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Chapter 31

Immunotoxicology: Effects of, and Response to, Drugs and Chemicals

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The immune system is a complex, multi-cellular organ system consisting of granulocytes, macrophages, lymphocytes, and dendritic cells with various functions and phenotypic characteristics, as well as various soluble mediators. These cells of hemopoietic origin are found in the peripheral blood, lymphatic fluid, and organized lymphoid tissues, including bone marrow, spleen, thymus, lymph nodes, tonsils, and mucosa-associated lymphoid tissue. The immune system is in a constant state of self-renewal involving cell proliferation, differentiation, and maturation. It exists to defend the body against invasion by infectious and opportunistic microorganisms, and spontaneously arising neoplasms. This network of cells and soluble factors is highly regulated and interdependent, must discriminate self from non-self, and can react to non-self with many different (pleiotropic) defensive responses (81).

IMMUNE MECHANISMS RESPONSIBLE FOR HOST DEFENSE

The host defense functions of the immune system are provided by two major mechanisms: a nonspecific (constitutive) mechanism that does not require prior sensitization with the inducing agent to elicit a response, and a specific (adaptive) mechanism directed against the eliciting agent to which the individual has been previously sensitized. Penetration of the skin or mucosal defense barriers by an invading microorganism results in nonspecific reactions by phagocytic cells (granulocytes and Mononuclear phagocytes [MØ]). If the microorganism is not controlled and persists, specific responses involving antibody production and the induction of effector lymphocytes follow. The effector lymphocytes respond through cytokine mediators to seek out and destroy the invading microorganism. Both antibody-producing lymphocyte responses (B-lymphocytes or B-cells) and thymus-dependent lymphocyte responses (T-lymphocytes or T-cells) are triggered by the presentation of foreign antigen to appropriate lymphocytes by MØ, dendritic cells, or other antigen-presenting cells. Following antigen-induced activation, B-cells proliferate and differentiate into plasma cells (PC), which subsequently produce large quantities of antigen-specific immunoglobulins (antibodies). Antibodies enter the plasma where they bind the foreign material and either

neutralize, lyse, or facilitate phagocytosis of the agent. Antibody–antigen interactions are expanded by actions of the complement (C') system and other inflammatory mediators (e.g., prostaglandins and leukotrienes). Fever, opsonization, and lytic factors released by activated lymphoid cells also contribute to this process of host defense.

Nonspecific and Specific Mechanisms of Immunity

Two categories of phagocytic leukocyte, the polymorphonuclear phagocyte (PMN) or granulocyte, and the MØ, are involved with nonspecific mechanisms of host resistance. Both cell types originate from myeloid progenitor cells in the bone marrow and normally pass through several maturation stages before entering the bloodstream. PMN readily traverse blood vessels and provide the primary defense against infectious agents. The inflammation associated with a splinter is typical of a nonspecific PMN and MØ response. Both PMN and MØ exhibit phagocytic activity toward foreign material, especially MØ in the presence of specific opsonic antibodies and complement, and can destroy most microorganisms. Macrophages are recruited to the site in the event that PMN either cannot contain or are destroyed by the infectious agent, as is the case with certain bacteria (e.g., Listeria). Macrophages can be activated to a state of enhanced bactericidal or tumoricidal activity by soluble lymphocyte products (e.g., cytokines) produced by T-lymphocytes sensitized to the invading microbe.

The immune responses that characterize adaptive host defense represent a series of complex events that occur following the introduction of foreign antigenic material into the body. There are two major types of specific immune response: cell-mediated immunity (CMI), which is initiated by specifically sensitized T-cells and is generally associated with delayed-type hypersensitivity (DTH), rejection of tumors or foreign grafts, and resistance to persistent infectious agents; and humoral immunity (HI), which involves the production of antibodies by cells following sensitization to a specific antigen.

Organization, Differentiation, and Function of Lymphoid Tissue

The cellular elements of the immune system arise from pluripotent stem cells, a unique group of unspecialized cells that have self-renewal capacity. The pluripotent stem cells are found in the blood islands of the embryonic yolk sac and in the liver of the fetus during fetal development, and later in the bone marrow. The pluripotent stem cell differentiates along several pathways, giving rise to either erythrocytes, myeloid series cells (i.e., MØ and PMN), megakaryocytes (platelets), or lymphocytes. Maturation generally occurs within the bone marrow, although lymphoid progenitor cells are disseminated through the blood and lymphatic vessels to the primary lymphoid organs where they undergo further differentiation under the influence of the humoral microenvironment of these organs (Figure 31.1).

The primary lymphoid organs include the thymus in all vertebrates and the bursa of Fabricius (in birds) or bursa-equivalent tissue in other vertebrates, the latter believed to be bone marrow and gut-associated lymphoid tissue in mammals (Table 31.1). Primary lymphoid organs are lymphoepithelial in origin, derived from ectoendodermal junctional tissue in association with gut epithelium. During the beginning of the second half of embryogenesis (days 12–13 in the mouse), stem cells migrate into the epithelia of the thymus and bursa-equivalent areas, where they differentiate independently of antigenic stimulation into immunocompetent T- and B-cells, respectively (Figure 31.1). The thymus, which is derived embryologically from the third and fourth pharyngeal pouches, is an organization of lymphoid tissue located in the chest, above the heart. Thymus development occurs during the sixth week of embryological development in humans and day nine of gestation in the mouse. The thymus reaches its maximum size at birth or shortly thereafter in most mammals and then begins a slow involution that is complete between the ages of 5 and 15 years in humans.

Histologically, the thymus consists of multiple lobules, each lobule containing a cortex (outer) and a medulla (inner). Lymphocyte precursors from bone marrow proliferate in the cortex of the lobules and then migrate to the medulla. In the medulla they further differentiate, under the influence of thymic epithelium and hormonal factors, into mature T-lymphocytes before emigrating to secondary lymphoid tissues. The neonatal/postnatal thymus has a significant endocrine function supported by non-lymphoid thymic epithelium cells. These cells produce a family of thymic hormones essential for T-lymphocyte maturation and differentiation. In contrast, B-cell differentiation occurs in the bursa of Fabricius in birds, a lymphoepithelial organ that develops from a diverticulum of the posterior wall of the cloaca. It is divided into a medullary region, containing lymphoid follicles and a cortical region. The mammalian bursa-equivalent is believed to be the fetal liver, neonatal spleen, gut-associated lymphoid tissue, and adult bone marrow, depending on age. Mature B-lymphocytes migrate from the bursa-equivalent tissue to populate the B-dependent areas of the secondary lymphoid tissues.

Neonatal removal or chemical destruction of primary lymphoid organs prior to the maturation of lymphocytes into T-or B-cells, or prior to their population of secondary

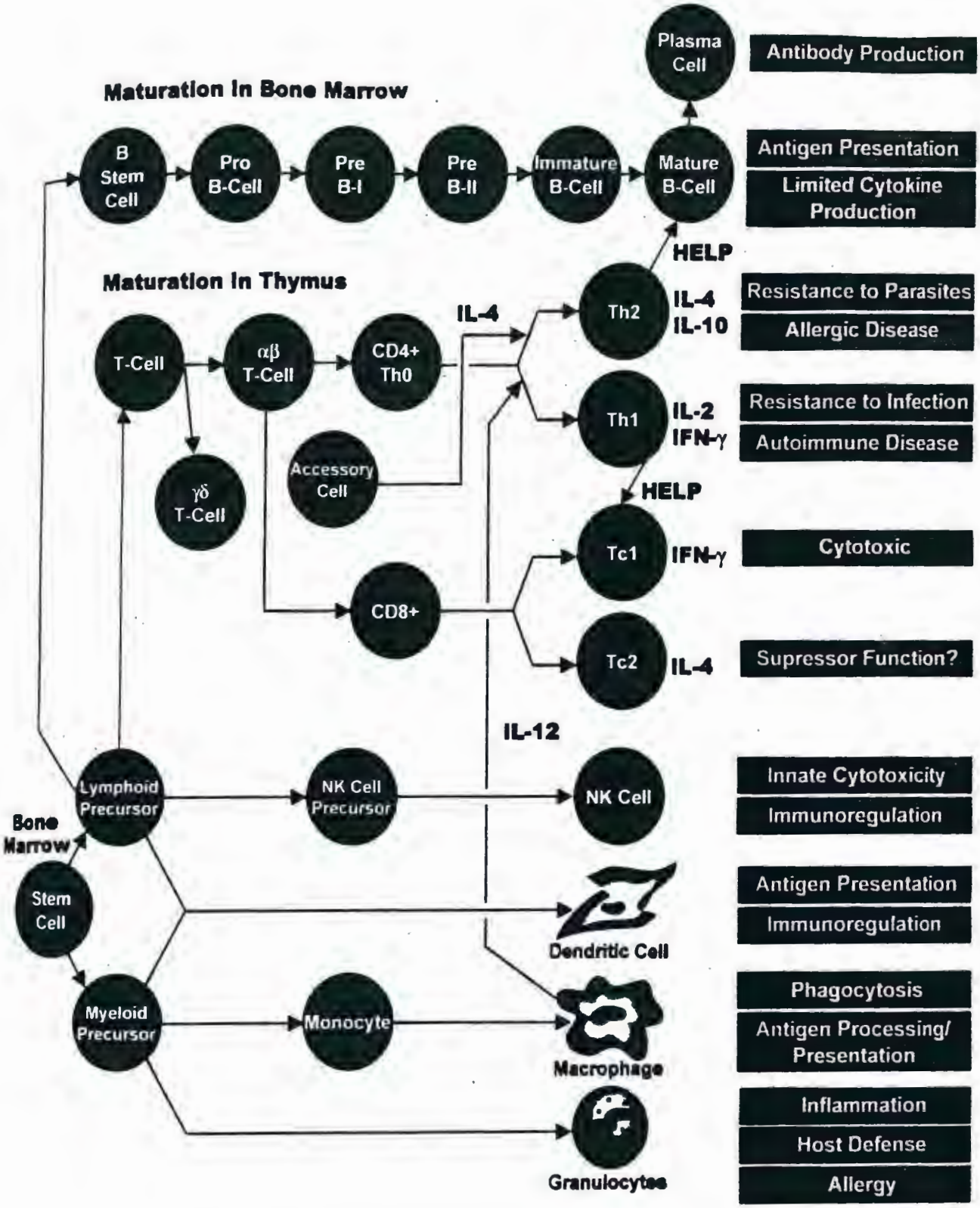

FIG. 31.1. Maturation and interaction of effector cells of the immune system.

Table 31.1
Origin and characteristics of primary and secondary lymphoid tissues

Function	Primary lymphoid organs	Secondary lymphoid organs
Generation and maturation of cells	Thymus Bursa of Fabricius (birds) Fetal liver (mammals) Adult bone marrow	Spleen Lymph nodes Gut-associated lymphoid tissue Bronchial-associated lymphoid tissue
Embryonic origin and development	Ectoendodermal junction Thymus: days 9–10 in mouse; week 6 in human Bursa-equivalent: days 10–13, mouse; week 10, human	Mesoderm
Lymphoid cell proliferation	Independent of antigenic stimulation	Dependent on antigenic stimulation
Germinal center formation	Nonexistent	Occurs after antigenic stimulation
Cells repopulating after depletion	Stem cells only	Differentiated lymphocytes
Early surgical or chemical removal	Depressed numbers of T- and B-cells; depressed immune responses	No significant effect on immune function

lymphoid tissue, dramatically depresses the immunological capacity of the host. However, removal of these same organs in adults has little influence on immunological capacity. In addition, neonatal thymectomy in mammals dramatically impairs the development of CMI but does not generally influence the generation of immunoglobulin-producing cells involved in antibody-mediated immunity (unless they strictly require T-lymphocyte help for the induction of antibody production). In contrast to the removal of primary lymphoid organs, removal of secondary lymphoid organs does not inhibit the development of immune competence, although it may suppress the magnitude or alter the tissue location of the responsive cells.

Organization and Function of Secondary Lymphoid Tissue

The organization and function of secondary lymphoid organs is extremely important for immune competence and host defense (Table 31.1). The organized areas of secondary lymphoid tissue are the spleen, lymph nodes, gut-associated lymphoid tissue (GALT), and bronchial-associated lymphoid tissue (BALT). The anatomical organization of these tissues provides a microenvironment for functional development of lymphoid cells and vital immune responses.

Lymph nodes are discrete, organized secondary lymphoid organs that serve as filtering devices for lymphatic fluid. Lymph nodes are divided structurally into three areas: cortex, paracortex, and medulla (Figure 31.2). Each lymph node is served by several afferent lymphatic vessels collecting lymphatic fluid (lymph) from distal tissue sites. Lymph may contain foreign antigens. The efferent lymphatic vessel, which drains lymph from the node, contains antibodies, cytokines, and lymphocytes produced in response to foreign antigenic stimulation. The cortex, located underneath the subcapsular sinus, receives the afferent lymph and serves as the major site of B-lymphocyte localization. The cortex consists of a narrow rim of small lymphocytes in the absence of antigenic stimulation. Also located in the cortex are aggregations of small lymphocytes, termed lymphoid follicles, which contain dendritic reticulum cells capable of retaining antigens on their plasma membranes. When the lymphocytes that make up the lymphoid follicles are stimulated by antigens, they proliferate, giving rise to dense aggregations of lymphocytes, termed germinal centers. These germinal centers serve as sites for differentiation of B-lymphocytes into PC capable of antibody production. Following antigenic stimulation, germinal centers are easily detectable as spherical or ovoid structures containing many large and medium-size lymphocytes, predominantly B-lymphocytes. The paracortex, lying between the cortex and the medulla,

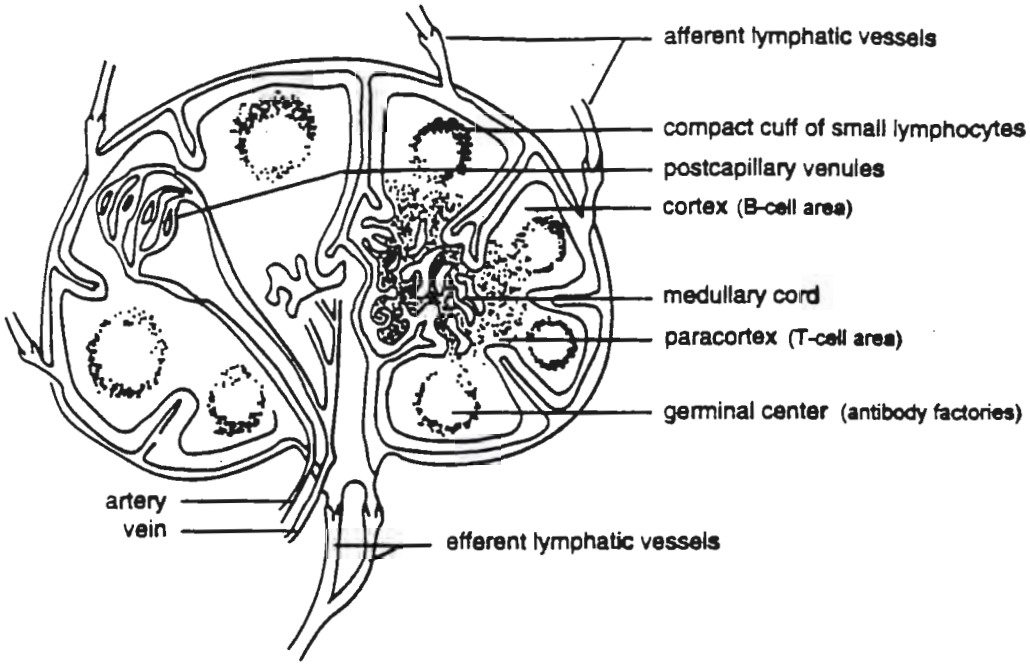

FIG. 31.2. Cross-section of a lymph node showing architectural organization.

is composed predominantly of T-cells and is a major site of MØ/T-cell interactions. Neonatal thymectomy or lymphocyte depletion by cytolytic drugs reduces the production of paracortical lymphocytes, leading to depressed immune capacity. In addition, the paracortex contains a specialized blood vasculature, termed postcapillary venules, that serves as a point of entry for recirculating lymphocytes from the bloodstream. The medulla of the lymph node is composed primarily of networks of cords and sinuses; it serves as an effective filter for removing particulate material from lymphatic fluid. Following antigenic stimulation, a majority of the antibody is produced by PC found within these medullary cords of nodes.

The spleen is the major filter of blood-borne antigens and the site of immunological responses to these antigens. In addition, the spleen is a site of extramedullary hematopoiesis (non-bone-marrow red blood cell production) and removal of damaged blood cells. There are two major histological regions within the spleen: the red pulp and the white pulp. These areas have been named for their colors in a freshly cut spleen. The white pulp consists of numerous white blood cell aggregates and lymphoid follicles. The red pulp contains cords and venous sinuses analogous to the medullary region of lymph nodes. The spleen has no afferent lymphatic vessels; thus, all antigenic material or cells enter the spleen through the blood vasculature. The marginal sinus in the spleen is structurally and functionally similar to the subcapsular sinus of the lymph node.

IMMUNE FUNCTION AND RESPONSES

Bone Marrow

The bone marrow functions as a primary lymphoid organ and serves as the principal source of uncommitted stem cells, including both myeloid and erythroid precursor cells. The bone marrow architecture is highly organized and complex, consisting of a matrix or cellular stroma derived from local mesenchymal cells, as well as cells of hemopoietic parenchyma that are descendants of circulating stem cells. The bone marrow matrix consists of reticular-dendritic cells, fibroblast-like cells, and immune cells within the bone marrow microenvironment.

Bone marrow stem and stromal cells have been shown to possess a significant capacity for metabolic activation because they contain cytochromes of the P450 and P448 families as well as peroxidases, and can generate reactive oxygen species, which could also activate xenobiotics via oxidant-dependent mechanisms (109). This metabolic activity is thought to contribute to the sensitivity of bone marrow elements to toxicants such as benzene, which is extensively metabolized within the bone marrow. In light of the cell proliferation and differentiation taking place within the marrow, this tissue is also one of the most sensitive to drugs or chemicals affecting cell division. Dose-limiting bone marrow toxicities are a significant problem with antiproliferative drugs including cytotoxic

agents, antifolates, AIDS therapeutics, and certain cytokines (28,93).

Mononuclear Phagocytic System

Whether an antigen induces CMI, antibody production, or both depends on the physical and chemical characteristics of the antigen, the mode of presentation of the antigen to lymphocytes, the pattern of antigen distribution within lymphoid tissue, and the molecular configuration of the antigen. In many instances, antigen is initially phagocytized and processed by MØ. Antigenic peptides are transported to the cell surface, where they are presented to lymphocytes through cell surface interactions via specific surface proteins (e.g., Class II molecule antigens).

Cells of the MØ/monocyte lineage are found in many tissues, including liver (Kupffer cells), lung (alveolar MØ), skin (Langerhans cells), and brain (astrocytes). These cells must cope with many xenobiotics because their proximity to portals of entry results in early interaction with drugs, chemicals, and physical agents entering the organism via air, food, or blood. The capacity of cells of the mononuclear phagocytic system (formerly known as the reticuloendothelial system) to carry out these functions is associated with their state of activation, which in turn is a function of both endogenous (e.g., interferon-gamma, IFN-γ) and exogenous (e.g., bacterial lipopolysaccharide, LPS) stimuli. Responsive MØ obtained from the peritoneal cavity are relatively quiescent and require extracellular signals or "priming," followed by a second signal induced by triggers such as LPS, to be fully activated.

Although the mononuclear phagocyte system is designed to protect the host, once a xenobiotic has gained entry, extensive tissue damage can paradoxically result from MØ-mediated responses to the agent. Silicosis and asbestosis are two examples of diseases that may result from MØ-induced injury (117), possibly due to inflammation. In this condition, tissue damage from infectious organisms or other agents results in the influx of phagocytic cells such as PMN. In most instances, these cells effectively eliminate the agents by digesting them in internal vacuoles. However, if the foreign particles are insoluble (as, for example, with silica crystals or asbestos fibers), a chronic inflammatory process ensues in which monocytes/MØs are the predominant effector cells. These cells release a variety of active molecules (cytokines, nitric oxide, amines, lipid mediators, etc.) which damage tissue, as well as recruiting other inflammatory cells into the local environment. This sometimes leads to the development of a granuloma, a collection of inflammatory cells surrounded by fibrotic tissue (92).

Humoral Immunity

The principal function of B-lymphocytes is production of specific antibody in response to antigenic stimulation. B-cells recognize antigen via the B-cell antigen receptor (BCR), comprising membrane immunoglobulins associated with various accessory proteins. Interaction of the BCR with its cognate antigen triggers transmembrane signaling, leading to activation of the B-cell. The antigen is subsequently internalized, where it is processed and associated with class II major histocompatibility complex (MHC) molecules. Antigen-derived peptides, along with MHC proteins, are then transferred to the cell surface, where they are free to interact with helper T-cells.

Within three to five days following antigen exposure, this T-/B-cell interaction results in the B-lymphocytes differentiating into blast cells, then into immature PC, and finally into antibody-secreting PC. The establishment of humoral immunity (HI) is characterized by an early rise in IgM antibody titer in the serum, followed several days later by the appearance of IgG antibodies. During this differentiation process, some of the lymphocytes develop into long-lived or memory cells (sensitized but nonblast cells), so that subsequent antigen encounters result in an enhanced response. This secondary response is characterized by a shorter latency for antibody appearance, as well as an increased production of antibodies. Antibody molecules react with specific antigenic determinants (epitopes) on their target, facilitating its removal (e.g., lysis or enhanced phagocytosis).

Based on chemical structure and biological function, the five classes of antibody molecules in mammals are IgM, IgG, IgA, IgD, and IgE; some of the physical and biological characteristics of each of these classes are listed in Table 31.2. Antibodies operate via several mechanisms to protect the host from infectious agents. Some of these mechanisms include virus neutralization, in which antibodies bind and prevent virus particles from infecting target cells; opsonization, the process by which antibody molecules react with infectious agents and thus enhance their phagocytosis; and antibody-dependent cellular cytotoxicity, the process whereby antibody-coated target cells are killed by Fc receptor-bearing lymphocytes.

Cell-Mediated Immunity

Cell-mediated immunity (CMI) refers broadly to any host resistance mechanism in which cellular elements play a direct role. This is in comparison to HI in which there are certainly cellular interactions, but in which the final host resistance mechanism is mediated by soluble factors such as antibody or C′. There are a number of host defenses mediated directly by cells, including MØ-

Table 31.2
Biological properties of immunoglobulin classes

Class	Serum concentration (mg/dl)	Molecular weight	Placental transfer	Half-life (days)	Biological function	Abnormalities
IgG	670 ± 33	150,000	+	23	Primarily synthesized during secondary immune response. Readily diffuses into extravascular tissue. Fixes complement.	Increased in liver disease chronic infection. Reduced in B-cell depression.
IgM	61 ± 5	890,000	–	5	Produced early in immune response. Isoagglutinins. Fixes complement.	Increased in infection. Reduced in B-cell depression.
IgA	40 ± 4	170,000	–	6	Major Ig in seromucous secretions.	Increased in liver disease. Increased or decreased in sinopulmonary infection.
IgD	—	150,000	–	2.8	Lymphocyte receptor	Decreased following thymectomy.
IgE	0.02	196,000	–	1.5	Mediator of allergic reactions and atopic diseases.	Increased in parasitic and allergic diseases, homocytotropic.

mediated cytolysis, antibody-dependent cellular cytotoxicity, and natural killer cell cytotoxicity. More specifically, however, CMI refers to acquired immunity involving primary and secondary immune responses.

Functions associated with CMI are commonly considered the province of T-lymphocytes, although research within the past decade has shown that other immune cells (e.g., B-cells and MØ) as well as nonimmune cells (e.g., fibroblasts and dendritic cells) contribute to the development of CMI. As the primary effector cell in CMI, the T-cell represents one of the most complex and multifunctional of immune cells. Antigens that generally elicit CMI include tissue-associated antigens, chemicals and drugs that covalently bind to autologous proteins, and antigenic determinants on persistent intracellular microorganisms. The route of exposure also plays a major role in the type of response generated. For example, sheep erythrocytes elicit antibody production (but not CMI) when injected intravenously in humans, but elicit both when injected intracutaneously. The induction of CMI proceeds when small lymphocytes differentiate into large pyroninophilic cells and ultimately divide, giving rise to cells responsible for effector function as well as immunological memory.

T-cells can differentiate into populations responsible for either regulatory or effector function. Regulatory function is provided by the T-helper cells (CD3/CD4 phenotype). T-helper function facilitates antibody responses by B-cells and assists in other T-cell responses. For most antigens, B-cells require assistance from T-cells for differentiation into plasma cells. T-helper cells are integral in the B-cell response by participating in two distinct mechanisms: major histocompatibility locus-restricted B-/T-cell collaborations; and cytokine-mediated differentiation. Helper function is a result of interactions between surface molecules on T-helper cells and B-cells, as well as the production and secretion of immunoregulatory cytokines.

Effector functions take the form of cytotoxic activity (CD3/CD8 phenotype), the so-called cytotoxic T-lymphocyte (CTL). These cells are able to specifically lyse target cells via the release of various bioactive molecules. Another effector function is the ability of T-cells to mediate suppressor activity for both T-and B-cell responses. Suppressor activity is also mediated by cells bearing the CD3/CD8 phenotype, although recent studies suggest that this activity may be the result, at least in part, of differential cytokine production by this population (see Figure 31.1). This responsibility for both helper and suppressor activities indicates the crucial role of T-cells in normal immune function.

The T-helper 1/T-helper 2 Cell Paradigm

An important recent conceptual breakthrough in immunology has been the hypothesis of Type 1 and Type 2 immune responses mediated by T-helper cells. The concept was first established by Mosmann et al. (73), who demonstrated that cloned murine T-cells exhibited differential patterns of cytokine production. One population, designated T-helper-1 type cells (Th1), were found to produce interleukin-2 (IL-2), IFN-γ, and lymphotoxin. The second major population (designated Th2 cells) produces IL-4, IL-5, IL-10, and IL-13. Both populations of T-cells produce IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF). Later, a third population, Th0, was described, and was found to exhibit an intermediate pattern of cytokine production. These cells are less well defined, but may be an early precursor of Th1 and Th2, or, alternatively, they may represent an intermediate stage in development of the other two populations.

Although there were initial doubts that human T-cells followed this paradigm, recent studies have demonstrated a similar (though not identical) pattern in human T-cells (89). The major differences appear to be in the profile of cytokine production, cytokine response (e.g., human Th1 and Th2 proliferate in response to IL-4), and cytolytic potential. Despite these differences, the human and rodent systems are similar enough to make experimental rodent models meaningful for understanding the human immune response.

Recent studies suggest that Th1 and Th2 cells may not necessarily represent distinct lineages descending from a common precursor, but rather may be seen as points in a continuum. For example, development of each population is influenced by type, location, and concentration of eliciting antigen and the cytokine milieu (see Figure 31.1). For example, the cytokines IL-12 and interferon gamma-inducing factor (from MØ) and IFN-γ (from NK cells) drive the development of Th1 cells, whereas IL-4 (from the ill-defined "T-accessory" cell, mast cells, or other sources) drives the development of Th2 cells (79). Interestingly, IL-4-driven development of Th2 appears to take precedence over IL-12-induced Th1 production; this may have ramifications in the etiology of some disease states.

The Th1/Th2 paradigm is especially important for immunotoxicology because certain immunopathologies have been associated with the predominance of one helper cell type over another, particularly in human disease states. For example, Th1 polarization has been associated with organ-specific autoimmune diseases such as multiple sclerosis and Hashimoto's thyroiditis, whereas systemic autoimmune conditions such as rheumatoid arthritis and Sjogren's syndrome lack a clear T-cell polarization (16). On the other hand, strong Th2-type responses appear to result in many hypersensitivity disorders. The extent to which cytokine polarization contributes to these pathologies, as opposed to being a sequela of other mechanisms, remains to be elucidated. It is possible, however, that assignation of Th1/Th2 patterns may eventually become much more important when designing and performing mechanistic immunotoxicology studies (99).

Natural Killer Cells

Natural killer (NK) cells are a population of non-B-, non-T-lymphocytes that exhibit cytotoxicity toward a variety of target cells, including tumor cells and virally infected cells. NK cells express a unique panel of cell surface markers and are morphologically distinct, being larger than other lymphocytes. In addition, they contain numerous granules, leading to their designation as large granular lymphocytes (LGL) (60). Unlike CMI, NK cell-mediated cellular cytotoxicity is MHC-unrestricted, and does not require prior exposure to the targets; thus, this form of cytotoxicity is generally referred to as "innate" or "natural" immunity.

NK cells have traditionally been seen principally as mediators of so-called "immune surveillance," the concept of a constant removal of spontaneously arising neoplastic cells (83,86). In fact, the standard methodology for assessing NK cell function relies upon the in vitro lysis of tumor target cells. However, an equally important, if not more important, role for NK cells is the control of infection (98,105).

In contrast to previous models in which NK cells were considered independent of the acquired immune response, recent studies have revealed an important role for these cells in the induction and regulation of acquired immunity (54,57,77). NK cells respond to, and produce, key immunoregulatory cytokines, and thus play an important role in the normal immune response. A more detailed understanding of these cells will certainly be crucial in future immunotoxicology study designs.

Other Immunoregulatory Circuits

Cytokines

Cytokines are glycoproteins which are generally produced in response to cellular activation. Most cytokines studied to date have multiple and overlapping actions, and they frequently function via cascading mechanisms referred to as the cytokine network, interacting with each other both synergistically and antagonistically. Two other important features of cytokines are that they usually act at a local level, and they are rapidly cleared

Table 31.3
The major classes of cytokines and chemokines

Class	Members	Functions
Interleukins (IL)	IL-1 (α and β), IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9 through IL-18	Primarily immunoregulatory, act on immune system cells (generally lymphocytes) in either stimulatory or inhibitory fashion
Colony-stimulating factors (CSF)	Granulocyte (G-CSF), Macrophage (M-CSF), Granulocyte/Macrophage (GM-CSF), IL-3	Involved in the proliferation of leukocyte progenitors. GM-CSF and IL-3 have some immunoregulatory functions
Interferons (IFN)		
• Type I	IFN-α, IFN-β, IFN-δ, IFN-ω	Primarily antiviral activity, some immunoregulatory functions
• Type II	IFN-γ	Primarily immunoregulatory
Tumor necrosis factors (TNF)	TNF-α, TNF-β, TNF-related apoptosis-inducing ligand (TRAIL)	Immunoregulatory activities; anti-tumor effector functions; apoptosis growth regulation
Hematopoietins	Stem cell factor, stem cell growth factor, erythropoietin, thrombopoietin	Involved in the regulation of bone marrow function and the production of hematopoietic cells
Miscellaneous	Oncostatin M, leukemia inhibitory factor, transforming growth factor(s)	Various pleiotropic functions
Chemokines		
C	Lymphotactin	Chemotactic for lymphocytes
CC	C10, eotaxin, I-309, leukotactin-1, MARC, MCP, MIP-1, MIP-3, MPIF-1, PARC, RANTES, TARC, TECK	Primarily active on monocytes/macrophages
CXC	IL-8, 6Ckine (Exodus), BLC, CINC-1, CINC-2, CRG, ENA-78, gro, KC, MIG, MIP-2, NAP-2	Primarily active on neutrophils and T-cells
CX3C	Fractalkine	Modulates calcium flux; involved in cell adhesion

from the circulation. This combination of features helps ensure that cytokines remain compartmentalized, undoubtedly an important consideration given the potent bioactivity of these molecules (22,62).

Cytokines serve as primary immune system mediators. They are produced in the greatest proportion by T-helper lymphocytes, as discussed earlier. On the other hand, cytokines are certainly not exclusive to the immune system. In fact, certain of these molecules are phylogenetically ancient and highly conserved. Furthermore, both IL-1 and TNF are intrinsically involved in apoptosis and cellular proliferation, both fundamental biological processes. Thus, cytokines should be recognized for their role as conveyers of bioinformation, rather than as simple effector molecules involved in a single physiological process such as immunity and host resistance. For convenience, cytokines may be grouped into several classes (Table 31.3). These classifications are necessarily arbitrary because of the overlapping activity of these molecules.

Chemokines

Another, related group of molecules are the chemokines. Chemokines are small peptide molecules that (like cytokines) were originally associated primarily with the immune system, but which now are recognized as being produced by almost all cells of the body, and which are involved in a multitude of biological functions (Table 31.3). Chemokines play a role in modulating the Th1/Th2 balance associated with autoimmunity and hypersensitivity (72), as mediators of allergic inflammation (3), and modulating the function of

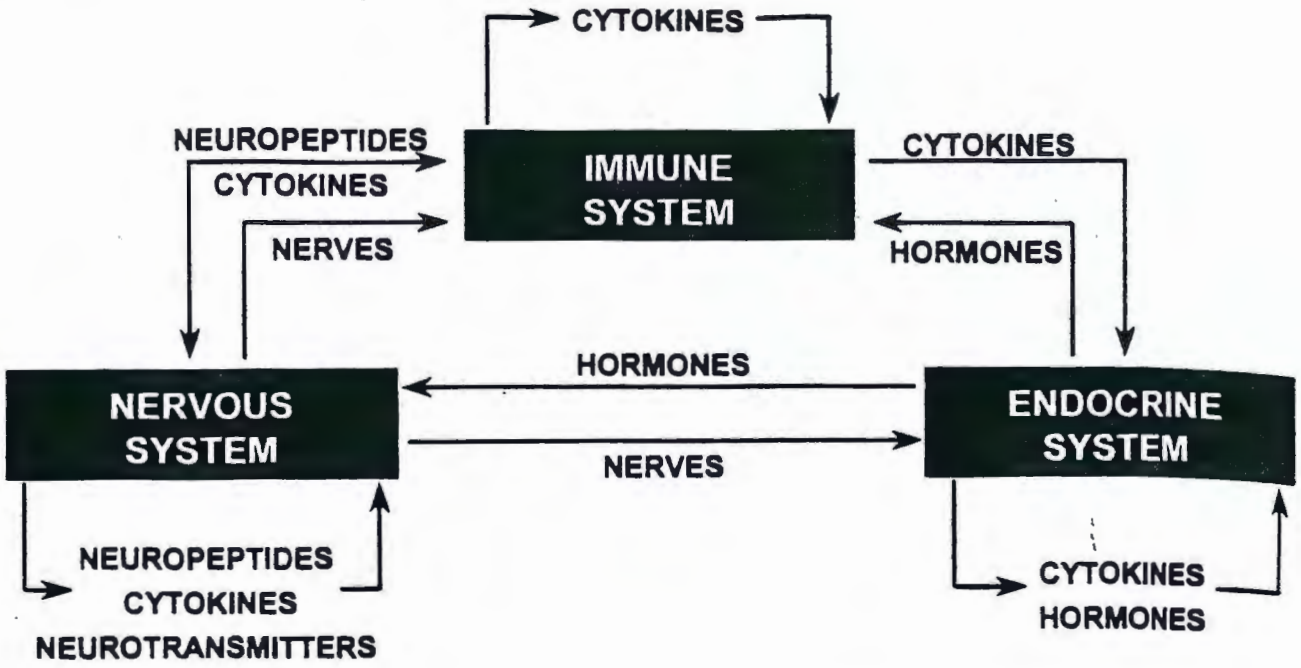

FIG. 31.3. Diagram of immune, nervous, and endocrine system axis.

leukocytes in disease states such as rheumatoid arthritis and asthma (104).

The Immune/Nervous/Endocrine System Axis

It has been recognized for some time that the nervous, immune, and endocrine systems, rather than being separate in function and structure, share many features and appear to cross-regulate each other's function (25,118). As illustrated in Figure 31.3, the soluble mediators used to regulate each of these systems (neurotransmitters, cytokines, and hormones) often serve multiple functions (31,84). The ramification for immunotoxicology of the existence of this neuro–immune–endocrine axis is that xenobiotics can affect the immune response indirectly by affecting other organ systems; conversely, modulation of the immune system may have secondary effects on other organ systems. To date, these interactions have not been extensively studied.

CONCEPTS AND APPROACHES FOR UNDERSTANDING DRUG- AND CHEMICAL-INDUCED IMMUNE DYSFUNCTION

Basic Considerations in Study Design

The value of incorporating immunological data for the toxicological evaluation of drugs, chemicals, and biologicals in human risk assessment has been increasingly accepted by regulatory agencies. For example, the U.S. Environmental Protection Agency (EPA) has established reference doses using immunotoxicity data for several compounds including 1,1,2-trichloromethane, 2,4-dichlorophenol, and dibutyltinoxide. The Agency for Toxic Substances and Disease Registry has derived "minimum risk levels" for arsenic, dieldrin, nickel, 1,2-dichloroethane, and 2,4-dichlorophenol from immune endpoints. More recently, this agency has established guidelines for testing chemical and biochemical agents.

On the other hand, rather than establish set guidelines, the U.S. Food and Drug Administration (FDA) has requested immunotoxicity testing of drugs and biologicals more on a case-by-case basis, with the recommended assays and testing approach suggested by the nature of the drug and its intended use (for previously untested agents), or based on the results of other toxicities observed during preclinical assessment (32,33). These guidance criteria have not been codified as yet for either drugs or biologicals, although much progress has been made for guidance on medical devices (2) and foods and food additives (36).

The sensitivity of the immune system to suppression by some drugs and environmental agents, that has been observed in experimental studies, is due as much to the general properties of the chemical (e.g., reactivity to macromolecules) as to the complex nature of the immune system (encompassing antigen recognition and processing; cellular interactions involving cooperation, regulation, and amplification; cell activation, pro-

liferation, and differentiation; and mediator production by various cell types and their products). Because of this complexity, the initial strategies among immunologists working in toxicology and safety assessment have been to select and apply a tiered panel of assays to identify immunosuppression or, in rare instances, immunostimulatory agents in laboratory animals (17,75,114). Although the configurations of these testing panels vary by laboratory and species, they generally include measures for

(a) altered lymphoid organ weights or histomorphology;
(b) quantitative changes in cellularity of lymphoid tissue, peripheral blood leukocytes, and bone marrow;
(c) impairment of cell function at the effector or regulatory level; and
(d) increased susceptibility to infectious agents or transplantable tumors.

There are a number of advantages and limitations to using such test panels. For example, although the sensitivity of these batteries in detecting immune system changes is well recognized, it is difficult to establish the clinical significance of subtle immune changes on neoplasia or infectious diseases, particularly in humans. Furthermore, some of the tests require invasive procedures such as immunization. These tests are not usually feasible or ethical for inclusion in human studies and, therefore, limit the potential for animal–human comparisons, although several recent immunotoxicology studies in humans have included primary immunization (e.g., hepatitis B) as a test measure. In this respect, assays that require in vivo primary antigenic challenge are generally accepted as the most sensitive and predictive of all immune function tests.

A variety of factors must be considered when evaluating the potential of an environmental agent or drug to adversely influence the immune system. Assessment requires validation of the endpoints to be measured (quality control and biological relevance) as well as knowledgeable selection of animal models, exposure parameters, and consideration of general toxicological parameters, including metabolism, distribution, and toxicokinetics. Treatment conditions should take into account the potential route and level of human exposure, biophysical properties of the agent (including protein-binding properties and toxicokinetics), as well as any available information on the agent's mechanism of action. Dose levels should be selected that attempt to establish clear dose-response curves as well as a no-observable-effect level (NOEL). Even though in some instances it might be beneficial to include a dose level that induces overt toxicity, any immune change observed at such a dose level should be interpreted cautiously, because severe stress and malnutrition are known to impair immune responsiveness. It is often recommended that the highest dose used be considerably lower than the LD_{10}. Although laboratories routinely employ three dose levels, dose range-finding studies are recommended prior to a full-scale immunotoxicology evaluation.

The selection of the exposure route should parallel the most probable route of exposure in humans, which is most frequently oral, respiratory, or dermal. Other routes of exposure may include parenteral, subcutaneous, or intraperitoneal exposure. Because the major routes of exposure (i.e., skin, lung, or gut) are also associated with local immunity, attention has been directed to the development of methodology for assessment of local immune responses, particularly in the lung.

Selection of the most appropriate animal model for immunotoxicology studies has also been a matter of great concern. Ideally, toxicity testing should be performed in a species that will elicit chemical-related pharmacology and toxicities similar to those anticipated in humans (i.e., the test animals and humans will metabolize the chemical similarly and will have identical target organ responses and toxicity). For most immunosuppressive therapeutics, rodent data on target organ toxicities and comparability of immunosuppressive doses have generally been predictive of what was observed in the clinic. Exceptions to the predictive value of rodent toxicological data are seen infrequently, but have occurred—for example, in studies of glucocorticoids, which are lympholytic in rodents, but not in primates (34,63). Although certain compounds may exhibit different pharmacokinetic properties in rodents than in humans, rodents still appear to be the most appropriate animal model for examining immunotoxicity. This statement is based on the established similarities of toxicological profiles across these models, as well as the ease of generating host susceptibility challenge and immune function data.

Both the quantitative and qualitative susceptibility of an individual animal to an immunotoxic agent can be influenced by its genetic composition (genotype), indicating a need to consider not only species but also strain. Rao et al. (87) described two approaches to the selection of genotypes for rodent toxicity studies. The first approach is to select genotypes that are representative of the animal species, in the hope that the choice will also exhibit sensitivities similar to humans. This can be accomplished by using randomly bred rodents. However, due to the variability in immune responses associated with outbred animals, it may be necessary to use a large number of animals to identify a sensitive population. A second approach attempts to identify genotypes that are uniquely suitable for evaluation of a specific class of chemicals. This requires considerable knowledge of the mechanisms of toxicity for the particular compound. One compromise would be to use F1 hybrids that contain

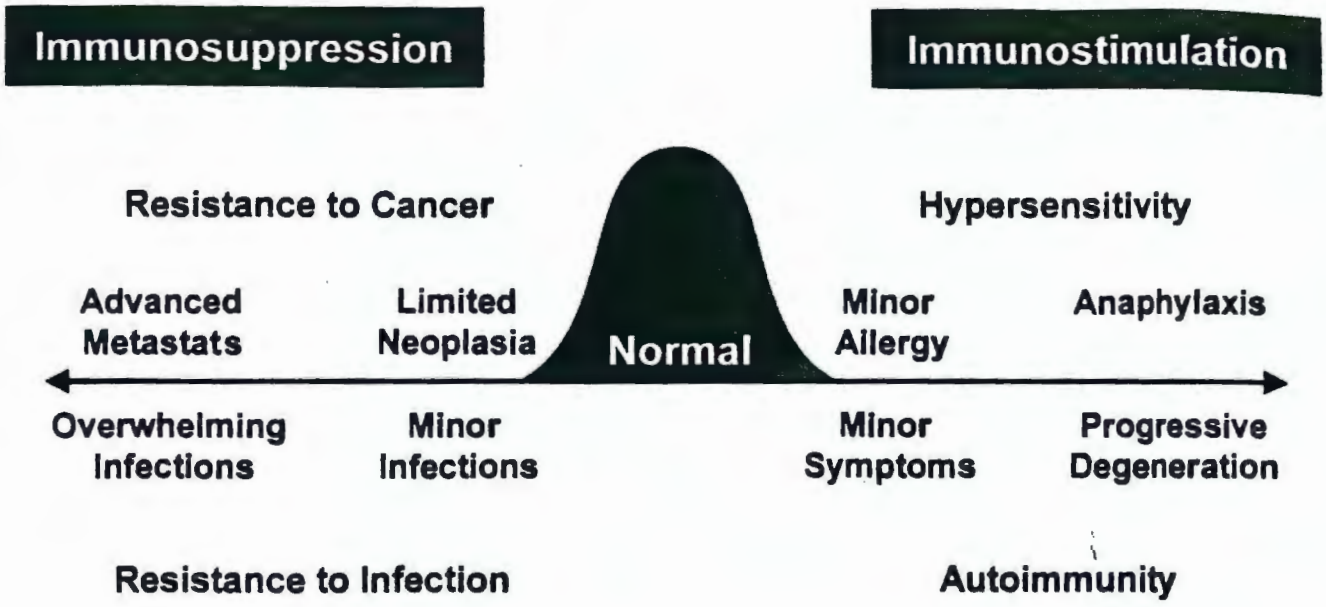

FIG. 31.4. Continuum of immune responsiveness and possible consequences.

the stability, phenotypic uniformity, and background information of an inbred animal, and yet have heterozygosity.

At present, it is impossible to determine how applicable these conclusions will be for immunotoxic compounds with different immune profiles. However, as more analyses become available, the ability to accurately estimate potential clinical effects from immunological tests should increase.

Immunosuppression

Introduction and Fundamental Concepts

Based on the preceding discussion of the important role that the immune system plays in protection of the host from infectious organisms and incipient neoplasia (Figure 31.4), it is logical to expect that disruption of this system following exposure to xenobiotics would have serious consequences. However, human exposure to many potential immunosuppressants is often difficult to assess given the uncertainties associated with dose, duration of exposure, and myriad other intervening variables. To better understand the potential for human effects following immunotoxic insult, it is instructive to examine a more controlled situation, namely the therapeutic use of agents designed specifically to suppress normal immune function.

Immunosuppressive therapy has been used to treat certain autoimmune, collagen, vascular, and chronic inflammatory diseases, as well as to prevent rejection of transplanted organs. However, therapeutic immunosuppression frequently causes complications from bacterial, viral, fungal, and parasitic infections. Another complication of immunosuppression observed in transplant patients has been a high frequency of secondary cancer. Partial or complete regression of the secondary cancers often occurs if the therapy is terminated. In a large sampling of renal transplant patients who survived 10 years, approximately 50% developed cancer (83). The types of tumors observed were heterogeneous and included skin and lip cancer (21-fold increase over the general population), non-Hodgkin's lymphoma (28- to 49-fold increase), Kaposi's sarcoma (400- to 500-fold increase), and carcinomas of the cervix (14-fold increase). Thus, it is clear that even controlled exposure to immunosuppressants may have severe consequences; this suggests that "uncontrolled" exposure to immunosuppressants (particularly when the mechanism of action is unknown) is of serious concern.

A large body of information has developed demonstrating that xenobiotic exposure can produce immune suppression and altered host resistance in experimental animals (Table 31.4) following acute or chronic exposure. Although only a limited number of reports indicate immune dysfunction following human exposure to xenobiotics, clinical data with a number of agents appear to demonstrate that immunotoxicity in rodents may form the basis for human risk assessment (115).

Given the complexity of the immune system (both natural and acquired) and the many potential target cells and molecules, it is impractical to enumerate all the potential targets of immunosuppressive agents. For this reason, a number of immune function assays have been developed

Table 31.4
Drugs and chemicals associated with immunosuppression

Pharmaceuticals	cytoreductive agents opiates	transplantation drugs AIDS therapeutics
Industrial Chemicals	organic solvents polychlorinated biphenyls glycol ethers	halogenated aromatic hydrocarbons polycyclic aromatic hydrocarbons
Environmental Agents	heavy metals ultraviolet light pesticides	air pollutants dusts (silica, asbestos)
Recreational Adjuncts	ethanol cannabinoids cocaine	tobacco (smoke) opiates

Table 31.5
Assays commonly employed to assess immunosuppression in laboratory animals

	Rodent	Nonhuman Primate
Initial Assessment ("Tier I")	Hematology	Hematology
	Bone marrow histomorphology	—
	Lymphoid organ weight and histomorphology	—
	Primary antibody response	Serum Ig level
	NK cell activity	NK cell activity
	Surface marker analysis	Surface marker analysis
Advanced Assessment ("Tier II")	CTL or DTH	—
	MØ function	MØ function
	Apoptosis	Apoptosis
	Cytokine analysis	Cytokine analysis
	Host resistance assays	-

Abbreviations: —: Not routinely performed; Ig = immunoglobulin; NK = natural killer; CTL = cytotoxic T-lymphocyte; DTH = delayed-type hypersensitivity.

and validated for evaluating immunotoxicity. These techniques and approaches are discussed in the following sections.

Techniques for Assessing Immunosuppression

The basic approach to immunotoxicity testing as it is currently practiced is based on the work of Luster et al. (64–66). This early work established the concept of the "tier" approach in which test materials are evaluated for effects on the immune system using a biphasic system of descriptive and functional assays. Tier I (screening) tests included hematology, body and selected organ weights, lymphoid organ cellularity and histology, evaluation of HI (using the IgM antibody-forming cell response and the B-cell proliferative response), evaluation of cellular immunity (T-cell proliferation in response to mitogens and alloantigens), and evaluation of NK cell activity. This group of tests provides a fairly comprehensive evaluation of immune structure and function (Table 31.5).

In situations where an effect was observed in one of the Tier I tests, the nature of the immune defect could be confirmed by using Tier II (comprehensive) tests. These included immunopathology (quantitation of T- and B-cell numbers), enumeration of IgG antibody response for HI, functional assessment of CMI using the cytotoxic T-lymphocyte (CTL) or DTH assays, and assessment of natural immunity using MØ assays. In addition, Tier II testing often included host resistance assays (bacterial, viral, parasite, or transplantable tumor) as a measure of whole-animal immune function.

Given the high predictive value of certain of these assays for immunotoxicity, as well as the time and expense involved in performing the entire battery of Tier I and Tier II tests, many practicing immunotoxicologists now use the AFC assay, in conjunction with more routine toxicological tests such as lymphoid organ weights and histomorphology, as an initial assessment for potential immunotoxicity of drugs or chemicals. In many cases, measurement of NK cell activity is also included in this initial assessment, since alterations in this effector of natural immunity would not normally be detected using the other assays. In the following section we, therefore, concentrate on these particular assays.

The following tests are commonly performed using the B6C3F1 mouse or the Fischer 344 rat, although they are readily applicable to other rodent strains. There are fewer well-developed immunotoxicology assays available for nonhuman primates at present, and even fewer tests available for use with canines.

Immunopathology. Routine histomorphology of bone marrow, thymus, spleen, and lymph node; a hemogram (complete blood count and differential); and determining spleen cellularity are useful for assessing the immunomodulatory activity of a drug or nondrug chemical, particularly when such data are combined with effects observed in lymphoid organ weights, such as the spleen, thymus, or lymph node (4,27,97). Because of the structural division of the spleen and lymph nodes into thymus-dependent and thymus-independent compartments, careful microscopic examination or immunocytochemical staining may indicate preferential effects for T- or B-cells. Likewise, microscopic examination of the thymus may reveal a compound that affects thymocyte viability.

IgM Antibody-Forming Cell Response. Within a few days following in vivo injection of a foreign antigen, antibody molecules of the IgM class are produced and released from PC into the systemic circulation. The antibody-forming cell assay (AFC, alternatively referred to as the plaque-forming cell, or PFC assay) quantitates the production of specific antibody through enumeration of antibody-producing cells in the spleen following a primary antigenic stimulus such as sheep red blood cells (SRBC). Although the AFC response to SRBC is a measure of B-cell function rather than T-cell function, it is an excellent functional parameter to examine, as this response requires cognate cell interaction and regulation by MØ, T-cells, and soluble regulatory molecules. The primary antibody response is currently measured using either a plaque-forming cell assay (15) or an ELISA (106). The steps involved in this assay are illustrated in Figure 31.5.

IgM Plaque Assay.

Materials and reagents required

- Earle's balanced salt solution (EBSS) supplemented with 25 mM HEPES buffer
- SRBC in Alsever's solution
- Guinea pig complement (GPC')
- Dulbecco's phosphate-buffered saline (DPBS)
- DEAE dextran, 30 mg/ml in saline, pH 6.9
- Bacto-agar
- Petri dishes and cover slips

Procedure

1. Four days prior to assay, immunize animals with an intravenous injection of washed SRBC in sterile saline. Recommended inocula are approximately 1×10^8 SRBC for mice and approximately 2×10^8 SRBC for rats.
2. Euthanize the animals, remove the spleens, and prepare a single-cell splenocyte suspension in EBSS. Prepare two dilutions of the cell suspension in EBSS.
3. Wash SRBC three times by centrifugation. After the final wash, retain approximately 100 μl of SRBC, then adjust the remaining cells to a final density of 10% in EBSS. Add GPC' to the reserved SRBC, mix well, and hold on ice until needed.
4. Prepare a solution containing 0.5% agar in DPBS, add DEAE-dextran (1.6 ml stock solution per 100 ml agar) and mix. Dispense the agar in 0.35 ml aliquots into polypropylene culture tubes, and maintain these tubes at 45°C.
5. For the assay, each tube contains 0.35 ml agar solution, 100 μl cell dilution(s), and 25 μl GPC'. Add SRBC first and then the cell suspension, and immediately remove the tube from the water bath. Add the GPC' and mix the contents of the tube. Dispense the contents into a Petri dish, then drop the cover slip so that an even layer of fluid forms underneath.
6. Incubate the plates at 37°C for approximately 3 h, and number the plaques. While the plates are incubating, determine cell number and viability of the original splenocyte suspensions.
7. Calculate the results as follows:

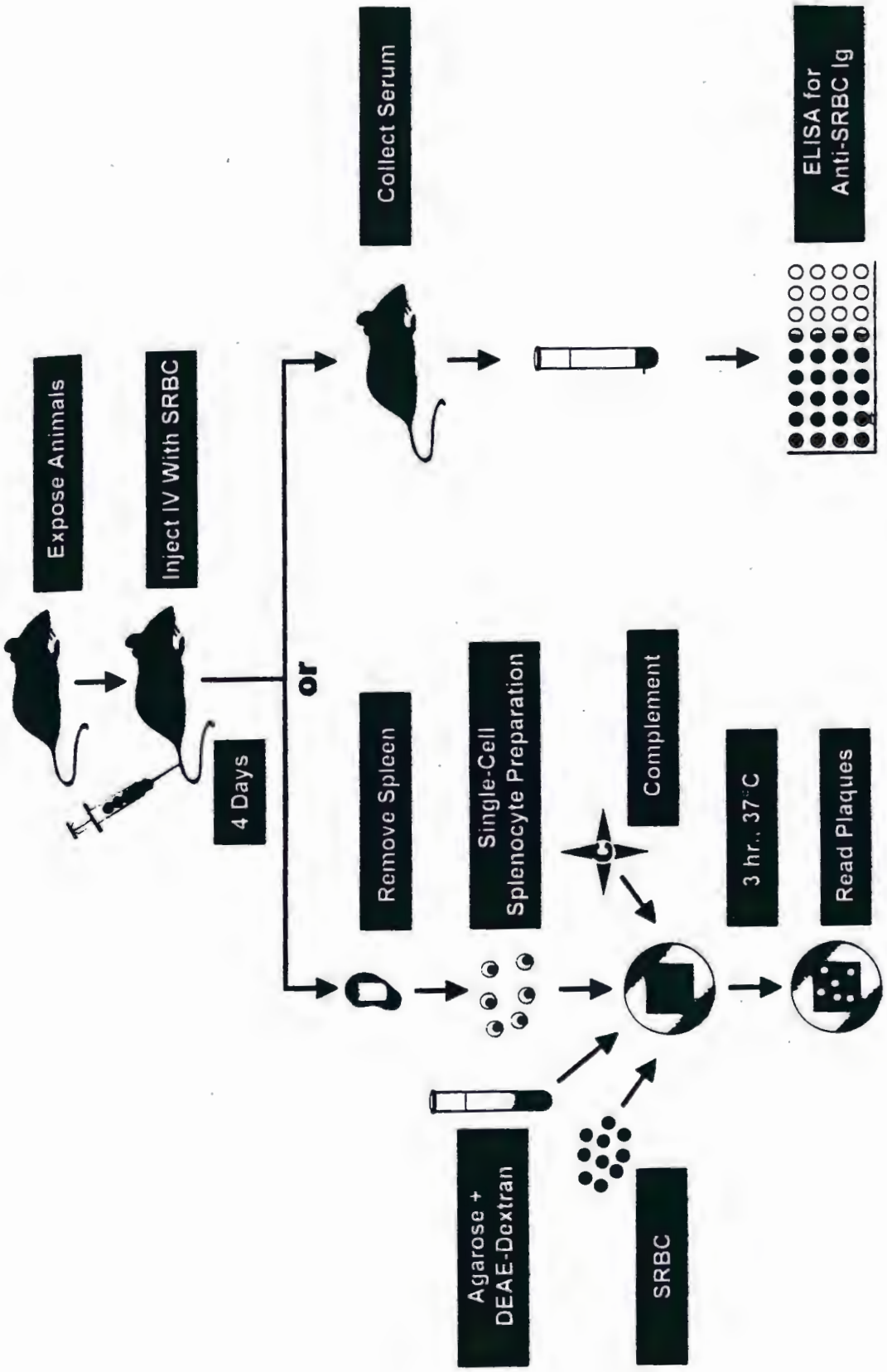

FIG. 31.5. Diagram of Antibody-Forming Cell (AFC) Assay.

a. Plaques counted under each cover slip × 10 × dilution factor = PFC/ml of the original cell suspension (since 0.1 ml of the cell dilution is counted);
b. PFC/ml × volume of original cell suspension = PFC/spleen;
c. PFC/ml/number of viable cells/ml = PFC/10^6 viable splenocytes.

Anti-SRBC IgM ELISA

Materials and reagents required

- SRBC in Alsever's solution
- Horseradish peroxidase (HRP)-conjugated, affinity-purified goat anti-mouse/anti-rat IgM antibody
- Peroxidase substrate [2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, ABTS]
- ABTS buffer (phosphate-urea-hydrogen peroxide)
- Phosphate-buffered saline (PBS)
- 96-well microplates
- general reagents and supplies for ELISA

Procedure

1. Immunize mice or rats with SRBC as for the plaque assay. Five days (mice) or six days (rats) later, obtain serum from both immunized and naive animals. Pool each as appropriate to use as standards or controls and freeze at −20°C.
2. Treat mice or rats with test material and vehicle (and a positive control, if necessary). On day 5 or 6 post-treatment (respectively), obtain serum from animals. Note: If serum is collected via the retro orbital sinus, additional samples may be collected later for time-course studies.
3. Prepare SRBC membrane antigen by lysis and solubilization (106). This antigen serves as the capture reagent in the ELISA.
4. Obtain anti-SRBC IgM monoclonal antibodies to use as standards. Note: Anti-SRBC must be of the appropriate species depending on the test animal (i.e., mouse or rat).
5. Dilute membrane antigen to 1.0 μg/ml in PBS and coat the wells of the microplates at approximately 4°C using 125 μl/well of the antigen preparation.
6. On the day of assay, wash the plates three times with 0.01% Tween-20 in water. Block any unbound sites on the plates by incubating the plates with 200 μl/well of PBS/0.05% Tween-20, 3% bovine serum albumin, or 3% powdered milk.
7. Prepare serial twofold dilutions of test sera and antibody standards. Add to the plates and incubate for at least one h at room temperature.
8. Wash the plates three times, then add HRP-conjugated secondary antibody. Incubate for at least one h at room temperature, then wash the plates three times.
9. Add peroxidase substrate (ABTS) and incubate the plates at room temperature for 45 min. Stop the reaction by adding 3% oxalic acid to all wells.
10. Read the plates at 405 nm and calculate the results based on curves prepared using the antibody standards.

Positive control. Cyclophosphamide is routinely used as a positive immunosuppression control for the AFC assay (either plaque or ELISA format). For mice, cyclophosphamide is administered intraperitoneally at 80 mg/kg once approximately 24 h prior to euthanasia. For rats, it is given ip at 20–25 mg/kg daily for four to five days prior to euthanasia.

Notes

1. The AFC response varies depending on the day of analysis following immunization. Each species and strain should also be evaluated for the optimum response, although for intravenous injection the optimum assay period is usually four days following immunization.
2. The dose and route of antigen exposure alters the peak AFC response. Intravenous injections shift the optimum response to an earlier time, whereas an intraperitoneal injection delays the peak response.
3. Each new test lot of complement should be tested and titrated prior to use.
4. The day of antibody induction relative to the last dose of chemical exposure should be considered when designing a study.
5. At the time of this writing, the SRBC membrane antigens are not commercially available, and must be prepared in-house. Monoclonal and polyclonal antibodies specific for SRBC are commercially available from a variety of sources.
6. The direct comparability of results between the plaque assay and the ELISA is the source of some discussion. The AFC assay measures antibody production in one organ (spleen) only, whereas the ELISA is a measure of systemic antibody production and may have a different time course.

Natural Killer (NK) Cell Assay. NK cell activity is measured in vitro by culturing single-cell suspensions of lymphoid cells with a tumor cell line known to be sensitive to NK-mediated cytotoxicity. The target cells are radiolabeled prior to the assay; thus, any cells which have been lysed will release their radioactivity into the culture medium, when it can subsequently be quantitated.

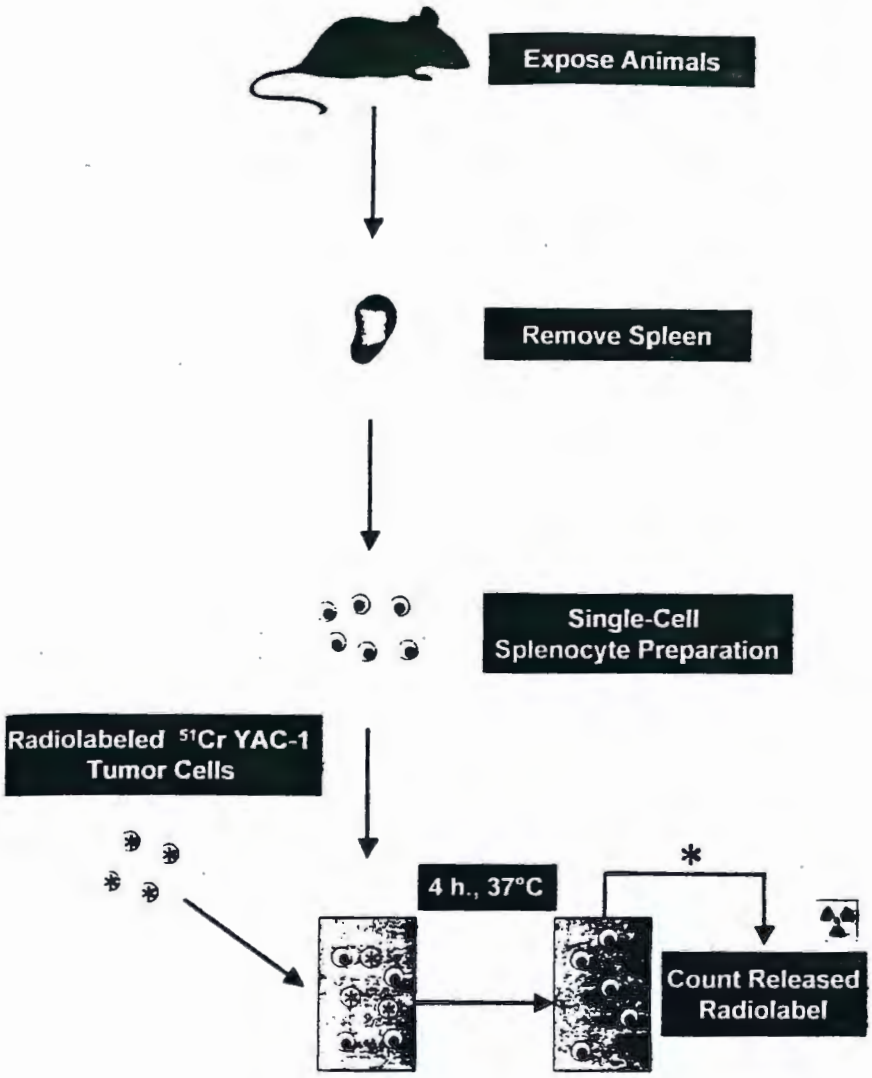

FIG. 31.6. Diagram of Natural Killer Cell Assay.

The procedure described below is modified from the microculture method described by Reynolds and Herberman (88), and is the standard approach for immunotoxicity assessment. The procedure for this assay is illustrated in Figure 31.6.

Materials and reagents required

- RPMI-1640 culture medium supplemented with 25 mM HEPES buffer, 10% FBS, 2 mM l-glutamine, and 50 μg/ml gentamicin
- Fetal bovine serum (FBS)
- DPBS
- Wash solution (DPBS/1% FBS)
- YAC-1 cell line (for rodent NK evaluation; ATCC #TIB 160) or K562 cell line (for primate NK evaluation; ATCC #243) maintained in log-phase growth in the culture medium described above
- 96-well round-bottom microculture plates
- 0.1% solution of Triton X-100 in distilled H_2O
- ^{51}Cr as sodium chromate in sterile saline; specific activity of 200–500 mCi/mg
- Supernatant collection system

Procedure

1. Prepare a single-cell suspension of the effector spleen cells, and adjust to a density of 5×10^6 viable cells/ml in culture medium.
2. Prepare two serial 1 : 3 dilutions of the cell suspension in culture medium. Dispense 100 μl of each dilution in quadruplicate wells of 96-well, round-bottom microculture plates.
3. Centrifuge a log-phase culture of target cells and suspend the cell pellet in 0.5 ml FBS. Add 200 μl ^{51}Cr to the cells, mix well, and incubate at 37°C for one h. Wash the cells three times.

4. Suspend the target cells in culture medium, determine cell number and viability, and adjust the cells to a final density of 5×10^4 viable cells/ml in culture medium. Add the target cells to all wells in a volume of 100 μl/well. Include a row containing 100 μl target cell suspension and 100 μl culture medium/well (spontaneous release) and one row consisting of 100 μl target cell suspension and 100 μl 0.1% Triton X-100/well (total release).
5. Incubate the plates at 37°C, 5% CO_2 for 4 h. Harvest all wells with a supernatant collection system, and determine radiolabel release in a gamma counter.
6. Harvest supernatant fractions either manually or by using a semiautomatic harvesting system. Quantitate radiolabel released into the supernatant fractions in a gamma counter, and determine percent cytolysis using the formula:

$$\text{Percent cytolysis} = (\text{experimental release} - \text{spontaneous release})/(\text{total release} - \text{spontaneous release}) \times 100$$

Positive controls

Immunosuppression control. Unless the laboratory has extensive experience, a positive suppression control of the NK response should be included. Approximately 24–78 h prior to euthanasia, a separate group of animals are injected intravenously with an optimum concentration of anti-asialo GM1 antibody. The exact amount to be given will vary from lot to lot, and between suppliers. Treatment with an optimum dose of anti-asialo GM1 will result in an essentially complete abrogation of the NK response in rodents (30).

Immunostimulation control. In some cases, it may be useful to include a positive control for NK cell augmentation. Although cytokines (IL-2 and IFN-γ) can enhance this response both in vivo and in vitro, an equally efficient, and more economical/reproducible option is the use of interferon inducers such as polyinosinic : polycytidilic acid (poly I : C) (23). Poly I : C is administered intraperitoneally at a concentration of 100 μg/mouse or 500–1000 μg/rat approximately 24 h prior to assay.

Notes

1. NK activity is highest in young mice, declining after 12 weeks of age. Basal NK activity may be highly variable or undetectable in mice over 20 weeks old.
2. The target cells must be in log-phase growth to achieve adequate labeling with ^{51}Cr. In addition, the target cell lines should be assessed for mycoplasma contamination at periodic intervals.
3. The assessment of NK cell activity has been utilized most extensively in rodents and primates. In instances in which evaluation of canine NK cell function would be useful, modified techniques have been published (53).
4. For laboratories unable or unwilling to use radioisotopes, alternative methods have been developed using colorimetric (78) and fluorometric (7) endpoints. A full comparison, however, has not been made between these alternative methodologies and the standard chromium-release assay.

Phenotypic Analysis of Cell Surface Markers by Flow Cytometry. The evaluation of both peripheral blood- and tissue-specific lymphocytes by cytometric analysis has become a relatively common clinical laboratory test for lineage assignment in leukemias and lymphomas, prognosis in HIV infection, and evaluation of immunodeficiency. The technique involves treating cells with monoclonal antibodies covalently bound to different fluorochromes. These antibodies recognize surface antigens, referred to as cluster of differentiation (CD), unique to different cell types. The availability of fluorochromes, which emit light at different wave lengths following excitation, combined with flow cytometers that are capable of performing multiple color analysis, provides a rapid and effective method of analyzing cell types. The most commonly examined CDs in the mouse are those that recognize pan T-cells (CD90 and TCR complex), T-helper cells (CD4), T-suppressor cells (CD8), and pan B-cells (CD45R/B220 or CD19). Nomenclature and immunophenotypes for mouse cell surface antigens have been recently updated (56). The ability of this technique to establish low-level immunodeficiency has not been established.

Materials and reagents required

1. Prepare single cell suspensions from the spleen (Ficoll-separated and whole blood have both been used occasionally). For washing and staining use DPBS (0.01 M).
2. Centrifuge conjugated reagents at $15 \times 10^3 \times g$ to remove aggregates.
3. Pipette desired concentration of antibody or control sera in 50 μl volumes to a small test tube or 96-well microtiter plates. For two-color analysis both conjugated antibodies can be added together.
4. Add 10^6 viable cells in a volume of 50 μl to the test tube or well containing the antibody.
5. Incubate 30 min on ice in the presence of 0.1% sodium azide.
6. Wash with 2.0 ml if in tubes or two 100 μl washes if in wells.

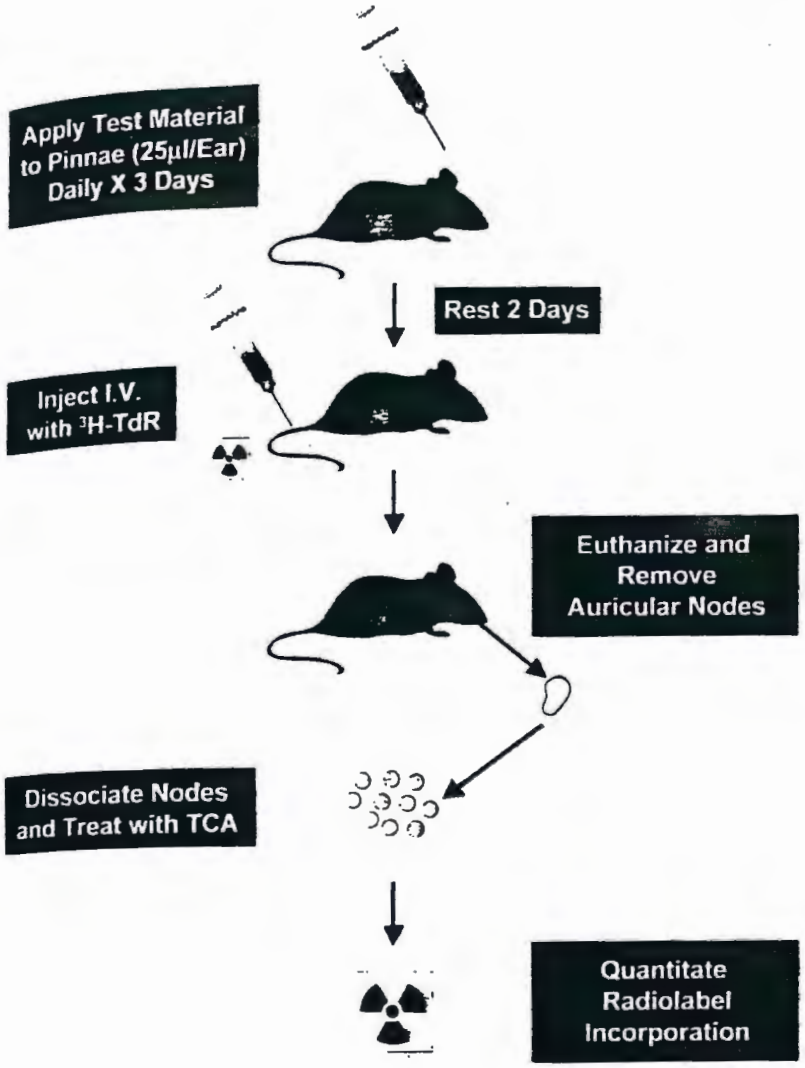

FIG. 31.7. Diagram of Murine Local Lymph Node Assay.

7. Suspend to a volume of $1–2 \times 10^6$ cells/ml in cold PBS containing 0.1% sodium azide and perform analysis.
8. Cell fluorescence and integrity can be preserved for up to five days by rapidly suspending the cell pellet in 50 µl cold PBS containing 1% paraformaldehyde.

Mechanistic Immunotoxicology Assays

From its beginnings, the discipline of immunotoxicology has constantly evolved and incorporated new techniques and paradigms to understand the nature of immunomodulation. The assays described previously in this section allow one to make a first-pass evaluation of drugs and chemical agents for generalized immunotoxicity. The assays will indicate that the immune system had been perturbed, although the cellular and molecular mechanisms involved will not necessarily be obvious. These assays are valuable for quick and relatively accurate identification of toxic agents.

The tools and concepts of immunotoxicology also are increasingly being utilized as research tools to understand the function of the immune system. For example, it may be useful to know not only whether or not an agent modulates the immune response, but why. This is especially important in the discovery and development of pharmaceutical agents, where therapeutic manipulation of the immune system may be a desirable goal. In response to these novel applications of immunotoxicology, assays are needed which will allow us to determine the precise mechanism of immunomodulation.

The methodology for Tier II-type assays such as T-cell-mediated immune function (CTL/DTH), MØ function, IgG antibody cell forming response, and host resistance models has been reviewed in detail elsewhere and will not be reiterated here (11,39,103,108). These assays are still valuable tools for understanding the mechanistic basis of immunotoxicity. In addition to these assays, several other methodologies are now

being included in the immunotoxicology armamentarium.

Assessment of apoptosis. Apoptosis (programmed cell death) is increasingly recognized as a fundamental process in both health and disease states, including the response to toxic insult (14). Apoptosis plays a vital role in the immune response, regulating the number and action of immune cells such as lymphocytes (41,74). Given the important role apoptosis plays in normal regulation of the immune system, as well as its implication in immune-related disease (41), it is a logical and potentially valuable endpoint for mechanistic immunotoxicology evaluation (80). Apoptosis has been found to play an important role in the immunotoxicity of a number of compounds including organotins (85), polychlorinated biphenyls (119), methylmercury (102), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (45).

Numerous techniques are available for assessing apoptosis, including analysis of DNA degradation, flow cytometry (10), morphological analysis, 3'-OH end labeling, and endonuclease analysis (101). More recently, a number of ELISAs have become available for assessing apoptosis; these ELISAs are based on the detection of Bcl-2 or histone-associated DNA fragments. The ELISA format offers a number of benefits over the other techniques including rapidity, simplicity, and cost-effectiveness.

Cytokine analysis. As described previously, cytokines represent an important mechanism not only for regulating the function of the immune system, but, also, for linking the immune system with other organ systems. Early studies employing cytokine analysis in immunotoxicology studies were more descriptive (38,67). However, as the intricacies of the cytokine/chemokine network become better understood, these assays are allowing us to assess the mechanisms responsible for a variety of immunomodulatory effects. As an example, a variety of nonbiological agents have been described which either specifically or nonspecifically alter cytokine production. These agents act via myriad mechanisms including direct toxicity to cytokine-producing cells (cyclophosphamide); inhibition of cytokine production (Cyclosporin, FK506, pentoxifylline); inhibition of cytokine release (pentamidine); induction of immunosuppressive factors (Leflunomide); alterations in cellular homeostasis (Tenidap); alterations in cellular activational or transcriptional mechanisms (Thalidomide); alteration of cell cycle progression (Rapamune); and miscellaneous or undefined mechanisms (glucocorticoids, phosphodiesterase isozyme inhibitors, metalloproteinase inhibitors, and p38 kinase inhibitors) (40).

There are currently four major types of cytokine assays: bioassays, immunoassays, mRNA gene expression, and flow cytometry. Sometimes used is what may be termed the "hybrid assay," employing elements of two or more of the main assay categories, and molecular biology assays to examine cytokines and cytokine receptors (111). Each of these assay types exhibits advantages and disadvantages, and no one type of assay is best suited for all applications. The type of assay chosen is subjective, and will depend on the capabilities of the laboratory, and the type of information to be gained.

Hypersensitivity and Allergy

Introduction and Fundamental Concepts

Acute hypersensitivity or allergy is a pathological state resulting from prior sensitization to a specific molecule or structurally related compound. In the context of immunotoxicology, hypersensitivity reactions are often considered to be the sequelae of immunostimulation, resulting in an inappropriately vigorous reaction to usually benign antigens or to chemical-modified (i.e., haptenated) self antigens. These antigens are processed by antigen-presenting cells (MØ or dendritic cells) and then presented to T-lymphocytes, resulting in the proliferation of lymphocytes and the subsequent release of various bioactive molecules (inflammatory cytokines, vasoactive peptides, etc.).

Exposure to any of a number of industrial chemicals and drugs, or their metabolites, has been associated with the development of allergy and hypersensitivity reactions (Table 31.6). These compounds are sometimes directly antigenic, but may also haptenate macromolecules present in the lung, gastrointestinal tract, bone marrow, or skin. In general, the route of antigenic exposure determines the ultimate type of hypersensitivity reaction; for example, dermal contact principally produces dermatological reactions (e.g., urticaria, rash, pruritus), whereas respiratory exposure results in airway reactions (e.g., bronchoconstriction). These reactions are not always localized, but may progress to systemic effects, with the most dramatic example being anaphylactic shock.

Individuals with potential occupational exposure (e.g., chemical manufacturing workers and farm workers) are at a higher risk than the general public for development of respiratory and cutaneous contact hypersensitivity to chemicals. Hypersensitivity is one of the most common and costly health problems in the United States, afflicting at least 35 million Americans. The indirect costs, such as wages lost because of illness, are estimated to be in excess of $800 million annually for asthma alone, with more than 35 million workdays lost to sickness each year (120).

Industrial processes utilize many materials capable of inducing occupational immunological lung disease or contact hypersensitivity in workers, and thus must be rigorously controlled to ensure worker safety. Studies

Table 31.6
Drugs and chemicals associated with hypersensitivity

Pharmaceuticals	phenylglycine acid chloride	ampicillin
	piperazine	spiramycin
	amprolium hydrochloride	antibiotic dust
	antihistamines	quinidine
	anesthetics	plasma substitutes
Foodstuffs	castor bean	pancreatic extracts
	green coffee bean	grain and flour
	papain	molds
Industrial Chemicals	ethylenediamine	phthalic anhydride
	diisocyanates (TMI, HDI, MDI)	trimellitic anhydride
	metallic salts	organic phosphorus
Miscellaneous Organics	cotton dust	animal products
	wood dusts	fragrance components

in the metal-refining industry, for example, suggested that many workers regularly exposed to the complex salts of platinum develop disorders of the respiratory tract. A study of workers exposed to toluene diisocyanate (TDI), a substance used in the manufacture of polyurethane, revealed that 5% of those surveyed developed occupational asthma in response to exposure to TDI. Studies of the detergent industry indicate that about 2% of employees exposed during manufacture developed asthma symptoms from inhaling enzymes used in detergents (120).

Drug allergy is also a significant problem and among the most common causes for new pharmaceuticals being withdrawn from the market after they are released. This allergic reactivity is not well predicted from the current battery of preclinical safety assessment methods (19). Drugs are unique in that they can provoke allergic and autoallergic reactions against blood cells including erythrocytes and platelets, as well as a variety of other antigens including the haptenated drug. Usually the reaction occurs to the drug or drug metabolites, in which case it is necessary for both the drug and the antibody to be present to produce the allergic or autoallergic reaction. The first observation of this type of drug reaction was made by Ackroyd, who observed thrombocytopenia purpura following administration of the drug Sedormid. Likewise, hemolytic anemias have been reported following the administration of a wide variety of drugs including penicillin, quinine, and sulphonamides. On rare occasions, drugs may induce allergic reactions where autoantibodies are raised that are directed against normal cellular constituents, as is seen against red blood cell antigens in 0.3% of patients given alpha methyl dopa. In addition to producing allergic manifestations or pathology under certain conditions, the presence of drug-specific antibodies can also alter the pharmacokinetics and clearance of the drug in plasma. Thus, drug-induced allergy reactions can come in many forms, producing either allergic or autoallergic phenomena. The autoimmune aspect of drug reactions is discussed in more detail in a later section.

Techniques for Assessing Contact and Respiratory Hypersensitivity

Given the potential economic and medical importance of hypersensitivity, the importance of sensitive and reliable assays for the detection of sensitizing potential for drugs and chemicals is obvious. For over 100 years, the guinea pig has served as the principal model for allergic reactions in humans because they demonstrate many similarities in their response to pulmonary hypersensitivity (response to histamine, demonstration of immediate and delayed allergic reactions, etc.), as well as dermal hypersensitivity. In addition, the lightly pigmented skin of albino guinea pigs, and their relatively small size and docile nature, make them manageable model animals. Thus, they have traditionally been used for assessing the human safety of drugs, as well as other chemicals, for contact and respiratory sensitization. Based on the specific experimental needs at hand, a variety of modifications have been described. In this section we will discuss only two, i.e., the Buehler assay and the guinea pig maximization test, which are probably the two most widely used tests for risk evaluation of contact sensitization at this time in the United States and Europe, respectively (70).

In recent years, the mouse has also been developed as an alternative model to the guinea pig. The impetus for this development has been the mouse's small size and reduced cost, more thoroughly understood immune system, and the perceived need of a more quantitative endpoint than the subjective degree of erythema that is the hallmark of most guinea pig assays. Two mouse models in particular have been developed: the mouse ear swelling test (MEST) of Gad (26), and the murine local lymph node assay (LLNA) first described by Kimber and Weisenberger (49). Of these two, only the LLNA has been fully validated and is described in this section.

As mentioned above, guinea pig models are also important for the assessment of potential respiratory sensitizers (95,112). The interested reader is directed to a number of excellent reviews of the use of these assays in risk assessment and drug development (13,69,112,113). In addition, a murine model for assessment of respiratory sensitizing potential, the Mouse IgE test, is currently being examined for its utility (21).

Buehler assay. The Buehler assay (8) was originally developed to evaluate strong and moderate contact sensitizers, leaving only negative or weakly positive compounds for testing in humans. The hallmark of the Buehler assay was the use of an occlusive patch to enhance or exaggerate exposure to test materials. This method also has the advantage of using an exposure method similar to the one that would be encountered in human exposure.

The specific technique for performing this assay is involved, and is only summarized below. A more detailed description of the assay is provided by Buehler (8,9).

Materials and reagents required

- young adult albino guinea pigs (Dunkin–Hartley strain)
- guinea pig restrainers
- patch delivery system (e.g., Hilltop chambers, Webril patch, PMP patch)
- dental dam

Procedure

1. On the day before induction exposure, clip the guinea pig's fur. Expose the skin to the selected test dose using a patch delivery system and then restrain the animal using a combination of guinea pig restrainer and dental dam. Duration of exposure, number of inductions, and induction regimens may vary, but are generally three 6-h induction exposures with an interval of 5–9 days.
2. Approximately 2 weeks after the last induction exposure the animals are exposed to the test material again, but at a different skin site that has not been previously exposed. Again, the timing and duration of exposure may vary.
3. If necessary, an animal may be rechallenged with test material between 6 and 14 days following the primary challenge.
4. The day after the challenge or rechallenge, depilate the animals using a commercial depilatory. Two h later the animals are ready to be scored.
5. Results are scored as 0 (no reaction), ± (slight patchy erythema), 1 (slight but confluent erythema), 2 (moderate erythema), and 3 (severe erythema, with or without edema). Scoring should be performed at 24 and 48 h after the challenge or rechallenge.
6. Scores of 1 or greater in the test group usually indicate that the test material is a sensitizer, if control scores are less than 1. Results of the challenge and rechallenge should be expressed as both incidence and severity.

Positive control. Dinitrochlorobenzene (DNCB) has been the traditional positive control for this assay. Suggested test concentrations are 0.3% DNCB in ethanol for induction and two concentrations of DNCB in acetone (0.05% and 0.01%) for challenge. Inclusion of two different challenge doses provides for a range of response (9).

Notes

1. An irritation screen is performed prior to the actual test. This assay requires an induction concentration that will not produce severe irritation or toxicity. The concentration used for challenge should produce only a slight degree of irritation.
2. Proper technique is essential to the success of this assay. In particular, animal restraint, test material occlusion, and consistency in scoring are all critical aspects. Whenever possible, it would be advisable to learn these techniques from a laboratory that has already demonstrated success with the assay.
3. Due to the relatively small number of animals and the nature of the readout, statistical analysis generally has not been practical for this assay. Rather, hazard assessment has been defined in terms of threshold levels.
4. It is important to maintain occlusion for the entire duration of the exposure in this assay. Proper use of the restraining devices is considered critical to obtaining consistent, meaningful results. Proper clipping and depilation of the animals are also important variables.

Guinea pig maximization assay. The maximization assay was described by Magnusson and Kligman in 1964 (68), and was developed to "maximize" the sensitivity

Table 31.7
Examples of drugs and chemicals implicated in autoimmune disease

Pathology	Agent	
Systemic lupus erythematosus/ Immune complex glomerulonephritis	Hydralazine Penicillamine Chlorpromazine Anticonvulsants Alfalfa sprouts (L. canavanine)	Heavy metals Isoniazid Organic solvents Procainamide
Hemolytic anemia	Methyldopa Penicillin Mefenamic acid	Diphenylhydantein Interferon α Sulfa
Thrombocytopenia	Acetazolamide Chlorothiazide Gold salts	p-amionsalicylic acid Rifampin Quinidine
Scleroderma-like disease	Vinyl chloride Silica	L-tryptophan
Pemphigus	Penicillamine	
Thyroiditis	PCBs Iodine	Lithium IL-2

of guinea pig tests. This was accomplished by intradermal injections of test material, inclusion of Freund's complete adjuvant, and the use of a pretreatment to irritate the skin at the site of exposure. These treatments enhanced the chance of a test material to penetrate the skin and subsequently produce allergic contact dermatitis (35).

Perhaps even more so than the Buehler assay, the Maximization assay is technically detailed, and only the basics of its performance are summarized below.

Materials and reagents required

- young adult albino guinea pigs (Dunkin–Hartley strain)
- Freund's complete adjuvant (FCA)
- Hilltop chambers or PMP patches
- hypoallergenic tape and elastic wrap
- dental dam

Procedure

1. Similar to the Buehler assay, a preliminary irritation/toxicity screen must be performed to determine the highest concentration of test material that can be tested. Both intradermal injection and occlusive patch tests are performed.
2. For the induction step, the animal's fur is clipped over the back on either side of the spine, and test material is injected intradermally in a volume of 0.1 ml. Each animal receives a total of six injections: two each of diluted adjuvant, two each of adjuvant containing test material, and two each of test material in vehicle. Control animals are treated similarly, but do not receive test material. Let the animals rest for 6 days.
3. If the test material being used is not an irritant, the injection sites should be treated with 10% sodium lauryl sulfate in petrolatum under an occluded patch for 24 h. This step may be skipped if the test material is a known irritant.
4. Place 0.8 ml of test material on a PMP patch (booster patch) and place this patch over the injection sites. Cover with dental dam and wrap the animal with elastic tape. Control animals should be treated with vehicle only. Remove the booster patch after 48 h.
5. Challenge the animals 10 days later by exposing to test material (or vehicle) under an occlusive patch (PMP patch or Hilltop chamber). Wrap the animals with dental dam and elastic tape. Note: animals

must be clipped prior to challenge, as the fur will have grown back.
6. Remove the patches 24 h later. Approximately 21 h later, remove any remaining fur with depilatory. Grade the reactions approximately 24 and 48 h after the challenge patches were removed.
7. Animals may be rechallenged with the same test material at the same or a different site within 2 weeks of the primary challenge. Naive test sites should be used on the animals.
8. The grading of this assay is similar to that of the Buehler assay.

Positive control. The positive control material generally used in the Maximization test is 1-chloro-2,4-dinitrobenzene, at a concentration of 0.1% in a vehicle of propylene glycol for the intradermal injections. The booster patch incorporates 0.1% (w/v) of test material in a 80:20 ethanol/water (vol/vol) vehicle.

Murine local lymph node assay. Although guinea pig models have proven exceptionally useful for assessing the potential of compounds to induce hypersensitivity reactions, they do have several drawbacks. For example, the endpoints are subjective and require skilled operators to evaluate the intensity of the reaction; in addition, this subjectivity precludes the use of statistical analysis. Moreover, the assays are relatively expensive and time-consuming. Finally, there are animal welfare issues regarding the use of agents such as adjuvant. Although none of these issues alone are major detriments, together they makes guinea pig assays less than ideal.

To address these concerns, Kimber and Weisenberger (49) reported the development of an alternative approach to assess potential contact hypersensitivity using the mouse as a model system; this model is known as the local lymph node assay (LLNA). The LLNA exhibits a number of advantages over guinea pig assays including a quantitative, objective endpoint, insensitivity to colored compounds, reduced turnaround time and cost, independence of specialized reagents or materials (adjuvant, wrapping material), and reduced animal welfare concerns. There is concern, however, that it loses predictability when weak sensitizers or irritants are tested.

The biological basis of the LLNA is simple. Test materials are applied epicutaneously to the dorsal surface of the pinna. From the skin, materials are transported by Langerhans cells to the draining (i.e., local) lymph node, where they are presented to T-lymphocytes. Contact sensitizers induce proliferation of these T-cells. By radiolabeling these proliferating cells in situ using a radioactive tracer, it is possible to determine the degree of proliferation. The LLNA does not utilize a secondary (challenge) exposure to the test material, as do the guinea pig assays. It differs fundamentally in that it evaluates only the induction phase of the hypersensitivity response.

The LLNA has been the subject of numerous validation studies, most recently a round of international validation studies employing a standardized protocol (51,52,61). The assay was consistently found to be robust, sensitive, and reproducible. In 1998, the LLNA was the first assay to be evaluated by the Interagency Coordinating Committee on the Validation of Alternative Animal Models (ICCVAM), and was found to provide an equivalent prediction of the risk for human contact dermatitis when compared to guinea pig assays (18,76).

The following protocol is the standard recommended by the ICCVAM Working Group (IWG):

Materials and reagents required
- Female CBA/J mice, 6–9 weeks old at initiation of assay
- Tritiated thymidine ([^{3}H]TdR), specific activity 5–10 Ci/mM
- Phosphate-buffered saline (PBS)
- Nylon mesh (100-micron opening size)
- 15 ml conical capped polypropylene centrifuge tubes
- 5 percent (w/v) trichloroacetic acid (TCA)
- Scintillation vials and scintillation mixture

Method
1. Apply vehicle, test compound, or positive control compound to the dorsum of each pinna (25 μl per ear), ensuring that the vehicle is evenly distributed on the pinna. Dose the animals daily for 3 consecutive days.
2. Rest the mice for 2 days, then inject each mouse iv with 20 μCi of [^{3}H]TdR in saline.
3. Five h following [^{3}H]TdR injection, euthanize the mice by CO_2 asphyxiation and remove the auricular lymph nodes. Place the nodes from individual mice in culture tubes containing 4 ml of PBS.
4. Transfer the nodes from the culture tubes to Petri dishes containing a 1-inch square of nylon mesh. Gently rub the lymph node cells through the mesh, then transfer the cell suspension back to the tube and allow it to settle for approximately 5 minutes.
5. Transfer the cell suspension to a 15 ml centrifuge tube containing 6 ml of PBS, taking care not to transfer the sedimented debris. Centrifuge the tubes for 10 minutes at approximately 200 x g. Wash the cells a second time in PBS.
6. After the second wash, suspend the cell pellet in 3 ml of 5% TCA and incubate at approximately 4°C for approximately 18 h.

7. Centrifuge the cell suspensions at approximately 200 × g for 10 min, discard the supernatant fluid, and suspend the pellet in 1 ml of 5% TCA. Transfer this suspension to a scintillation vial. Rinse the culture tubes with an additional 1 ml of TCA and add this to the scintillation vial. Mix the contents of the vial thoroughly.
8. Count the samples in a scintillation counter for 5–10 min, and record the counts as disintegrations per min (DPM).
9. Using the results obtained with the vehicle controls as baseline, calculate the stimulation index (i.e., mean experimental results divided by mean control results). Test compounds inducing a stimulation index of 3 or greater at any concentration evaluated in this assay, along with DPM values are statistically different from control DPM, are considered to be contact sensitizers.

Positive control. Hexylcinnamaldehyde (a contact sensitizer of moderate activity) in a solution of 20% serves as a useful positive control for this assay.

Notes

1. The LLNA is technically straightforward, and has been demonstrated to be forgiving of technical modifications (51,61). Perhaps the only difficulty a new investigator might have is in locating the lymph nodes draining the pinnae. A relatively simple way to determine this is to inject the ears with a dye (e.g., Evans Blue), and subsequently identify the nodes incorporating the dye.
2. Nonradioactive endpoints have been investigated, but at present their comparability to the radioactive endpoint has not been established.

Autoimmunity

Introduction and Fundamental Concepts

Autoimmunity comprises two processes. Initially, an immune response occurs to normal components of the host, and, second, a pathological condition may ensue in which the response causes structural or functional damage. As often noted by our colleagues in immunology, this immune response does not necessarily reflect disease, although it is a prerequisite for disease to occur, as autoimmune disease is multifactorial in nature. The autoimmune response can be cellular in nature, mediated by CD4 and/or CD8 T-cells. More often, however, it arises from antibody, mediated by specific B-cells that are driven by cytokines. Autoimmune disease is complicated by the fact that it is not a single disease, but rather represents more than 25 different diseases which can be either systemic or organ-specific. The most common autoimmune diseases are rheumatoid arthritis and those associated with the thyroid, such as Graves' disease (44). In total, they represent a significant and chronic morbidity problem, with recent estimates indicating that 1 in 31 individuals in the United States is affected, with women at 2.7 times greater risk than men (44).

The mechanisms responsible for autoimmune diseases are not clear. It is believed that the failure of any one of several immune processes can result in their development. However, the key process involves the loss of self-tolerance, such as the missed deletion or activation of autoreactive lymphocyte precursors. This process may be exacerbated by altered immunoregulation, such as over-expression of the immunoregulatory cytokine IL-4, or under expression of IFN-γ. Autoimmunity may also occur in the absence of an aberration in the immune system, such as when microbial agents express cryptic determinants (100). Although autoimmunity is a disease of the immune system, non-immunological genetic and epigenetic factors play a major role in disease development. For example, autoimmunity is influenced strongly by infectious agents, stress and diet (epigenetic), and polymorphisms in the T cell receptor and drug metabolizing phenotypes (genetic). The association of autoimmune diseases with certain haplotypes of the major histocompatibility complex (MHC), such as HLA-DR3 in systemic lupus, is striking. A detailed description of the potential mechanisms and the influential factors for autoimmune disease is beyond the scope of this section, and the reader is referred to recent reviews (59,90,100,107).

Autoimmune diseases have been associated with exposure to specific chemicals and, in particular, certain drugs. These diseases differ somewhat from their idiopathic counterparts in terms of their clinical spectrum or their specific immunological response (91). In addition, chemical/drug-induced autoimmune diseases normally remit when the agent is removed. In contrast to most agents, certain biologics, such as IFN-γ, while not themselves etiological agents for autoimmune disease, are believed to exacerbate preexisting disease (6). This occurs through their immunomodulatory properties rather than their ability to unmask novel antigenic determinants or to affect tolerance. The most common examples of drugs which produce autoimmune disease are those that cause hematoiogical disorders such as neutropenia, thrombocytopenia, and immune hemolysis and include a variety of antibiotics as well as anticonvulsants such as phenytoin (Table 31.7). Another autoimmune disease commonly associated with drug exposure is systemic lupus erythematosus (SLE). Approximately 10–20% of patients receiving procainamide and 5–20% receiving hydralazine develop drug-induced SLE (6). Individuals with the low acetylator phenotypes are associated with

Table 31.8
Examples of potential experimental and screening models for autoimmunity

Experimental models to study autoimmunity
Organ-specific autoimmunity
• Induced by immunization (EAE, AA)
• Spontaneous mice (NOD, transgenics)
• Toxicant-induced (streptozotocin, Cd)
Systemic autoimmunity
• Allogeneic reactions
• Neonatal thymectomy
• Spontaneous mice (NZM)
Models to evaluate the potential of xenobiotics to induce autoimmunity
• PLNA with reporter antigens
• Increased titers of antibodies to self constituents
• Examination of Ig complexes/deposits (immunohistochemical staining for immune complexes)
• Spontaneous animal models

Abbreviations: PLNA: popliteal lymph node assay; Ig: immunoglobulin; EAE: experimental autoimmune encephalitis; AA: autoimmune arthritis; NOD: nonobese diabetic (develop immune diabetes); Cd: cadmium; NZM: New Zealand Mixed (prone to develop lupus).

drug-induced SLE and the relative risk for developing autoimmunity from gold salts increases 32-fold in individuals who possess the HL-A DR3 allele (for review see Miller [71]).

Although not as well demonstrated as for drugs, considerable evidence exists that autoimmune diseases can also be induced by substances found in food or the environment. Regarding food consumption, strong associations have been found to exist between the consumption of iodine and autoimmune thyroiditis, L-5-hydroxytryptophan and scleroderma, and alfalfa seeds and SLE. Exposure to occupational agents has also been linked to autoimmune diseases. Scleroderma-like skin diseases can result from exposure to vinyl chloride, silica, or aniline derivatives, the latter presumably the active agent resulting in the "toxic oil syndrome" (46). Agents such as heavy metals and nitrofurantoin and organic solvents such as trichloroethylene are associated with SLE or glomerulonephritis. Like their idiopathic counterparts, xenobiotic-induced autoimmune diseases are also associated with certain genetic backgrounds. Experimental studies of mercury-induced autoimmunity in the Brown–Norway rat and B.10 mice suggests the same genetic influences apply in animals as in humans (82).

Techniques for Assessing Autoimmunity

Although there is general consensus within the toxicology community that there is a major need to screen drugs or chemical agents for their potential to induce autoimmunity, suitable validated models do not, as yet, exist. This is despite the fact that a large number of experimental animal and in vitro models are available to study the mechanisms of autoimmune disease. The primary reason for the lack of validated assays probably stems from the complexity of the disease. First, and as mentioned earlier, autoimmune disease is not one disease but a group of over 25 diseases affecting distinct organs, often through different mechanisms. Unless a common early process is identified, a single test would be unlikely to provide an adequate degree of concordance to be useful for predictive risk assessment. Secondly, although almost all diseases are affected by genetic and epigenetic factors, the degree of influence in autoimmune diseases is such that it could drastically alter the outcome of a test. Lastly, when using animal models, there is some uncertainty regarding what actually constitutes autoimmunity. This is also reflected by a lack of well-defined diagnostic tests for identifying autoimmune disease in humans.

Despite these challenges, attempts to develop predictive screening assays for detecting xenobiotic-induced autoimmunity have been undertaken in several laboratories. Currently, four screening approaches, which are clearly different from those used for mechanistic studies, have been suggested, each having received varying levels of attention (Table 31.8). These include: (1) monitoring changes in the frequency or rate of autoimmune disease using autoimmune-prone rodents (55); (2) identifying immunoglobulin complexes or immunoglobulin deposits using immunohistological procedures; (3) monitoring for increased levels of serum autoantibodies; and (4) the use of the popliteal lymph node assay (PLNA) with reporter antigens (1).

The successful use of exacerbating disease by chemical exposure in autoimmune-prone rodent species has been illustrated by administering streptozotocin in diabetic mice (58) or $HgCl_2$ in glomerulonephritis-prone rodents (42). Less studied has been the monitoring of Ig deposits or autoantibody production (48). The approach that has received the most attention is the PLNA with reporter antigens (1). In this model, the "autoimmunogenicity" of chemicals, like GVH reactions, is determined by their ability to stimulate specific IgG responses to TNP–Ficoll and TNP–ovalbumin in the popliteal lymph node. Although more an indicator for adjuvancy than for disease, it may prove extremely valuable as a "first-tier" screen, as it is independent of the nature of the neo-antigens and eliminates many of the potential genetic

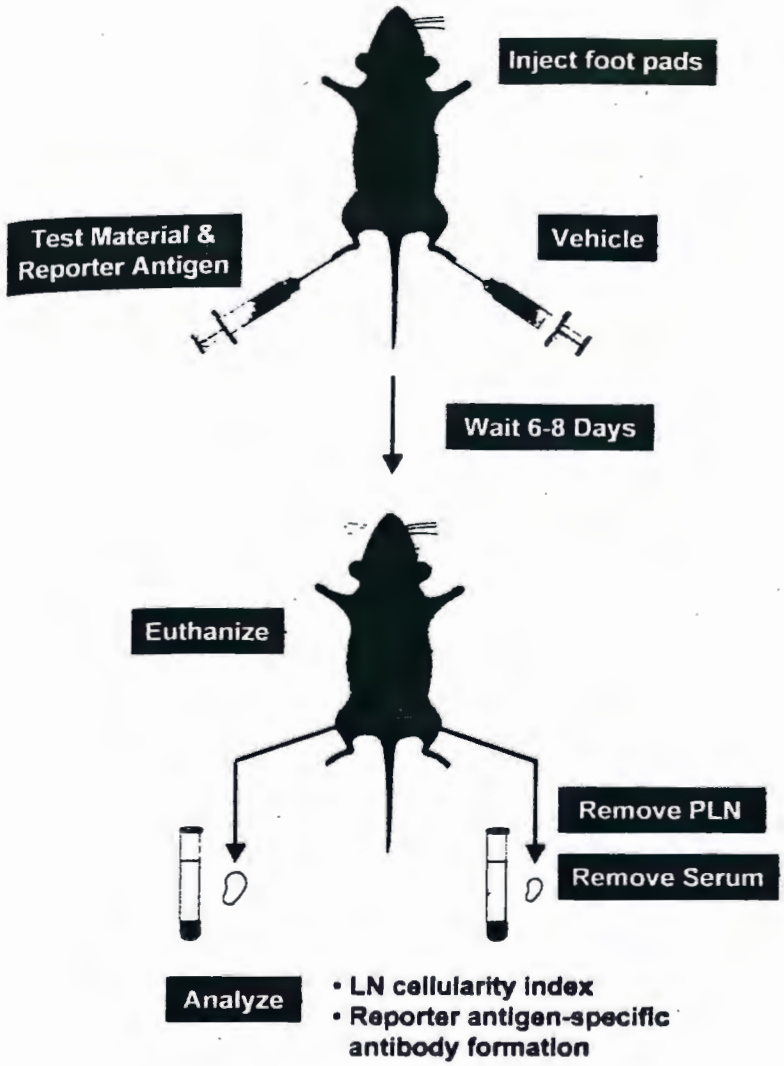

FIG. 31.8. Diagram of Popliteal Lymph Node Assay with Reporter Antigen.

confounders. In this assay the test compound is co-injected with 10 μg TNP-Ficoll or 10 μg TNP-ovalbumin subcutaneously into the right hind paw of BALB/c mice. The amount of test substance injected can be equimolar to a related compound or, if known, a concentration demonstrated to be stimulatory in the PLNA. Seven days following treatments, the thickness of the paw is measured using a micrometer and the draining popliteal lymph node (PLN) is isolated. Specific antibody-forming cells from the PLN are quantitated by any one of several methods such as ELISPOT. For memory responses, mice are similarly treated and then challenged in the right paw with 10 μg antigen, 4–5 weeks following the primary immunization, and antibody-producing cells from the PLN determined 6 days later. Serum samples can also be collected weekly following the primary immunization and serum antibodies determined using commercial procedures (illustrated in Figure 31.8).

EVALUATION OF IMMUNOLOGICAL CHANGES IN HUMANS

Introduction and Fundamental Concepts

Increased susceptibility to infectious disease, autoimmunity, and allergy is generally considered characteristic of altered immunity (116). In the case of increased susceptibility to infectious disease, the type of infectious agent or persistence of the infection often signals the nature of the immune defect. For example, individuals who suffer from recurrent infections with

Table 31.9
Classification of immune assessment tests for humans

I. Basic tests	General indicators	Procedures
Should be included with general health panels along with immune status questionnaire	• Assay methods are standardized among laboratories • Results are clinically interpretable • Reference ranges established	1. Complete blood count and differential 2. Acute phase proteins (CRP) 3. HI: serum IgG, IgA, IgM levels 4. CMI: delayed type skin test
II. Confirmatory tests	**More specific immune tests**	**Procedures**
Should be included when indicated by clinical findings or prior test results	• Assay methods are less standardized • Results are difficult to interpret • Reference ranges less well established	1. Surface marker analysis: assessment of phenotypes for major lymphocyte subsets (CD3, CD4, CD8, CD2). 2. HI: primary antibody response to immunogen; total serum IgE; secondary Ab response to proteins and polysaccharide antigens 3. Non-specific: auto-antibodies (ANA, DNA, mitochondria, RA); granulocyte/leukocyte function (oxidative burst) 4. Bank serum sample for additional analysis

encapsulated bacterial pathogens (e.g., Pneumococcus and *Haemophilus influenza*), often have associated B-cell deficiencies. These patients may present with a chronic sinopulmonary infection, bacteremia, or bacterial meningitis. In contrast, patients with defects in cellular immunity are predisposed to a wide variety of infections and opportunistic agents, including disseminated viral diseases caused by herpes simplex, varicella zoster, and cytomegalovirus; fungal agents such as mucocutaneous candidiasis; and parasitic agents including *Pneumocystis carinii*, an agent often associated with AIDS.

There are many clinical tests available to assess immune status in humans (91). A systematic approach based on simple screening procedures followed by more specialized tests of immune function usually provides the best overall assessment. This approach should include the functional evaluation of cellular immunity (T-cell), an antibody response (B-cell), and nonspecific resistance (e.g., PMN function). Recently, it has been suggested that immunization through vaccination, to elicit a primary immune response would, as in rodents, be the best criterion to establish immunotoxicity (110). Many of the screening tests employed in the past were established to define the location of a defect in either the B- or T-cell systems, or the effect of the defect on cell maturation or regulation. These tests may not be sufficiently sensitive or too specific to detect subtle or modest immune system changes that might result from exposure to toxic environmental agents. Sensitivity may also be impaired by the wide variation in test results in normal individuals. Therefore, a modest variation from the normal range in a single individual for one of these immunological tests might be expected in the normal population of subjects. Because of concern about individual variation, a confirmatory evaluation and a cross-sectional or longitudinal study design can be employed using randomized normal, non-exposed individuals. It is also imperative to document the nature of the exposure and to obtain a careful medical history that covers the clinical features of immune dysfunction.

The ability to identify individuals or populations affected by toxic compounds is also confounded ("assay confounders") by the signal-to-noise ratio of the test procedures, artifacts introduced by sample transportation, lack of well-established normative values for certain populations and age groups, lack of

Table 31.10
Comparative evaluation of the ICCVAM Peer Review Panel's revised LLNA database

Comparison	Number of comparisons	Sensitivity		Specificity		Accuracy	
		%	#	%	#	%	#
LLNA vs GPMT/BA	97	91	(62/68)	83	(24/29)	89	(86/97)
LLNA vs GPT	126	87	(81/93)	82	(27/33)	86	(108/126)
LLNA vs HUMAN	74	72	(49/68)	67	(4/6)	72	(53/74)
GPMT/BA vs HUMAN	57	70	(38/54)	100	(3/3)	72	(41/57)
GPT vs HUMAN	62	71	(42/59)	100	(3/3)	73	(45/62)

Abbreviations: LLNA = local lymph node assay; GPMT = guinea pig maximization test; BA = Buehler assay; GPT = nonstandard guinea pig tests; HUMAN = human maximization test (HMT) plus human patch test allergen (HPTA)

well-characterized or standardized reagents for some assays, different methods of data analysis between laboratories, and inherent interlaboratory variation. Clinical confounders for such studies include preexisting viral diseases, certain prescription and over-the-counter medications, alcohol and drug abuse, and preexisting HIV infection or AIDS.

Basic Test Panel

Complete Blood Count and Differential

The simplest screen to be included in the basic panel (Table 31.9) is a complete white blood cell count (WBC) and differential that is recommended for all individuals whose immune status is being evaluated. The data should be expressed as absolute lymphocyte count. Higher absolute lymphocytes counts should be expected in children than in adults and in certain ethnic groups. Lymphocyte counts consistently below 1500/mm^3 are indicative of lymphocytopenia and may signal a defect in the T-cell system. Lymphocytopenia can be associated with primary immune deficiency disease, but also can occur secondary to viral infections, malnutrition, severe stress, autoimmune diseases, and hematopoietic malignancy. When lymphocytopenia is repeatedly observed, a bone marrow biopsy is recommended as an important adjunct for exclusion of other diseases and for identification of normal plasma cells, pre-B-cells, or diagnosis of bone marrow depression or dysplasia. Individuals with lymphocytopenia should be reevaluated and further assessed for changes in CMI.

Humoral Immunity

The assessment of humoral or antibody-mediated immunity should involve the measurement of the concentration of serum immunoglobulins (IgG, IgM, IgA, and IgE), the assessment of antibody formation following immunization (i.e., using a standard recall antigen or childhood immunogen), or the measurement of naturally occurring isohemagglutinins (blood type antibodies). However, as indicated previously, measurement of primary antibody would represent the gold standard (110).

Immunoglobulin concentration. As a screen for HI competence in the basic panel, it is recommended that the serum concentration of the major immunoglobulin classes IgG, IgM, and IgA be measured. There are several standardized laboratory methods and reagents available for measuring these major classes of immunoglobulin. These methods include single-radial diffusion, double diffusion in agar gel, immunoelectrodiffusion, radioimmunoassay, enzyme-linked immunoassay, and automated laser nephelometry. Single-radial diffusion is the most widely used method. As a screen for HI competence in the basic panel, it is recommended that the serum concentration of the major immunoglobulin classes IgG, IgM, and IgA be measured. Because serum Ig concentrations may vary with age, ethnic group, and environmental factors, appropriate norms must be used with any type of population assessment. Patients with humoral immunodeficiency can manifest a decrease in all Ig classes or only in a single class or subclass.

Cell-Mediated Immunity

A complete blood count with differential is one of the least expensive and most comprehensive ways to quickly survey for the presence of absolute number of PMN and lymphoid cells in the circulation. In addition, several tests are commonly used in clinical medicine to specifically assess cellular immunity. These include tests that identify delayed-type skin reactions, flow cytometry to enumerate T-cells, B-cells, and T-cell subsets, and quantitative cytokine production.

Delayed-Type Skin Testing. Skin testing is a commonly used procedure (Basic Tests Panel, Table 31.9) to assess cellular immune competence because delayed cutaneous hypersensitivity, a localized immunological skin response, depends on functional T-cells and the production of inflammatory cytokines. Antigens commonly employed to elicit a positive skin response include: purified protein derivative PPD of mumps, trichophyton, *Candida*, tetanus, or diphtheria. These antigens usually are employed in a panel and are administered by intradermal injection at the appropriate dilution. Skin responses are read at 48 and 72 h for maximal diameter of erythema and induration. The test is not considered very sensitive unless very severe immunosuppression is suspected, which is unlikely to occur.

Confirmatory Tests

Some of the tests that follow are less well established or not as well standardized for routine application, but may have utility for a confirmatory or research application.

Specific Antibody Assessment

In this procedure the antibody response is measured to a primary, novel antigenic challenge or to specific recall antigens to which most normal adults are commonly immunized following re-immunization (Confirmatory Tests, Table 31.9). Examples of recall antigens include diphtheria–tetanus and poliomyelitis. For diphtheria–tetanus vaccine and poliomyelitic vaccine it is recommended that blood be taken for antibody determination two weeks after the last immunization. Another approach is to measure the antibody response following a primary immunization with a novel antigen, such as the *Haemophilus influenza* capsular polysaccharide polyribose phosphate (PRP) or hepatitis B.

Phenotypic Analysis by Flow Cytometry

Modern flow cytometers have multiple photomultiplier tubes (four or more) and are capable of measuring three-color fluorescence, 90-degree light scatter, and forward light scatter. When highly specific fluor-labeled monoclonal antibodies are used in these instruments, very quantitative measurements can be made of T-, B-, and T-cell subsets. The most commonly used procedure for processing peripheral blood samples for immunofluorescence is first to stain an aliquot of whole blood with fluorescent-conjugated monoclonal antibodies, and then to lyse the erythrocytes. The proportion of circulating T-cells is then determined by immunofluorescence with fluor-labeled CD2 or CD3 monoclonal antibodies in a flow cytometer. Normally, T-cells constitute 55–80% of peripheral blood lymphocytes. Normal values reported for absolute numbers of circulating T-cells are 590–3090/mm^3 for individuals older than 18 months (24). If an immune defect is suspected, the ratio of CD4 to CD8 T-cells can also be beneficial. Although this method is quite quantitative, the ability of this test method to detect subtle immune changes in populations of individuals has recently been challenged (43).

Nonspecific Measurements

Neutrophil Function. Neutropenia may be observed and has many causes, often associated with bacterial abscesses and bacterial infections. Apart from neutropenia, there are also defects in PMN and monocyte function that contribute to increased susceptibility to bacterial infections. The measurement of nitroblue tetrazolium dye reduction by actively phagocytosing PMN is a method that should be considered if a PMN defect is suspected.

Autoantibodies. It is often stated that the immune system is established on a principle of self/non-self recognition. In some cases tolerance of self antigens breaks down and autoantibodies are produced which, in some cases, are manifested by autoimmune disease. Antibodies to cellular components and nuclear antigens (ANA, DNA, mitochondria) and to rheumatoid factor (RA) and their frequency in a population may reflect an immune alteration. Standardized diagnostic kits are available to detect the presence of these autoantibodies in sera. It is a good practice to establish a freezer bank of an aliquot of each test subject's sera for later evaluation when new research questions or test methods are developed.

RISK ASSESSMENT CONSIDERATIONS

Data used in risk assessment for immunotoxicology are derived primarily from animal toxicology studies. When data are available, epidemiological or controlled clinical exposure studies take precedence. The results obtained from in vitro studies, structure activity relationship (SAR), or mechanistic investigations are used normally as supportive information. Mechanistic studies, however, are important, as without them the "10-fold classical defaults" in the risk assessment process, such as inter-individual variability and species differences, are assumed valid (96). In toxicology, human clinical studies, of course, represent the gold standard. Questionnaires offer some value, particularly as they provide information on reportable diseases such as autoimmunity. They provide less utility in studies of immunosuppression. The National Institute for Occupational Safety and Health (NIOSH) has recently prepared questionnaires directed to immunotoxicology (5).

Animal studies that evaluate agents for immunosuppression are becoming increasingly more common

in risk assessment. Because of the complexity of the immune system, the initial strategy devised by immunologists working in toxicology was to select and apply tiers of assays to identify hazardous agents (64). Among these are consideration of induced changes in the weight, composition, and histology of lymphoid organs, immunophenotyping, generally performed by cytometric analysis, and various functional assays. The latter were designed specifically to evaluate B-cell, T-cell, MØ and NK cell function to in vitro or in vivo antigen challenge. These tests were usually accompanied by an additional tier which included host resistance assays to help establish whether the immune changes observed translated into increased susceptibility to infectious or malignant diseases. This testing battery has been conducted in a number of laboratories and the results analyzed to improve the testing configuration in order to accurately identify immunosuppressive chemicals with the least number of tests and to help establish the quantitative relationship between immune function and host resistance (65,66).

Although a number of limitations exist, the following conclusions were drawn from these analyses:

(1) Examination of only two or three immune parameters is required to accurately predict an immunosuppressive agent. In particular, results from the T-cell-dependent antibody response appear to provide excellent concordance;
(2) Altered host resistance is closely associated with immune function although changes in immune function often occur at lower dose levels;
(3) No single immune test is predictive for altered host resistance although some tests showed relative good concordance (>70%);
(4) Logistic and standard modeling, using a single data set indicated most immune function-host resistance relationships follow a linear-quadratic model rather than a true threshold.

This would suggest that even very small changes in the immune system can alter host resistance, although due to the frank nature of most susceptibility tests, a large group size might be required to achieve statistical significance. Thus, at an individual level, small changes in immune function would likely have little impact in combating infectious disease. However, such changes may have significant impact when considering large populations or those already at increased risk such as the elderly or the very young. The consequence of an immune alteration may be difficult to definitively establish in humans because of the inability to detect small increases in the frequency or severity of infectious diseases resulting from the immune changes is exceedingly difficult.

As ethical considerations usually prevent the use of human patch testing for establishing the potential of agents to induce allergic contact dermatitis, animal models, particularly the Buehler occluded and Magnuson–Kligman maximization tests in guinea pigs have been used as predictive tests. Several graded doses of antigen may be examined simultaneously and an entire dose-response curve can be generated by comparing skin reactions in individual animals. However, it is expensive to purchase as well as maintain guinea pigs, there are few inbred strains, and immunological reagents are not widely available. Furthermore, there is some suggestion, although never fully substantiated, that these models are overly sensitive when compared to studies in humans and, thus, may present false positives.

A more quantitative and objective assay than the guinea pig tests, the LLNA (49) has successfully undergone a series of exercises which support its use as a "stand-alone" test to assess allergic contact hypersensitivity. Even though the strengths and weaknesses of this test have been discussed earlier (see section on murine local lymph node assay), much like tests for immunosuppression (64–66), the assay has undergone a series of examinations to provide technical refinement and assess inter- and intra-laboratory reproducibility, as well as relative sensitivity and specificity, referred to as concordance (50–52,76). Concordance for a new assay should be established to previously used test models as well as to available human data. Such data for the LLNA are shown in Table 31.10, where the assay is first compared to guinea pig tests and then to human studies. The analyses indicate that the LLNA is highly comparable to guinea pig tests (concordance almost 90%), but only about 70% accurate when compared directly to human studies. Because this is similar to results obtained when guinea pig tests are compared to human studies, in terms of risk assessment, the LLNA can be used in lieu of guinea pig tests, but an alternative assay which could provide higher concordance with humans would be desirable.

In contrast to predictive tests for allergic contact hypersensitivity, the identification of proteins and chemicals to induce respiratory hypersensitivity is in its infancy. As these tests are difficult to undertake, often involving respiratory exposure and lung function tests, efforts to develop and validate new methods are limited. Although the guinea pig has significant immunological differences compared to humans (e.g., IgG1 versus IgE reagenic antibodies), it appears to be a predictive model for humans given the limited comparative data available, and has been used to test for high and low molecular weight sensitizers. This test requires a systemic or inhalation sensitization phase and an aerosol challenge and both immediate and delayed-onset responses are measured, although this does not distinguish between nonspecific pulmonary hyperreactivity and specific immune responses (47). The latter can be established

by examining sera for the presence of reagenic antibodies. An IgE test has been proposed for the prospective identification of chemical respiratory allergens in the mouse (20).

General agreement exists among the regulatory and pharmaceutical communities that predictive tests for autoimmunity or systemic allergy are in most need of development to improve risk assessment in immunotoxicology (19). Although many models exist to study autoimmune processes, they do not readily lend themselves to use in risk assessment because they do not consider the multifactorial nature of the disease. To improve the risk assessment process, screening models need to be developed and validated, not only incorporating mechanistic information into the assessment process, but allowing for the consideration of the genetic, physiological, and environmental influences that lead to the loss of self-tolerance, autoimmune disease, or systemic allergy. Despite the challenges in developing such screening tests, the considerable amount of data generated by immunologists and pharmacologists pertaining to basic mechanisms of chemical-induced autoimmune diseases have provided a conceptual framework which allows the establishment of potential structure–activity relationships. These structure–activity relationships are by no means definitive and, as the database increases, no doubt some will not be supported while others will be added. In all cases these relationships are supported by basic understanding of immunological and pharmacological processes. For example, estrogens are known to be a major factor in classical autoimmune diseases presumably due to their ability to stimulate certain components of the immune system (37), and, as such, agents of concern.

Laboratory studies have also shown that thymolytic chemicals, such as cyclophosphamide and cyclosporin A, can induce autoimmunity when given neonatally by altering normal patterns of autoreactive T-cell deletion (94). In this respect, the thymus has been shown to be a target for many toxic chemicals. As in the case of halothane, chemicals that form protein adducts or damage tissue in such a way to allow expression of cryptic determinants would provide novel host antigens which could now be recognized by T cells. Agents that have adjuvant activity, or biologicals which stimulate certain cytokines, may shift the balance of Th1 and Th2 cells and allow exacerbation of preexisting autoimmune disease (12). Common features associated with many drugs that induce autoimmune diseases are that they serve as myeloperoxidase substrates and/or cause changes in methylation (29). The explanation for the latter association is less clear, but may require identification of the specific antigenic epitopes responsible for the autoimmune response. In the case of the association with myeloperoxidase substrates, it has been suggested that many of the chemicals require metabolism in proximity to immune cells in order to be antigenic, and immune cells such as monocytes contain high levels of myeloperoxidase.

CONCLUSIONS AND FUTURE DIRECTIONS

The discipline of immunotoxicology has grown in importance in toxicology since its inception in the mid-1970s. It has progressed from the early identification of immunotoxic chemicals, through the validation of sensitive and quantitative assays that serve as biomarkers of immune system alterations in animals and humans. More recently, academic, industrial, and government scientists have taken a more mechanistic approach to define how therapeutic and environmental agents alter immune function at a cellular and molecular level. Immunotoxicity data (e.g., hypersensitivity) now play a key role in establishing health standards and defining permissible levels of toxic chemical exposure in humans. Most new pharmaceuticals are being studied for immunotoxicity on a case-by-case basis to define their margin of safety (19).

What is still needed is better correlation between animal data with known immunotoxicants and epidemiological or clinical studies to ascertain the predictive value of the immune evaluation methods for human populations that may be occupationally or environmentally exposed. Well-controlled studies are still needed in human subjects exposed to environmental chemicals to establish concretely the relationship between documented exposure and immune-mediated effects. With pharmaceuticals where exposure is well documented, the correlation of the prediction value of animal studies for immuno-alterations (e.g., immunosuppression or allergy) in humans are more clearly defined.

QUESTIONS

1. The immune system exists to protect the body against:
   a. Specific invading pathogens and microorganisms.
   b. Neoplastic cells.
   c. Non-self antigens.
   d. Transplanted foreign antigen.
   e. All of the above.
   f. A, B, and D.

   Answer: e

2. Which of the following statements is false?
   a. Macrophages and leukocytes are types of phagocytic cells derived from the bone marrow.

b. Cell-mediated immunity represents a type of nonspecific immune response.
c. The two major mechanisms of immunity are nonspecific and specific.
d. Humoral immunity is associated with the production of antibody.
e. Pluripotent stem cells are found in the bone marrow and give rise to megakaryocytes and lymphocytes.

Answer: b

3. Which of the following statements are true?
a. The primary lymphoid organs are represented by the thymus and bursa-equivalent tissues.
b. Lymphoid tissue is derived from ectoendodermal junctional tissue.
c. Secondary lymphoid tissue is found in the spleen, lymph nodes, gut-associated lymphoid tissue (GALT), and bronchial-associated lymphoid tissue (BALT).
d. a and c
e. all of the above

Answer: e

4. Autoimmunity is best defined as:
a. an immune response to normal components of the host.
b. being mediated by IgE.
c. can best be measured in guinea pigs.
d. reflects a single organ.

Answer: a

5. Immunotoxicity assessment is most often conducted using:
a. epidemiology studies.
b. in vitro studies.
c. animal studies.
d. combinations of SAR and clinical trials.

Answer: c

6. Chemical- or drug-induced autoimmunity differ from their idiopathic counterparts in that they:
a. usually remit when the drug is withdrawn.
b. only target the kidney.
c. only target blood elements.
d. are more common in females.

Answer: a

7. Validation of animal models for immunotoxicology studies requires:
a. laboratory validation.
b. establishment of specificity.
c. establishment of sensitivity.
d. reproducibility.
e. all of the above.

Answer: e

8. The most appropriate animal model for evaluating immunotoxicity appears to be:
a. rodents.
b. mini-pigs.
c. guinea pigs.
d. nonhuman primates.

Answer: a

9. Allergic reactions to drugs may result from:
a. direct antigenicity of the drug moiety.
b. activation of complement proteins.
c. haptenation of self proteins by the drug.
d. bone marrow ablation.

Answer: a and c

10. Macrophages are an important potential target of immunotoxicants because:
a. They are capable of metabolizing xenobiotics.
b. They are potent immunoregulatory cells.
c. They secrete large quantities of inflammatory antibodies.
e. a and b.
f. b and c.

Answer: e

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