyly, *t*elangiectasis.

Cross-priming Presentation of cellular antigens from a nonpresenting cell by antigen-presenting cells.

Cross reaction Reaction of an antibody to an antigen structurally similar to the antigen that induced the antibody.

Cryofibrinogenemia Presence of reversibly cold-precipitable fibrinogen in blood.

Cryoglobulins Gamma globulins that precipitate in the cold.

Cryopathies Cold-related clinical syndromes that arise secondary to cold agglutinins or cryoglobulins.

Crypts Invaginations in the epithelium of lymphoid tissue in Waldeyer's ring.

Cytokine Substance, usually of low molecular weight and not antigen-specific, that is secreted by one cell (a monocyte or lymphocyte) and acts on another cell.

Cytophilic antibodies Immunoglobulins that bind to cells by their Fab or Fc regions.

Delayed hypersensitivity Prototype inflammatory reaction initiated by specific antigen and the cognate T cells leading to a cellular infiltrate within 24 to 48 hours.

Dendritic cells (DCs) Nonlymphoid bone marrow-derived cells that are efficient in antigen presentation.

Dermographism Urticarial lines appearing on skin along the course of a stroke with a sharp object.

Desmoplakins Intracellular plaque proteins of desmosomes.

D gene region Diversity segment of the genome that encodes part of the hypervariable region of the immunoglobulin heavy chain.

Direct agglutination Clumping of erythrocytes, bacteria, or other antigenic particles by antibody.

Direct immunofluorescence Detection of antigens by antibody labeled with a fluorescent dye.

Domains Segments of heavy or light chains showing homologous structure; stabilized by disulfide bonds.

Dutcher bodies Intracytoplasmic inclusions due to cytoplasmic Ig.

Dysgammaglobulinemia Condition in which one or several (but not all) immunoglobulin classes are over-or underproduced.

EA or EAC rosette Cluster of *e*rythrocytes coated with *a*ntibody and/or *c*omplement adhering to cells with receptors for immunoglobulin Fc or complement.

EBER Small nonpolyadenylated RNAs expressed in EBV-infected lymphoblasts.

EBV p62 nuclear antigen (EBNA) Epstein-Barr virus nuclear antigen. A set of molecules encoded by the herpes virus Epstein-Barr virus found in the nucleus of infected cells.

ECF-A (eosinophil chemotactic factor of anaphylaxis) Peptide, derived mainly from basophils and mast cells, that is chemotactic for eosinophils.

Electroimmunodiffusion Precipitation method in semisoft medium in which antigen precipitates with antibody during electrophoresis. Also called “rocket electrophoresis.”

Electrophoresis Separation of molecules, according to their net charge, in an electrical field.

Endocytosis Carriage of external substances into a cell by pinocytosis, phagocytosis, or both.

Endogenous pyrogens Substances produced by macrophages and other cells that cause fever by acting on the thermoregulatory center in the hypothalamus. Includes IL-1 and IL-6.

Endotoxins Lipopolysaccharides derived from cell walls of gram-negative bacteria.

Enhancement Increased survival of tumors or allografts in animals immunized by those materials, presumably working via antibody.

Enzyme-linked immunosorbent assay (ELISA) A more convenient development of the (radioactive) solid phase radioimmunoassay, whereby the presence of bound antibody is detected by a second antibody that binds to the first and is linked to an enzyme; this enzyme acts on a substrate to generate a color change.

Episome Extrachromosomal covalently closed circles of DNA.

Epitopes Antigenic determinants.

Epstein-Barr virus (EBV) A member of the herpes virus family; associated with a number of human malignancies.

Equilibrium dialysis Method of measuring the affinity by which a small molecule binds to a larger one.

Equivalence Ratio of antigen and antibody concentration that produces maximal precipitation.

E rosette Cluster (rosette) of erythrocytes adhering to a T cell.

Exon Coding strip of DNA.

Experimental allergic encephalomyelitis (EAE) Autoimmune disease produced by immunizing an animal with brain tissue or substances derived from brain tissue together with adjuvant.

Extravasation The process by which white blood cells cross the endothelium to leave blood vessels and enter tissues.

Fab Antigen-*b*inding fragment of an immunoglobulin molecule containing the NH_2-terminal ends of the H and L chains.

F(ab)'$_2$ Fragment of an immunoglobulin molecule containing the NH_2-terminal portions of two H-L chains.

Fc fragment Crystallizable portion of an immunoglobulin molecule containing the C-terminal portions of the H chains.

Fc receptor Receptor present on various cells for the Fc (C-terminal) portion of immunoglobulin molecules.

a-Fetoprotein (AFP) b-Globulin, similar to albumin, that is present in one species.

F_1 generation First generation of offspring after a designated mating.

F_2 generation Second generation of offspring, usually from the mating of two F_1 individuals.

Fibronectin Protein with adhesive properties that is important in the extracellular matrix of connective tissue.

Fluorescence Emission of light of one wavelength after irradiation by light of a different wavelength.

Fractional turnover (or catabolic) rate Percentage of the pool of a substance catabolized in unit time.

Freund's complete adjuvant Emulsion produced by mixing oil, an aqueous phase, and an emulsifier with killed mycobacteria. When mixed with antigen, it amplifies the immune response.

Freund's incomplete adjuvant Emulsion similar to Freund's complete adjuvant but without the mycobacteria.

Gell and Coombs classification A method for classifying mechanisms of immunopathologic damage: Type I, IgE-mediated hypersensitivity; Type II, antibody directed to cell-associated antigens; Type III, immune complex-mediated injury; Type IV, delayed hypersensitivity or cell-mediated immunity.

Germinal center Collection of macrophages and lymphocytes in lymph nodes and other organized peripheral lymphoid tissues.

b_2-Glycoprotein I (b_2-GPI) A phospholipid-binding plasma protein of unknown function that is the antigen for so-called “anticardiolipin” antibodies and some lupus anticoagulants.

Gm marker Genetically determined polymorphic marker on the heavy chain of human IgG.

Graft-*versus*-host disease (GVHD) Cell-mediated reaction mounted by donor T cells against histocompatibility antigens of the host (recipient).

Granulocyte colony-stimulating factor (G-CSF) Cytokine promoting the production of granulocytes from their precursors.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) Cytokine promoting the production of granulocytes and macrophages from their precursors.

Haplotype Set of alleles on a single chromosome that are inherited as a closely linked set, generally considered in terms of MHC genes.

Hapten Antigenic determinant of low molecular weight that can act as an immunogen when coupled to an immunogenic carrier molecule.

Hassall's corpuscles Collections of thymic epithelial cells that may function in the *in situ* death of thymic lymphocytes.

Heavy chain diseases Heterogeneous group of paraproteinemias in which an aberrant clone of B cells makes monoclonal, but often incomplete, heavy chains without light chains.

Heavy chain (H chain) One of two identical polypeptide chains in an immunoglobulin molecule. It contains a V (variable region) at the NH_2 terminus and a C (constant region) at the carboxy terminus.

Helper T cells (Th) Subset of T cells, usually bearing CD4, which functions by cooperating with B cells or other T cells.

Hemagglutination inhibition Sensitive test for antigen in which the agglutination of antigen-coated particles by specific antibody is inhibited by specific antigen.

Hemolysin Antibody or other substance able to lyse erythrocytes.

Heterodimer Molecule composed of two different components.

High-dose tolerance Specific immunologic unresponsiveness induced by large amounts of specific antigen.

High endothelial venules (HEVs) Specialized vessels in the lymphoid system that permit passage of lymphocytes from the vasculature into the tissues.

Hinge region Segment between the first and second constant region domains in an H chain.

Histamine Potent bioactive amine produced by mast cells and basophils; important in IgE-mediated hypersensitivity and other reactions.

Histamine-releasing factors Heterogeneous collection of molecules able to promote histamine release by mast cells and basophils.

H-2 locus Main genetic (MHC) locus in the mouse controlling transplantation reactions and genetic control of the immune response.

Homocytotropic antibody Antibody that binds to cells of the same species.

Homopolymer Molecule composed of repeating units of the same chemical fragment.

Homozygous typing cells (HTCs) Cells from an individual who is homozygous at the class II MHC locus. These cells are used for mixed lymphocyte reaction (MLR) typing.

Horror autotoxicus Concept proposed by Ehrlich indicating that an individual cannot usually mount an immune reaction against his own tissues.

Human leukocyte antigen (HLA) Human MHC region and its products.

Humoral Term applied to soluble substances in body fluids. In immunology, it generally refers to immunoglobulins or complement components (or both).

Hybridoma Transformed cell in which somatic fusion of two parental cells results in a cell with genetic material from both parents.

Hyperacute rejection Accelerated form of graft rejection mediated by antibodies to donor (graft) MHC antigens.

Hypervariable regions Genetically determined areas of amino acid sequence variability in the V regions of H and L chains that determine the unique binding sites for antigen.

Hypogammaglobulinemia Quantitative deficiency of all immunoglobulin classes; often misnamed agammaglobulinemia.

Hyposensitization (desensitization) Production of allergen-specific immunologic hyporesponsiveness in sensitive individuals by repeated exposures.

Ia antigens (I region-associated antigens) Antigens produced by genes in the Ir region.

Iatrogenic Effects induced in patients as a consequence of treatment.

Idiotope Unique configuration on the antigen-combining site of an immunoglobulin or T cell receptor considered as an antigen (epitope).

Immediate hypersensitivity Rapidly developing immune reactions caused by antigen combining with IgE or another class of mast cell–sensitizing antibody. (Type I hypersensitivity of Gell and Coombs.)

Immune adherence Agglutination of particles (including microbes or cells) facilitated by antibody or complement (or both).

Immune complexes Protein complexes composed of antigen and antibody, with or without complement.

Immune elimination (clearance) Enhanced removal of antigen from the circulation by combining with its antibody; it leads to rapid clearance of antigen-antibody complexes by cells of the reticuloendothelial system.

Immune privilege The phenomenon whereby there are sites in the body that appear to preclude cell-mediated immune responses. In transplantation, the prolonged survival of allogeneic or xenogeneic tissue grafts in certain anatomic places.

Immune response (Ir) genes Genes in the MHC that control responses to specific antigens.

Immune surveillance Hypothesis stating that an important function of the immune system is to detect and destroy tumor cells bearing distinct antigens.

Immunization Induction of an immune response by natural exposure or deliberate stimulation of the immune system.

Immunocytoadherence Method for identifying antigen or antibody by the adherence of cells or particles coated with those reagents.

Immunoelectrophoresis Method of detecting antigen-antibody reactions that involves electrophoretic separation of antigens and subsequent immunodiffusion against antibodies.

Immunofixation electrophoresis Method of identifying proteins by electrophoretic separation and subsequent binding to specific antibodies.

Immunofluorescence Technique by which fluorescent molecules are coupled to immune reactants or cells to permit localization of antigens or antibodies.

Immunogen Substance capable of inducing an immune response.

Immunogenicity The ability of a substance to elicit an immune response.

Immunoglobulin Protein composed of H and L chains; it functions as an antibody.

Immunoglobulin class Division of immunoglobulin molecules that depend on unique structures in the constant region of the H chain. There are five immunoglobulin classes in humans: IgG, IgA, IgM, IgD, IgE.

Immunoglobulin isotype switching (class switch) Genetic process by which B cells change the heavy chain isotype of the antibody they produce during the continuation of the antibody-forming process.

Immunoglobulin subclass Further subdivision of immunoglobulin molecules within a class according to structural differences in the H chain. There are four IgG subclasses in humans: IgG1, IgG2, IgG3, and IgG4, and two IgA subclasses: IgA1 and IgA2.

Immunoglobulin supergene family Group of genes that code for a number of immunologically relevant and structurally related proteins, including immunoglobulins, T cell receptors, and class I and II MHC.

Immunosuppression State of diminished immune responsiveness produced by disease or by therapy.

Immunotherapy Treatment of disease by immunologic methods, including hyposensitization with antigen and passive administration of antibodies.

Immunotoxin Compound containing an antibody and an inactive toxin or radioactive isotope; the antibody “delivers” the material to a target cell containing the antigen (a tumor cell) so the toxin or isotope can specifically kill that cell.

Indirect immunofluorescence Three-component system in which antibody binds to the substrate antigen, and fluorescein-labeled anti-immunoglobulin is added to detect the complex.

Innate immunity The immune system that consists of effector cells and soluble mediators that respond rapidly to a variety of insults and that use constantly present germ line recognition mechanisms of broad specificity to accomplish self *versus* non-self discrimination.

Integrins A family of proteins that promote cell adherence.

Interferons (IFN) Diverse group of low-molecular-weight polypeptides having multiple antimicrobial and immune regulatory functions. The group includes IFN-b, IFN-b, and IFN-g.

Interleukins (IL) A heterogeneous group of low-molecular-weight substances having diverse activities in immunologic and inflammatory processes. At the time of writing, IL-1 through IL-12 have been identified and characterized.

Intrabodies Single-chain antibody fragments expressed intracellularly.

Intron Noncoding region of a DNA molecule. *See also Exon.*

Inv marker Genetically determined polymorphic marker on the light chain of human IgG.

Ir region Region of the MHC (usually class II) containing genes that control various immune responses.

Isoagglutinin Antibody able to agglutinate cells of individuals of the same species.

Isograft Tissue or organ graft placed in a genetically identical recipient.

Isohemagglutinins Anti-erythrocyte antibodies able to bind to erythrocytes of other individuals of the same species.

Isotype Characteristic of a class or subclass of immunoglobulins.

J chain Glycopeptide that is part of polymeric immunoglobulins of the IgM and secretory IgA classes.

J region genes Genes coding for the J segment of heavy chains.

Kappa (k) chains One of the two types of immunoglobulin light chains.

K cell Lymphocyte bearing Fc receptors and capable of carrying out antibody-dependent cellular cytotoxicity (ADCC).

Keratoconjunctivitis sicca A condition marked by hyperemia of the conjunctiva, lacrimal deficiency, thickening of the corneal epithelium, itching and burning of the eye.

Kinin system Humoral system linked to the coagulation pathway. The enzymatic activation of kallikrein results in the cleavage of bradykinin from kininogen.

Knock-out animals Animals in which a gene has been selectively eliminated.

Kveim test Diagnostic test for sarcoidosis in which extracts of sarcoid tissue are injected into the skin. A subsequent delayed hypersensitivity response is a positive test.

Lambda (l) chain One of the two types of immunoglobulin light chains.

Langerhans cell Dendritic bone marrow-derived cell in the epidermis that has antigen-presenting capabilities.

Late-phase reaction Inflammatory response occurring 4 to 8 hours after an immediate IgE-dependent allergic reaction.

Latex fixation test Passive agglutination reaction in which latex beads, coated with IgG or other antigens, are used to detect antibody.

LE cell test (or phenomenon) Phagocytosis of nuclear material by leukocytes *in vitro* (rarely *in vivo*) mediated by anti-DNA antibodies and seen in patients with systemic lupus erythematosus and other rheumatic diseases.

Lectin Substance, usually of plant origin that agglutinate cells, usually by binding to sugar moities, and which may have nonspecific mitogenic properties.

Leukotriene (LT) Product of the 5-lipoxygenase pathway of arachidonic acid metabolism.

Ligand Molecule capable of binding to another molecule.

Light chain disease Monoclonal gammopathy in which the B-cell clone produces only light chains.

Light chain (L chain) Polypeptide that is part of all immunoglobulin molecules. Two classes exist: kappa (k) and lambda (l).

Linkage disequilibrium Tendency of certain genes on a chromosome to be inherited as a group.

Lipopolysaccharide Group of molecules derived from gram-negative bacteria, which frequently are proinflammatory. *See also Endotoxins.*

Liposome Artificial particle surrounded by a lipid membrane.

5-Lipoxygenase pathway Enzymatic pathway of arachidonate metabolism leading to leukotrienes.

Local anaphylaxis IgE-mediated reaction that occurs in a specific target organ or tissue.

Low-dose (low-zone) tolerance Production of specific acquired unresponsiveness by small doses of antigen.

Lupus anticoagulant (LA) Autoantibodies that prolong the time to clot formation of *in vitro* coagulation assays; often found in the antiphospholipid syndrome and systemic lupus erythematosus (SLE).

Lymphocyte activation (stimulation, transformation, or blastogenesis) Process of activating resting small lymphocytes via an interaction with specific antigen, superantigen, antibody to surface molecules, or mitogen.

Lymphomas Neoplasms of lymphocytic cells of the immune system.

Lymphoreticular Term referring to tissue containing lymphocytes, mononuclear phagocytes, and stromal elements.

Lymphotropic An infectious agent that preferentially infects lymphocytes.

Lysosomes Cytoplasmic vesicles that contain proteolytic enzymes.

Lysozyme Naturally occurring low-molecular-weight enzyme in mucosal fluids that has antimicrobial activity. *See also Muramidase*

Macroglobulinemia The presence of elevated levels of monoclonal IgM in plasma.

Macrophages Tissue phagocytes derived from bone marrow promonocytes.

Major basic protein (MBP) Cationic protein found in eosinophil granules that, when released, is toxic to tissues.

Major histocompatibility complex (MHC) Cluster of genes responsible for most transplantation reactions and associated with many aspects of immune responsiveness and autoimmunity.

Mast cell Cell with secretory granules containing histamine, neutral proteases, and proteoglycans and having high-affinity Fc receptor for IgE on its surface.

Mastocytosis A relatively rare disease characterized by mast cell hyperplasia in organs such as bone marrow, skin, lymph nodes, liver, gastrointestinal tract, or spleen.

M cells Specialized epithelial cells overlying lymphoid aggregates in the small intestine that are involved in antigen passage and presentation.

Membrane attack complex (MAC) A complex formed of the late acting components of the complement activation cascade (C5-C9) responsible for damaging membranes, including the lysis of cells.

b_2-Microglobulin (b_2M) Small monomorphic protein; member of the immunoglobulin supergene family that is one of the two chains of class I MHC molecules.

Migration inhibition factor (MIF) Cytokine, perhaps related to interferon, that was detected in the first *in vitro* test for delayed hypersensitivity.

Mitogen Substance capable of activating a resting cell to undergo mitosis.

Mixed lymphocyte culture or reaction (MLC or MLR) *In vitro* technique in which non-T cells of one genotype stimulate resting T cells of another genotype to divide.

Molecular mimicry Cross-reactivity between an antigen and a tissue component, so that an immunologic reaction against the former leads to tissue damage; a mechanism for autoimmunity. Formation of a molecular structure, usually by a microorganism, that resembles a normal host component, thereby protecting the organism from host defense mechanisms or facilitating tissue invasion.

Monoclonal antibodies (mAb) Set of identical immunoglobulins produced by one B cell alone.

Monoclonal gammopathy Condition in which a hyperplastic or malignant clone of B cells overproduces a monoclonal immunoglobulin.

Monoclonal protein Homogeneous product of a single clone of cells.

Monokine Cytokine produced by mononuclear phagocytes.

Monomer Unit of a multimeric molecule.

Mononuclear phagocytic system Collection of promonocytes, monocytes, macrophages, and histiocytes – the main components of the reticuloendothelial system.

M protein Surface antigen of streptococci. *See also Monoclonal protein.*

Mucosal immune system Lymphoid tissues associated with the gastrointestinal and respiratory mucosa.

Multiple myeloma (MM) A malignant disease affecting the most mature cell of the B-cell lineage, the plasma cell.

Myelin basic protein (MBP) Component of myelin used as an antigen in experimental allergic encephalomyelitis (EAE).

Myeloperoxidase (MPO) Enzyme in granules of neutrophilic phagocytes.

Natural antibody Antibody produced without apparent antigenic stimulation.

NBT (nitroblue tetrazolium) test Test for the activity of the hexose monophosphate shunt, which is defective in individuals with chronic granulomatous disease.

Neoantigen Appearance of a new antigen. Also, modification of an existing (usually self) antigen by mutation or chemical means.

Neoplasia Tumors or their formation.

Nephelometry Technique to measure antigen–antibody reactions that cause turbidity or light scattering in solution.

Nephritic factor An immunoglobulin capable of reacting with and stabilizing components of the alternative complement pathway; found in the serum of some patients with glomerulonephritis and partial lipodystrophy.

Network hypothesis Jerne's theory of immune regulation involving idiotype-anti-idiotype reactions.

Neutralization Process by which the toxicity or infectivity of toxins or microbes is neutralized by antibody.

NK (natural killer) cells Cells able to kill certain target cells without previous immunization.

Nonresponder Subject who is unable to respond to an ordinarily immunogenic stimulus, usually for genetic reasons.

Northern blotting Process used to identify RNA fragments by electrophoresis and subsequent binding to labeled DNA or RNA oligonucleotides.

Nude (nu) mouse Strain of mouse lacking body hair, thymus, and almost all T cells.

Null cells Lymphocytes that do not bear the typical surface markers of T or B cells.

NZB (New Zealand black) or NZW (New Zealand white) Strains of mice used in studies of spontaneous autoimmunity.

Oligoclonal bands Immunoglobulins of restricted electrophoretic mobility seen in cerebrospinal fluid of patients with multiple sclerosis and other inflammatory diseases of the central nervous system.

Oncofetal antigens Antigens present on normal fetal cells (but not normal adult cells) and that reappear on malignant cells.

Oncogene Gene causing malignant transformation of normal cells.

Ontogeny Developmental history of a group of individuals.

Opportunistic organism Organism of limited virulence that can cause disease in an immunocompromised host.

Opsonin Protein able to enhance phagocytosis of a particle.

Oral tolerance Process by which orally administered antigen causes specific acquired unresponsiveness.

Osteoclast activating factor (OAF) Cytokine that enhances bone resorption.

Ouchterlony test or analysis Method of detecting precipitating antigen-antibody reactions in which both reactants diffuse in a gel-supporting medium.

Palindrome Length of DNA bases that displays an equivalent sequence, whether read from the 5' to the 3' end or vice versa.

Paracrine Effects of a hormone or cytokine on neighboring (rather than distant) target tissue.

Paraproteinemia Situation in which an abnormal monoclonal immunoglobulin or fragment is present in plasma or urine.

Passive agglutination Clumping of particles or erythrocytes coupled with antigens by specific antibody.

Passive cutaneous anaphylaxis Method for detecting biologically active IgE *in vivo.*

Passive immunity State in which a nonimmune recipient has been provided with specific immunity via the transfer of antibody or immunocompetent cells.

Peripheral lymphoid organs or tissues Lymphoid aggregates or tissues excluding the thymus, bursa of Fabricius (in birds), and bone marrow, the latter considered to be the source of lymphoid stem cells.

Peritoneal exudate cells Cells present in peritoneal fluid after injection of an inflammatory stimulus.

Peyer patches Specialized collections of lymphocytes and antigen-presenting cells in the submucosa of the small intestine.

Phagocytes Cells able to ingest particles, including microbes.

Phagolysosome Vesicle formed by fusion of a lysosome and a phagocytic vesicle (phagosome).

Phylogeny Evolutionary history of a group of organisms.

Phytohemagglutinin (PHA) Plant lectin capable of stimulating virtually all T cells.

Pinocytosis Process of cellular ingestion of soluble material.

Plaque-forming cells Individual antibody-forming cells seen in an assay for hemolytic antibody.

Plasma cell myeloma Monoclonal gammopathy characterized by paraproteinemia and often paraproteinuria and lytic bone lesions.

Plasma cells Mature B cells capable of intensive antibody production and secretion.

Plasmapheresis Removal of plasma from the vascular system, usually with the return of the formed elements.

Plasmin Proteolytic enzyme capable of cleaving fibrin and complement components.

Plasminogen activator Enzyme capable of converting plasminogen to plasmin.

Platelet-activating factor (PAF) A phospholipid mediator with proinflammatory properties.

Plectin Plaque protein of hemidesmosomes.,

Pokeweed mitogen (PWM) Plant lectin able to stimulate both T and B lymphocytes.

Polyclonal hypergammaglobulinemia Increase of various isotypes of immunoglobulins of different specificity; characteristic of chronic inflammatory states.

Polymerase chain reaction (PCR) Method of amplifying DNA or RNA segments.

Prausnitz-Küstner (PK) reaction A wheal-and-flare reaction produced by injection of serum containing specific IgE antibodies into the skin of a nonallergic person followed by local antigen injection.

Pre-B cells Precursors of mature B cells, having cytoplasmic but not surface IgM.

Precipitation reaction Formation of an insoluble lattice by the specific interaction of soluble antigen and soluble antibody.

Primary follicles Aggregates of lymphoid cells within lymph nodes or splenic white pulp.

Properdin system Proteins comprising the alternative complement pathway, including factors B, D, properdin, and C3.

Prostaglandins (PG) Naturally occurring aliphatic acids, mostly derived from arachidonic acid via the cyclooxygenase pathway.

Prothrombin Zymogen precursor of thrombin and the antigen for some lupus anticoagulants.

Prozone phenomenon Incomplete lattice formation in a precipitin reaction in the region of antibody excess.

Pyogenic microorganisms Microbes, often called high-grade pathogens, that elicit reactions rich in polymorphonuclear leukocytes.

Pyrogens Substances that cause a rise in body temperature, acting either directly or indirectly on the hypothalamus. *See also Endogenous pyrogens.*

Pyroglobulins Proteins, usually monoclonal, that precipitate when heated to 56°c.

Radial immunodiffusion Precipitation technique in which antigen diffuses radially from a well into semisolid medium containing antibody.

Radioallergosorbent test (RAST) Radioimmunoassay in which antigen is coupled to a solid phase; often used to detect IgE *in vitro.*

Radioimmunoassay One of a group of tests for detecting antigen or antibody where one of the reagents is labeled with a radioactive isotope.

Raji cell test Method of measuring immune complexes by their ability to bind to the Raji cell line.

RANTES Stands for Regulated on Activation Normal T Expressed and Secreted.

Raynaud's phenomenon Intermittent attacks of ischemia mainly of the fingers or toes that is marked by pallor and often is accompanied by paresthesias and pain.

Reagin Early name for skin-sensitizing (IgE) antibody. Also the name for the antibody detected in Wassermann and similar nonspecific serologic tests for syphilis.

Respiratory burst Process by which phagocytic cells generate oxygen radicals.

Retrovirus RNA virus that uses reverse transcriptase for replication and cellular integration.

Reverse transcriptase Enzyme in microorganisms that catalyzes the transcription of DNA from RNA.

Rheumatoid factor (RF) Immunoglobulin, usually IgM, directed against the Fc portion of IgG, often seen in patients with rheumatoid arthritis or other rheumatic diseases.

Schirmer's test Tear flow measurement by wetting of filter paper strip.

Schultz-Dale test In vitro method to demonstrate cytotropic antibody (e.g., IgE) by its ability to bind to smooth muscle tissue and cause contraction when bound to specific antigen.

Second set graft rejection Accelerated graft rejection that occurs after previous sensitization to the same alloantigens.

Secretory IgA Specialized form of IgA, found mainly in mucous secretions, composed of a dimer of IgA molecules together with a J chain and secretory piece.

Secretory immune system That part of the peripheral lymphoid system associated with the upper and lower respiratory, gastrointestinal, and genitourinary tracts in which the major immunoglobulin isotype is secretory IgA.

Secretory piece (component) Polypeptide (95 kd) produced by mucosal epithelial cells that facilitates transport of secretory IgA across the epithelium.

Sequestration The entry of pathogens into intracellular or extracellular compartments not accessible to all components of the immune response.

Serotonin (5-hydroxytryptamine) Catecholamine stored in granules of rodent (not human) mast cells and platelets; a neurotransmitter.

Serum amyloid P component (SAP) Normal plasma protein of the pentraxin family that binds to all types of amyloid fibrils and therefore accumulates specifically in amyloid deposits.

Serum sickness Clinical syndrome that includes fever, arthritis, urticaria, and lymphadenopathy; due to deposition of circulating antigen-antibody complexes.

Sicca symptoms Symptoms of dryness of eyes and mouth. *See Xerostomia* and *Keratoconjunctivitis sicca.*

Slow-reacting substances of anaphylaxis (SRS-A) Early term denoting nonhistamine substances released by an anaphylactic reaction and eliciting a smooth muscle contraction; now known to contain the cysteinyl leukotrienes.

Slow virus Virus that produces a protracted illness, usually after a long incubation period.

Solid phase radioimmunoassay Form of radioimmunoassay in which one of the reactants is adsorbed to a solid surface.

Southern blotting Technique for DNA analysis that includes digestion with restriction endonucleases, electrophoresis, and then probing with complementary oligonucleotide fragments.

SS-A (Sjögren's syndrome A; anti-Ro) Autoantibody directed to nuclear RNA, often found in Sjögren's syndrome. Also known as *anti-Ro.*

SS-B (Sjögren's syndrome B; anti-La) Autoantibody directed to nuclear RNA, often found in Sjögren's syndrome. Also known as *anti-La.*

Suppressor T cells Subset of T cells able to inhibit T cell or B cell reactivity. May be antigen-specific or antigen-nonspecific.

S value Svedberg unit. Sedimentation coefficient of a homogeneous protein as determined in an analytic ultracentrifuge.

Syndesmophyte A bony excrescence.

Syngeneic Genetically identical.

T-cell antigen receptor (TCR) Antigen-specific complex on a T cell, composed or b and b chains or d and g chains, that is able to recognize a specific antigenic peptide in the context of an MHC molecule.

T cells (T lymphocytes) Thymus-derived lymphocyte.

Tetramer Artificial association of four peptide MAC molecules on strep evidence for identification of specific antigen binding T cells.

Thermal amplitude The temperature range within which an antibody will react with target cells.

Thymus Central lymphoid organ in which bone marrow-derived stem cells mature into immunocompetent clones of T cells.

Thymus-dependent antigen Antigen that requires T cell “help” for its specific B cell to produce antibody.

Thymus-independent antigen Antigen that can directly stimulate B cells to produce antibody without “T cell help.”

Tissue factor pathway (extrinsic pathway of coagulation) The component of the coagulation pathway activated by the interaction of tissue factor with Factor VIIa.

Tolerance Unresponsiveness to antigenic stimulation. Tolerance can be genetic or it can be acquired by special methods of antigenic exposure.

Toxoid Toxin that has been chemically modified so it is immunogenic but not toxic.

Transcription Synthesis of RNA from a DNA template.

Transfection Transfer of an extra gene (or genes) to a cell or (via the embryo) to an individual.

Transfer factor Extract of leukocytes from an immune person capable of passively transferring delayed hypersensitivity to a nonimmune recipient.

Transgenic Situation in which a foreign gene (or genes) is present in an individual or cell.

Translation Synthesis of a polypeptide from an RNA template.

Transplantation antigens Antigens present on cell surfaces that produce rejection reactions when introduced into genetically dissimilar recipients.

Trophoblast Embryonic tissue containing the fetus and interfacing with maternal tissue in the uterus.

Tropism Preferred target cell for infection by a virus.

Tryptic peptides Peptide fragments of a polypeptide derived by tryptic digestion.

Tumor necrosis factor (TNF) Cytokine able to cause tumor cell lysis and other effects, including inflammation. TNF-b and TNF-b (lymphotoxin) exist.

Tumor-specific antigens Cellular antigens expressed on malignant but not on normal cells.

Urticaria (hives) Pruritic skin eruption with edematous wheals, often (but not always) with an allergic etiology.

Vaccination Immunization with microbial antigens to prevent infectious disease (originally, the production of immunity to smallpox by introduction of cowpox [vaccinia] material).

Variable (V) region Amino-terminus end of an H or L chain where the amino acid sequence differs from one antibody to another.

Variolation Immunization by inoculation with the live virus of smallpox (variola).

Vasculitis This term refers to clinical disorders characterized by inflammation of, and damage to, blood vessels.

V-beta (Vb) region Variable (polymorphic) section of the b-chain of the T cell receptor.

Virion A viral particle.

Waldeyer's ring A series of lymphoid tissues and organs surrounding the nasopharyngeal region, including the tonsils and the adenoids.

Western blotting (immunoblotting) Method to detect antigens in a complex mixture resolved for size of components (SDS-PAGE) followed by overlaying with antibody.

Xenogeneic Relation between different species.

Xenograft Graft of organs, free tissue, or isolated cells between members of different species.

Xenotransplantation The transplantation of organs, tissues, or cells between individuals of different species.

Xerostomia Dryness of the mouth from salivary gland dysfunction.

Zone electrophoresis Electrophoretic separation of molecules on a solid substrate according to the net electrical charge.