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Immune Markers in Epidemiologic Field Studies

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Introduction

The mammalian host defense system is a complex network of cells and mediators with recognition and response functions throughout most tissues of higher organisms. The primary functions of the host defense system are repairing injured tissue, identifying and removing foreign substances, destroying or containing infectious agents, and, in some cases, eradicating cancer cells. These functions are carried out through both nonspecific mechanisms of innate or natural immunity and through specific mechanisms of acquired immunity that develops as the organism encounters environmental agents or antigens. The term "immune system" will be used to refer to all components of nonspecific innate immunity and antigen-specific acquired immunity, since their components and activities are invariably intertwined.

Over the last few decades, the cellular and molecular basis for many host defense functions has been uncovered through the use of emerging laboratory technologies. The use of these laboratory methods to detect biologic markers in epidemiologic studies already has provided critical information in many areas of basic and public health science. Their future potential is even greater. However, this potential can be realized only by applying scientifically valid and logistically feasible markers in field studies. The absence of either characteristic can result in flawed or failed research through the use of markers that are not measured properly or cannot be interpreted with respect to exposure, susceptibility or health effects (NRC, 1992).

This chapter outlines the major considerations involved in using laboratory tests for immune markers in epidemiologic studies. Although it cannot

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provide a complete description of the host defense system, for which many excellent texts and reviews are available (e.g., Hood *et al.*, 1990; Roitt, 1990; Paul, 1992), it discusses many of the cellular and molecular components of the immune system and their use as biologic markers.

General Aspects of Biologic Markers of the Host Defense System

The benefits and limitations of immune markers in epidemiologic studies may be appreciated best by understanding their relationship to the basic biology of host defense function. Biologic considerations are especially important for the effective use of immune markers in epidemiologic studies because of the dynamic and variable nature of the immune system, its complex organization, the large number of components, and its decentralized location in the body. Since the immune system is designed to change with environmental exposures, markers that are adaptive must be distinguished from those that are pathognomic. Moreover, pathologic alterations of the immune system may result in disease through over-reactivity (autoimmunity, hypersensitivity) or impairment (immune deficiency). Familiarity with this range of normal and abnormal perturbations is required before tests for markers of the immune system can be used effectively. The numerous confounding factors that can influence the immune response also must also be considered.

Almost all markers used as tests of immune status are active participants in protective, regulatory, or pathogenic processes of the immune system. This direct biologic relevance provides special opportunities to learn about the mechanisms of host injury and response through tests for immune components. However, this relevance also can make interpretation more difficult, since physiologic interactions among markers can mask changes or create internal confounders. Moreover, the continual changes that occur as the immune system senses and responds to environmental influences make the normal range of variability for immune constituents very large among individuals and even within individuals over time. Finally, the immune system of each individual continues to evolve throughout life, its course determined by a combination of inherited influences and acquired exposures. Normal ranges of immune cells and mediators therefore may be very broad in the general population, and relatively clustered in genetically or environmentally restricted populations. This characteristic has important implications for the use of immune biomarkers in epidemiologic studies, in which subpopulations may have their own "normal" immunologic values.

A final point for emphasis concerns the biologic material used to test for immune components. Human studies often are limited to sampling peripheral blood, which does provide a convenient source of cells and mediators. However, peripheral blood by no means represents the immune system as a whole. Host defense activities take place primarily in the lymphoid tissues (spleen, lymph nodes, epithelial-associated lymphoid tissues) and in intersti-

tial tissue at local sites of injury and infection. Cell traffic and recirculation through the blood is controlled carefully (Figure 16.1), and activated cells and molecules are removed quickly. In contrast, some cells and mediators persist within or outside the bloodstream for days and even years (Figures 16.1 and 16.2). Therefore, blood samples often may represent an inappropriate surrogate of the actual immune system components being evaluated.

Components of the Host Defense System

Table 16.1 presents a summary of the major molecular and cellular constituents of the host defense system. The following synopsis [adapted from Vogt (1991)] elaborates briefly on these components.

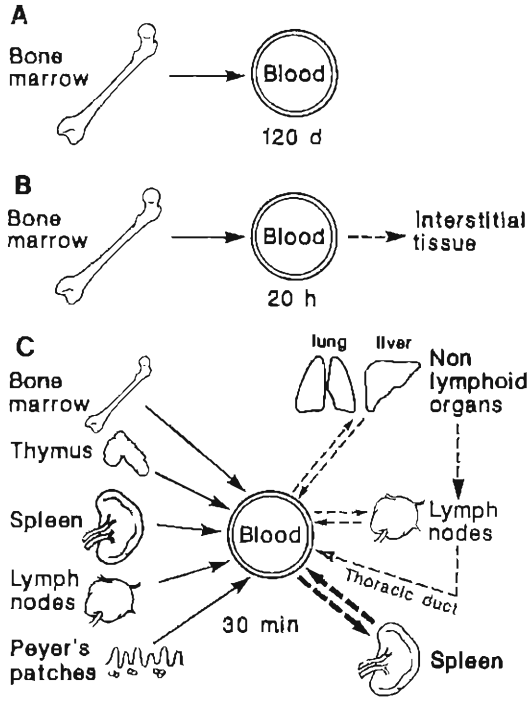

FIGURE 16.1 Comparison of (A) erythrocyte, (B) granulocyte, and (C) lymphocyte kinetics. Lymphocytes differ greatly from erythrocytes and granulocytes because they are produced in many organs. They have a very short mean transit time through the blood and can migrate. Interpretations based on the number of lymphocytes in the blood are much more difficult to make than those based on the number of erythrocytes or granulocytes. The solid arrows indicate the organs releasing newly formed lymphocytes into the blood; the broken arrows indicate migration routes. Note the outstanding role of the spleen in lymphocyte migration. (From Westermann and Pabst, 1990.)

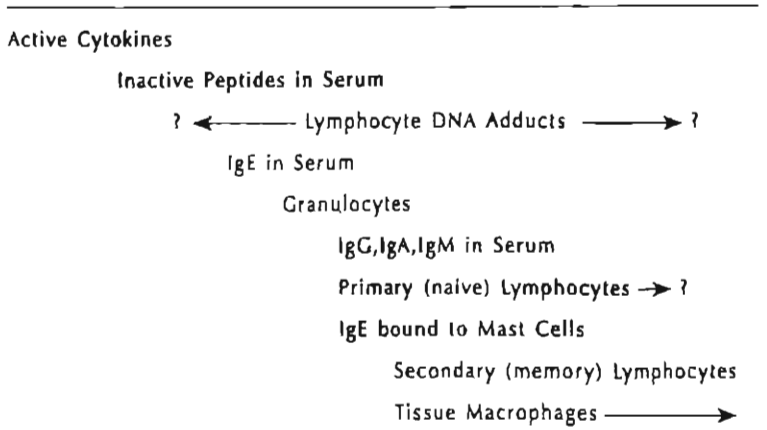

FIGURE 16.2 The persistence of immune markers varies widely among the different humoral mediators and cellular components, and may also depend on conditions within the organism. Highly reactive humoral mediators that act locally (such as cytokines) often are inactivated within minutes of their formation, but their inactivation products may circulate in serum much longer. Serum IgE is cleared more quickly than serum IgG, but IgE bound to mast cells or basophils persists much longer than any serum immunoglobulin. Primary lymphocytes with new specificities do not survive long, but if they are stimulated by contact with antigen, their clonal offspring colonize the organism and persist indefinitely.

Chemical Mediators of the Immune System

Many of the defense and regulatory functions of the immune system are conducted by chemical mediators released from its cells. Antibodies (also called immunoglobulins, Igs) are the only antigen-specific mediators; they are secreted by stimulated B lymphocytes and comprise several major classes with different functional capacities. IgM and IgG antibodies, the most general-purpose types, facilitate phagocytosis, antigen clearance, and destruction of parasites. IgA antibodies are secreted at the mucous membranes, where they help prevent attachment and invasion by microbes and parasites that come in contact with these surface tissues. IgE antibodies, bound to the outer membrane of mast cells and basophils, help initiate immune responses and are involved particularly with immunity against worms and mites; they are also the antibodies responsible for allergic reactions such as hay fever.

Cytokines are extremely potent peptide molecules that activate or suppress target cell populations that express the appropriate receptors. More than a dozen immune cytokines (many called interleukins) have been identified as participants in the complex network of immune regulation (Figures 16.3 and 16.4).

Complement is one of several plasma proteins involved with acute nonspecific responses to tissue injury and invasion. Complement is actually a

TABLE 16.1 Major Components of the Host Defense System

Molecular mediators	
Component	**Function**
Proteins	Viral inactivation; antigen clearance; complement activation; opsonization
Immunoglobulins (antibodies)	
Cytokines	Intercellular signaling
Interferons	
Interleukins	
Growth factors	
Complement (interacting with the kinin, fibrin, and plasmin systems)	Parasite destruction; chemotactic stimulation; acute inflammatory reactions
Heat shock	Protein binding and preservation; cross-reactive antigenicity
Lipid-derived	
Prostaglandins	
Leukotrienes	Intercellular signaling

Molecular cell surface receptors	
Component	**Function**
Immunoglobulins; T-cell antigen receptor	Specific antigen recognition on lymphocytes
Immunoglobulin E	Specific antigen recognition on mast cells and basophils
Class I histocompatibility proteins	Cell–cell interactions
Class II histocompatibility proteins	
Immunoglobulin-related proteins (CD4, CD8, β_2-Microglobulin)	
Cytokine receptor proteins	Receptors for the various cytokines
Cell adhesion molecules	Cell traffic and migration control

Cell lineages and subsets	
Component	**Function**
Granulocytes	
Neutrophils	Phagocytosis and antigen destruction
Eosinophils	Parasite destruction; regulation
Basophils	Parasite destruction; regulation
Monocytes/macrophages	Phagocytosis and antigen destruction; antigen processing and presentation; regulation
T-Lymphocytes	
Helper (CD4) cells	Activation of antigen-specific responses
Suppressor (CD8) cells	Suppression of antigen-specific responses
Cytotoxic (CD8) cells	Destruction of virus-infected and neoplastic cells
B-Lymphocytes	Antibody production; regulation
Plasma cells	
Natural killer cells (NK)	Destruction of virus-infected and certain neoplastic cells
Dendritic cells	Antigen presentation
Platelets	Blood clotting; activation

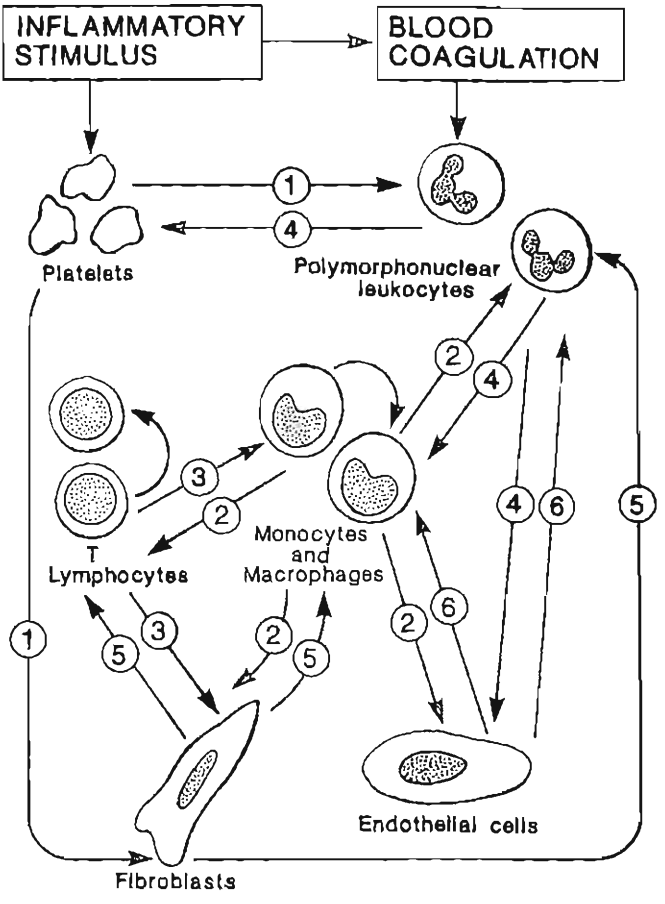

FIGURE 16.3 Diagrammatic representation of the complex series of interactions between numerous cell types, depicted as a series of arrows, involved in such events as inflammation, wound healing, tissue repair and tissue formation. These interactions are made possible through the release and recognition of cytokines acting in autocrine and paracrine fashions. The arrows are numbered and the cytokines involved in the interactions are listed below illustration. (From Pugh-Humphries *et al.*, 1991.) (1) *Platelet-derived factors:* PDGF-A and -B, TFG-β, bFGF; (2) *Monocyte/macrophage-derived factors:* PDGF-A and -B, TFG-α and -β, IL-1, IL-6, IL-8, TNFα, INFα, INFβ, GM-CSF, bFGF, EFG, IGF-1; (3) *T-helper-derived factors:* IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF, IFNγ, TNF-α and β; (4) *PMN-derived factors:* Many noncytokine factors, including arachidonic acid metabolites; (5) *Fibroblast-derived factors:* IL-1, IL-6, PDGF-A and -B, GM-CSF; (6) *Endothelial-cell-derived factors:* IL-1, IL-6, TNFα, PDGF-A and -B, GM-CSF, bFGF.

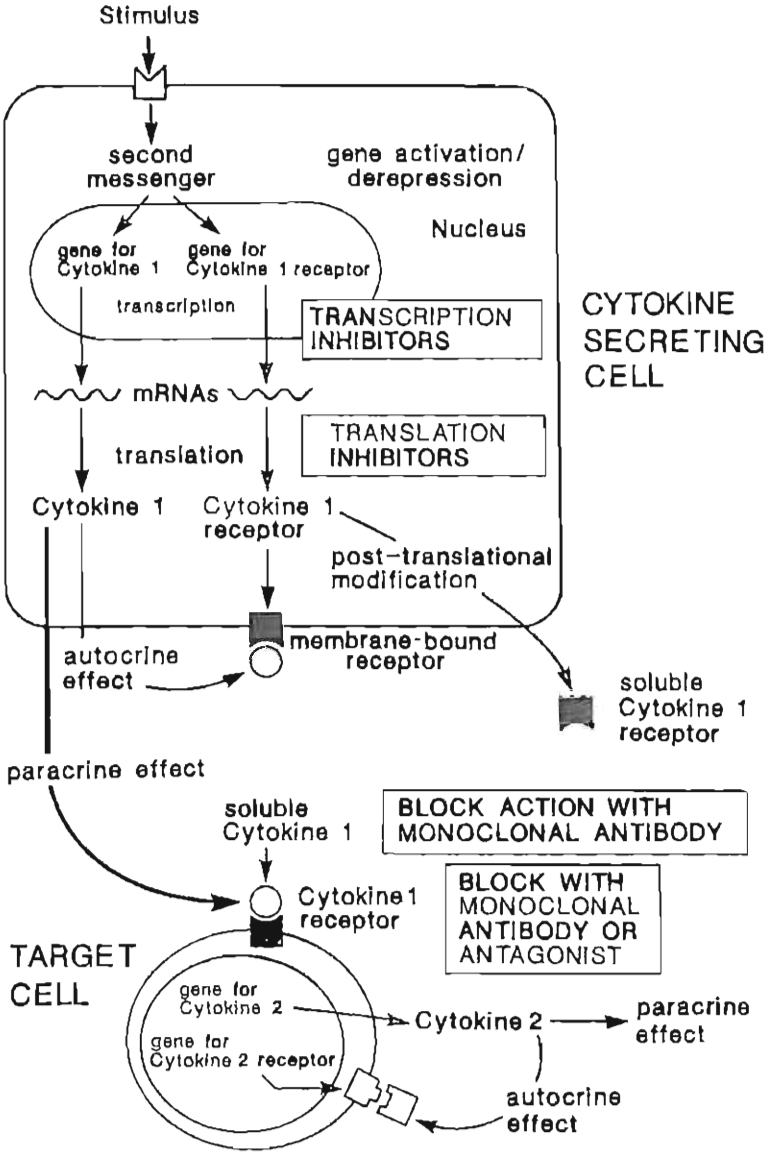

FIGURE 16.4 Diagrammatic representation of the actions of cytokines on cells within the cytokine network. The activation of cytokine and cytokine receptor genes within cytokine-secreting cells, and the sites of interference with gene expression using transcription and translation inhibitors, are indicated. Cytokine-secreting cells not only release cytokines which can have autocrine or paracrine actions, but they can also synthesize cytokine receptors, parts of which can be cleaved extracellularly and released as soluble cytokine receptors. The interactions of cytokines with cells can be blocked using antibodies directed either against the cytokines and/or against their receptors. Cytokines can induce the release of other cytokines within target cells through activation of the relevant cytokine genes. (Adapted from Pugh-Humphries *et al.*, 1991.)

cascading system of different protein molecules that can be activated by a variety of stimuli, including antigen–antibody complexes, blood clotting proteins, and other mediators. Complement activation products have a number of activities, including chemotaxis, clearance, and destruction of cells. Other such "acute phase" serum proteins include transferrin and plasmin.

Several nonprotein molecules are also important immune mediators. They include different lipid-derived chemicals (such as prostaglandins) that have a wide variety of effects on many different tissues, including the activation or suppression of immune cells and the dilation or constriction of blood vessels and airways. Histamine, which is stored in the granules of mast cells and basophils, causes dilation and leakage in small blood vessels and has effects on immune cells and other tissues; it is responsible for many of the symptoms of allergy.

Several other chemical mediators influence cells of the immune system, although they are not central to its function. These include catecholamines (such as adrenalin), endorphins, and insulin.

Cells of the Immune System

Most of the several different types of cells that constitute the immune system spend at least part of their lifetime in the peripheral blood, where they constitute the white blood cells or leukocytes. The major types of leukocytes are lymphocytes, monocytes, and granulocytes.

Lymphocytes (B cells and T cells) are the specific recognition cells of the immune system. Each family (clone) of lymphocytes has unique recognition molecules on its surface. If the lymphocyte is activated by recognizing a foreign protein (antigen), a specific immune response is initiated. Activated lymphocytes proliferate and engage in a variety of host defense functions, such as producing antibody (B cells) and killing virus-infected cells or regulating immune activities (T cells).

Monocytes are immature cells that differentiate into macrophages after they emigrate from the blood. Macrophages are distributed throughout many tissues including the lung, liver, skin, brain, and bone marrow. Their innate activities of phagocytosis and digestion are nonspecific, but they become part of the specific immune response when they "present" processed fragments of foreign protein to lymphocytes.

Granulocytes are important auxiliary cells with activities that are critical to host defense but also may contribute to disease processes. Neutrophils, like macrophages, are avid phagocytes, but are short-lived and less versatile. Mast cells, basophils, and eosinophils are involved in immunity to larger parasites such as worms, and are the primary participants in the allergic responses to pollens, foods, and other substances. They also appear to be involved in inflammatory reactions to certain toxic and sensitizing chemical exposures (Vogt *et al.*, 1984).

Laboratory Measurements of Immune Cells, Mediators, and Functions

Laboratory tests can be used to measure the concentrations of many immune mediators, the types and numbers of immune cells, their functional capacities, and factors influencing disease susceptibility (Tables 16.2, 16.3). However, measurements for most immune components are not well standardized, so the proper choice and evaluation of analytical methods is critical to the success of epidemiologic studies using such measurements. The number and type of tests employed depend on the purpose of the field study and on indications that an immunologic end point (biomarker) may be involved. Clearly, no single immunologic test can evaluate the entire immune system; rather, a comprehensive panel of assays should be selected carefully. Method evaluation has become even more important since many assays have evolved from simple dichotomy (positive/negative results) to semiquantitative (weak/

TABLE 16.2 **Immune Markers Associated with Health Effects and Exposures**

Effects or Exposures	Marker
Inflammatory disease	
Autoimmune disorders	Antibodies to tissue antigens; histocompatibility genotypes
Allergic (hypersensitivity) reactions	Antibodies to environmental antigens; *in vivo* reactions (e.g., skin tests)
Immunoproliferative disease	
Chronic lymphocytic leukemia	Peripheral blood lymphocyte counts
Multiple myeloma	Monoclonal serum immunoglobulin
Lymphoma	Monoclonal lymphocyte infiltration
Infectious disease	
Parasitic	Antibodies to parasite antigens; peripheral blood eosinophil counts
Bacterial	Antibodies to bacterial antigens; peripheral blood granulocyte counts; *in vivo* reactions (e.g., TB skin tests)
Viral	Antibodies to viral antigens; cellular cytotoxic responses; peripheral blood lymphocyte subset counts
Neoplastic disease	
Solid tumors	Antibodies to tumor-specific antigens
Environmental exposures	
Infectious agents	Antibodies and cellular responses to specific antigens
Other biologic antigens	
Chemical antigens	
Volatile organics (benzene)	Peripheral blood leukocyte counts
Volatile irritants	Leukocyte infiltration of mucous membranes

TABLE 16.3 Susceptibility Factors

Factors	Immune related		Nonimmune
	Antigen specific	Nonspecific	
Genetic	Antibody variable-region genes T-cell receptor variable-region genes	Antibody constant-region genes T-cell receptor constant-region genes Histocompatibility genes	Metabolism related Gender related Other
Environmental	Sensitizing exposures	Adjuvant inflammatory reactions	Behavioral (smoking, etc.) Concurrent illness Neurogenic/ psychogenic

strong reactions) and fully quantitative (concentration units). The important parameters are analytical accuracy, precision, sensitivity, and specificity.

Analytical accuracy, the extent to which the measurement gives the "true value" for the analyte, must be evaluated to prevent the false impression of biologic differences actually caused by bias among methods. Accuracy cannot be assessed for most immune markers because no true reference standards exist. However, some indication of accuracy is provided by the degree of consensus between values on reference materials obtained from different laboratories and methods. Consensus evaluation of reference materials is of critical importance to epidemiologic studies, since only unbiased methods can be compared among different studies and different times, or pooled into databases to determine reference ranges and long-term predictive values. Unfortunately, consensus evaluation is available for only a few immune markers, such as serum immunoglobulins and complete blood counts. Even these standard reference values are often subject to method-related biases and changes with time (see Chapter 4). Reference ranges based on consensus evaluation are beginning to emerge for the major lymphocyte subsets measured by flow cytometry (Kidd and Vogt, 1989), although considerable work is still needed in this area. Little, if any, consensus evaluation exists for functional immune assays such as cell proliferation or cytoxicity.

Analytical precision, the reproducibility of a measurement, is a critical aspect of laboratory assays and becomes even more important in the absence of an acceptable accuracy base. Imprecision generally indicates some inher-

ent weakness in the assay methodology and lowers predictive value by blurring the distinction between true differences in results. The smaller the true differences between distributions in the populations tested, the more detrimental is the effect of imprecision on predictive value. The broad distributions of many immune parameters make small differences difficult to detect. Analytical imprecision can add to this problem by concealing biologically significant differences between groups. Unfortunately, many tests for immune markers (particularly cellular and functional measurements) have poor reproducibility. In any event, study designs for any biologic marker should include assessment of analytical imprecision to account for all the sources of variability in the final distributions of marker results.

Analytical sensitivity may be considered the lowest level of an analyte that can be measured with reasonable precision and accuracy. If the laboratory method is not able to detect the analyte at levels important to pathophysiology, the test is likely to have little value.

Sensitivity is especially important for immune markers because most testing is performed on peripheral blood, whereas host defense functions are based primarily in localized tissue reactions. Although active immune mediators and cells may become very concentrated in a microscopic area of tissue, the overall amount of analyte in peripheral blood or serum samples is often too low to measure. In fact, activated cells and molecules are removed from circulation rapidly to prevent harmful systemic reactions. Analytical sensitivity, therefore, becomes a critical issue in developing tests to probe active immune processes.

Analytical specificity is the extent to which other influences alter the result of a biomarker measurement. If the measurement of the biomarker is subject to interference from other substances, the results will not be correct. Interference is a common problem with assays in which antibodies are measured or used as reagents for other analytes because of nonspecific binding and cross-reactivity. For instance, some recipients of a standard influenza vaccine have shown a false-positive serologic reaction for antibodies to HIV. If not detected, such false results obviously can be disconcerting to the individuals tested and also can lead to inappropriate public health decisions. For this reason, assays that measure only antibody binding do not by themselves establish the presence of antigen-specific antibodies. Specificity must be documented by competitive binding assays and, preferably, by purification (or at least concentration) of the specific antibody population. Nonspecific influences also can interfere with *in vitro* and *in vivo* cellular assays. For instance, some skin reactions attributed to nickel hypersensitivity are more likely to be caused by nonspecific irritation (deBoer *et al.*, 1988; Staberg and Serup, 1988). Moreover, because of the potential for immune cross-reactivity between similar antigens (and even some dissimilar antigens), analytical specificity in immunochemical assays still does not prove biologic specificity.

Applications of Immune Markers for Determining Exposure, Health Effects, and Susceptibility

Exposure

Because the immune system responds specifically to a variety of foreign material, immune markers may be used to detect exposures to many environmental substances, including infectious agents, chemical and biologic materials that cause allergic reactions, and foreign tissue proteins from blood transfusions, organ transplants, or developing fetuses. The foreign material is called an antigen. A specific immune response may be demonstrated by (1) chemical assays that detect antibodies specifically bound to the antigen, (2) cellular function assays that show lymphocytes reacting specifically to the antigen, or (3) specific hypersensitivity reactions in tissues exposed to the antigen (e.g., skin tests).

Changes in host defense components may occur without specific immune responses to particular antigens. For instance, many chemicals will cause an irritative inflammatory response characterized by redness, swelling, heat, and pain, although no antigen-specific immune response occurs (Vogt *et al.*, 1984). Such nonspecific acute inflammatory activity might be detected by local tissue reactions or by increases of peripheral blood granulocytes and acute-phase reactant serum proteins. Other exposures (such as antiproliferative agents) may damage host components nonspecifically and suppress the functional capability of the system, as is true for cancer chemotherapeutic drugs. Tests for cellular and molecular events that accompany these nonspecific changes also may serve as markers of exposure.

Health Effects

Immune System Disorders

The most obvious health effects that might be revealed by changes in immune cells and mediators are those involving the immune system itself. Three general types of disorders of the immune system may have adverse health consequences: immune deficiencies, inappropriate immune reactivities, and unregulated immune proliferation.

Immune deficiency disorders are those in which the immune system fails to mount adequate protective responses against infection or certain forms of cancer. Depending on the nature of the deficiency, the health consequences can range from almost undetectable (increases in the incidence of mild infections) to life threatening (overwhelming sepsis). Immune deficiencies may be indicated by low or absent levels of serum immunoglobulins, low or absent numbers of immune cells, or decreased functional responses.

Immune reactive disorders are those in which immune activity damages host tissues because of inappropriate or poorly regulated responses. Again, depending on their cause and nature, such disorders can be very mild or very

severe. Common allergies are caused by inappropriate responses that release histamine and lipid-derived mediators. These allergic reactions often are directed against airborne antigens and may contribute to the pathogenesis of asthma. Depending on the causative antigen, *in vitro* tests for IgE serum antibodies may be good markers for allergies. Autoimmune diseases are debilitating immune reactive disorders in which the immune system reacts against its own body's tissues. Autoimmune reactions can damage the skin, liver, kidneys, various glands, joints, and other tissues, leading to diseases such as rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosis, thyroiditis, multiple sclerosis, myasthenia gravis, and some types of diabetes. Autoimmune reactions often are associated with antibodies that react to self-proteins in particular tissues or cell components, and can serve as excellent markers of health effects.

Immune proliferative disorders include lymphoma, multiple myeloma, and some types of leukemia. Like other forms of cancer, they involve the relentless expansion of one family (clone) of cells. However, since clonal expansion is normally an essential part of immune function, immune proliferative disorders often have distinctive characteristics that can be detected by their cell-surface protein phenotypes and molecular genotypes (Figure 16.5) (Waldmann, 1987; Fey *et al.*, 1991).

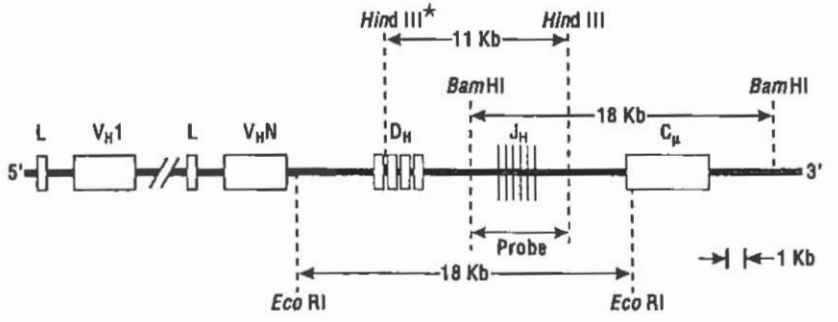

FIGURE 16.5 Genetic restriction map of the immoglobulin heavy chain locus on human chromosome 14. Lymphocytes are the only known cells in which somatic recombination is used to provide a source of diversity within an individual organism. In each lymphocyte family (clone), one of the many variable region genes (V genes to the left of the configuration) has combined with one of the few constant region genes (C genes to the right of the configuration) to form a single gene that encodes the heavy chain of an antibody molecule. The recombination site particularly involves regions designated D (for diversity) and J (for joining). Use of appropriate restriction enzymes and complementary probes can identify the recombinant "signature" unique to each lymphocyte clone. Similar V gene/C gene recombination occurs for the kappa light chain locus (human chromosome 2), the lambda light chain locus (human chromosome 22), the alpha T-cell receptor locus (human chromosome 14), the beta T-cell receptor locus (human chromosome 7), the gamma T-cell receptor locus (human chromosome 7), and the delta T-cell receptor locus (human chromosome 14). (Reprinted with permission from Cossman, *et al.*, 1991. Copyright © 1991 by J. B. Lippincott.)

Health Effects in Other Tissues

Infectious diseases that involve any organ tissues are likely to cause changes in the host defense system. In fact, many of the symptoms associated with infections are caused not by the infectious agents themselves but by cellular and molecular activities of the host response. Some solid tumors that release tumor-specific antigens may elicit autoimmunogenic responses that could serve as markers of the malignancy (Mavligit and Stuckey, 1983). Malnutrition, stress, pregnancy, and a variety of other factors all can influence the immune system (Table 16.3). Immune markers can be used as indicators of such health effects; conversely, these effects can be confounding variables when immune markers are used in attempts to characterize the host defense system itself.

Susceptibility

Susceptibility may be considered the relative propensity of an individual or population to develop dysfunction or disease. Increased susceptibility to infectious diseases and to certain cancers often is associated with deficiency or suppression of the immune system, as discussed earlier. Here we consider the susceptibility of the immune system itself to influences that alter its normal function, as in allergic, autoimmune, and immunoproliferative diseases.

Some well-characterized susceptibility markers have been established for certain immune-related disorders (Nowell and Croce, 1988; Clayton *et al.*, 1989; Wordsworth, 1991). Many of these markers may be detected by direct probes for the gene or by characterization of the gene-related protein products. Most of the genetic markers useful for assessing susceptibility to autoimmunity encode proteins directly involved with the immune system itself, but certain genetic markers for ancillary systems also may be helpful in assessing immune status. Genetic markers concerned directly with the immune system may be related to antigen-specific functions or to nonspecific activities.

In addition to these genetic factors, susceptibility to immune disorders may be influenced by a variety of behavioral and environmental factors such as stress, smoking, and preexisting disease. Thus, susceptibility markers can fall into one of several categories (Table 16.3, on page 416).

Immune-Specific Genetic Factors

The antigen specificity of every immune response depends on two genetically derived systems of recognition proteins: variable genes (V genes) that encode antibodies (Figure 16.5) and V genes that encode T cell receptors. These genes are expressed as the variable regions of antibody proteins or T-cell surface receptor proteins, respectively. The repertoire of these pro-

teins clearly influences immune reactivity and could, therefore, reveal biomarkers of susceptibility. One pathologic process in which susceptibility can be attributed directly to V genes is experimental allergic encephalitis (EAE), an animal disease model with similarities to human multiple sclerosis. EAE is caused by immunization with nerve tissue protein; the pathogenic response depends on a particular V gene in the T-cell receptor repertoire (Clayton *et al.*, 1989). Multiple sclerosis may involve similar interactions between V genes and histocompatibility antigens (Sinha *et al.*, 1991).

Assays for specific V genes or their protein products (idiotypes) are not performed readily at this time, nor is the understanding of V gene biology comprehensive enough to permit focused use of such assays. This is especially true for V genes of the T-cell receptors, which have been characterized only recently (Marrack and Kappler, 1986, 1987, 1990) and already have been implicated in human diseases such as toxic shock syndrome (Choi *et al.*, 1990). Further examples of susceptibility related to the V gene repertoire no doubt will be uncovered as gene probe techniques become more common and our knowledge of idiotypes increases. This area of research should be fruitful ground for development of assays to identify susceptible populations.

Nonspecific Immune Genetic Factors

Two major gene families that also can have strong influence on susceptibility to immune disorders encode immune proteins other than specific antigen recognition structures. One is the family of constant-region genes (C genes), which encode parts of the antibody and T-cell receptor molecules that are not involved in antigen recognition. The second is the family of histocompatibility genes that encode the so-called "transplantation" antigens.

The C genes for antibodies determine the isotypes (class and subclass) of antibodies (IgG, IgA, IgM, IgD, or IgE), which are found in all individuals, and also determine certain allotypes that differ among individuals. The C genes for the T-cell receptor determine whether the T cell expresses receptors of the alpha–beta type or the gamma–delta type. Both alpha–beta and gamma–delta T cells are found in all individuals, but some reports suggest that differences in lymphocyte compartmentation and antigen specificities within individuals are related to the type of T-cell receptor (Davis and Bjorkman, 1988).

The most well-known susceptibility factor related to antibody C genes is the IgE antibody isotype. Individuals who respond to allergens by producing primarily IgE (reaginic) antibodies may become sensitized for allergic reactions, whereas those who produce IgG (blocking) antibodies generally avoid such reactions. The mechanisms of IgE isotype regulation are only partly understood (Ishizaka, 1987, 1988; Vercelli and Geha, 1989; Maggi *et al.*, 1989). At this time, there are no clear tests to determine susceptibility to reaginic sensitization. However, it has been well established that individuals

with relatively high IgE levels are likely to be atopic. The impact of atopy on risk of disease in occupational exposures has been demonstrated using a case-control design to shown that atopy was a predisposing factor in the development of work-related hand eczema. Further elucidation of IgE isotype regulation will be required before more informative susceptibility factors can be found.

Other antibody C-gene susceptibility factors may lie among the different isotype subclasses and among different allotypes, particularly in association with certain allotypes of the major histocompatibility complex (MHC). C genes encoding the gamma–delta T-cell receptor proteins may be associated with susceptibility to particular autoimmune disease because of their interactions with heat-shock proteins (Winfield and Jarjour, 1991).

The genes of the MHC are the second family of nonspecific constitutive genetic factors known to influence susceptibility to a variety of immune-mediated diseases. The proteins that these genes encode are involved in presentation of antigen to T cells (Figure 16.6), a critical aspect of immune function. These genes are clearly major influences in the susceptibility to autoimmune disorders (Sinha *et al.*, 1990). Evidence has been reported for MHC regulation in the development of contact dermatitis to nickel (Braathen, 1988; Emtestam *et al.*, 1988) and the development of the scleroderma-like illnesses caused by vinyl chloride (Black *et al.*, 1983) and Spanish toxic oil (Vicaro *et al.*, 1982). Other susceptibilities have been identified among certain combinations of MHC and the C-gene allotypes discussed previously.

Ancillary Genetic Factors

Other genetic factors that are not directly involved with the immune system could act as ancillary markers of susceptibility to immune-mediated disease. The metabolism of xenobiotics may provide ancillary susceptibility markers; for instance, the probability of developing autoimmune illness from procainamide exposure is influenced by the genetically determined rate at which the chemical is acetylated (Reidenberg and Drayer, 1986). The XX karyotype (i.e., female gender) definitely predisposes to autoimmune disease (Bias *et al.*, 1986). The activation of certain oncogenes (Haluska *et al.*, 1986; Nowell and Croce, 1988) also may signal an increased susceptibility, especially to immunoproliferative disorders.

Nongenetic Susceptibility Factors

A number of preexisting conditions as well as behavioral and environmental factors also can influence susceptibility to immune-related health effects. Perhaps the most obvious example of a preexisting condition related to immune effects is previous sensitization by an antigen causing susceptibility to hypersensitivity disorders. Nonspecific inflammation also can influence susceptibility by enhancing immune responsiveness to specific antigens, the so-called "adjuvant effect." Air pollutants, such as ozone and diesel ex-

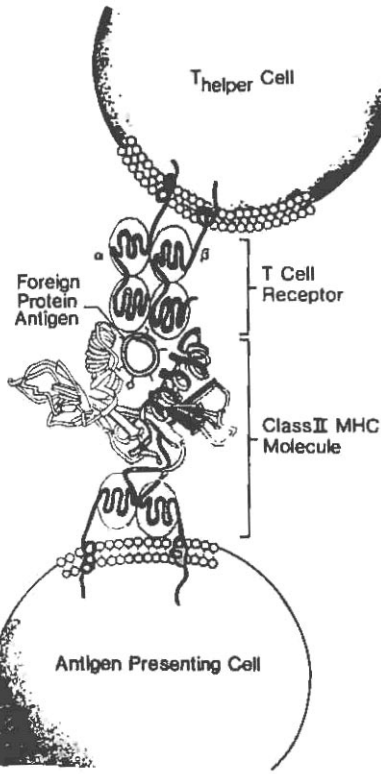

FIGURE 16.6 The three-way (ternary) complex that forms between an antigen-presenting cell (APC) and a T-lymphocyte. The APC binds antigen in its Class II protein of the major histocompatibility complex (MHC), and the T-lymphocyte binds to the antigen–MHC II complex via its specific receptor. This type of receptor-mediated cell–cell interaction is essential for many immune functions. (Reprinted with permission from Sinha, 1990. Copyright 1990 by the AAAS.)

haust particulate matter, can act as adjuvants, increasing the susceptibility to specific hypersensitivity reactions (Muranaka *et al.*, 1986; Koenig, 1987; Bascom *et al.*, 1990).

A number of behavioral factors, include smoking and drug use, can influence immune parameters and susceptibilities. Neurogenic and psychogenic factors also can influence immune function (Figure 16.7). Stress, for instance, can modulate immune reactivity and increase the propensity to develop active autoimmune or allergic disease. Neurogenic factors probably are involved in the pathogenesis of atopic reactions (Greene *et al.*, 1988). Conditioning also can cause changes in immune status and function. Suppressive and reactive immunologic changes have been observed in response to conditioning stimuli such as taste, odor, and audiovisual cues (Kusnecov *et al.*,

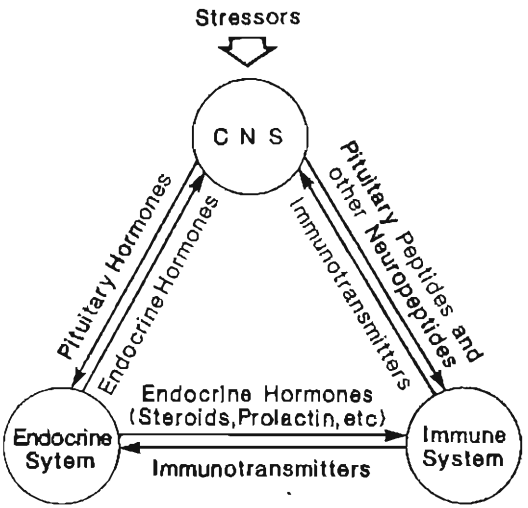

FIGURE 16.7 The direct influence of stress on the immune system may occur via hypothalamic pituitary peptides and the sympathetic branch of the autonomic nervous system. Stress also may influence the immune system through bidirectional communications between the CNS, the endocrine system, and the immune system. Thus, the influence of stressors on the immune response can be viewed as feedback regulatory loops between the CNS, the endocrine system, and the immune system. (Reprinted with permission from Greene *et al.*, 1988.)

1989; MacQueen *et al.*, 1989). Therefore, under some circumstances, the perception of exposure may trigger the appearance of a specific immune reaction (such as allergy) in a conditioned animal without any actual exposure to antigenic material, or it may inhibit an immune response to a sensitizing agent.

Confounding Variables

As the preceding discussion on susceptibility indicates, the cells and mediators of the immune system may be influenced by a wide variety of pathophysiologic processes, some of which are completely unrelated to immune function. For instance, hypergammaglobulinemia may be secondary to dehydration. Malnutrition affects a variety of systems, including the immune response. Other confounding factors that may affect immune parameters include aging, pregnancy, stress, genetic polymorphisms, prior or concurrent disease, previous toxicant exposures, psychoactive drugs (tobacco, alcohol, illicit drugs), therapeutic medications, and neurologic and endocrine influences.

The multiplicity of confounding factors adds another layer of complexity to the statistical analysis of multiple immune parameters. The com-

plex interactions of the immune system require that a judgment be made about whether immunologic markers are biologically independent, so they can be treated correctly as statistically independent or statistically correlated. Proper control groups (to control for confounders and to assess variation between populations) and longitudinal studies (to assess variation within populations) are the keys to successful evaluation. Confounding factors may be controlled in epidemiologic study designs by restriction or matching and in analyses by stratification or multivariate methods. When genetic factors are confounding, the choice of inheritance models is important for interpreting results.

Host defense activities involve many transitional stages, so the distinction between an intermediate factor and a confounding factor may be difficult to determine. When there is uncertainty about the mechanism, handling a potential confounding factor as both confounding and not confounding in different analyses may be justified (Rothman, 1986). If the variable is actually an intermediate in the process under study, then controlling for it in the analysis as a confounding factor may lead to serious underestimation of effect.

Guidelines for Using Immune Markers in Field Studies

General Approaches

Tests for immune markers may be included when searching for exposure to biologics or chemicals, susceptibility to particular health effects, or biologic effects from exposure or disease processes. Biologic effects range from the earliest stages of pathogenesis or exposure, through stages of subclinical disease, to overt health effects (NRC, 1992).

General applications for immunologic biomarkers are presented in Table 16.4 under two categorical end points: antibodies and immune response. Antibodies can be used in two general ways to effectively estimate internal dose, preclinical biologic responses, or clinical health effects from chemical exposures. The first approach is to exploit antibodies as powerful molecular *probes* to detect a compound of interest, made feasible by scientists' ability to produce purified antibodies with known specificity. For example, specific antibodies commonly are used in forensic toxicology to detect the presence of specific abused drugs, such as morphine, in biologic fluids. Another growing use of antibodies as specific probes is to incorporate them into immunoassays that can detect the presence of biomolecules, such as cytokines or hormones, with exquisite sensitivity. The second approach for using antibodies in biologic monitoring is to measure antibody *titers* (levels of immunoglobulins that are able to react with a specific antigen of interest) in biologic fluids (usually serum). Antibody titers, like probes, also can provide specific and sensitive assessments of exposures to chemicals and the resulting early biologic responses or adverse effects on health. For example, positive

TABLE 16.4 Approaches for Using Immunologic Biomarkers[a]

Exposed individual	Biologic end points		
	Antibodies		Immune response function
	Probes	Titers	
Susceptibility	Genotype, metabolism[b]	Hypogammaglobulinemia[c]	SCID, Atopy[d]
Internal dose	Xenobiotics, toxicants[e]	—	—
Early response	—	Specific serum Ig[f]	Immune profile[g]
Adverse effects	Biologic molecule[h]	Allergy(IgE)[i]	Resistance to disease[j]

[a] Table adapted from presentations developed by Gerry Henningsen, NIOSH, showing immunologic indicators of environmental exposure, internal dose, early preclinical responses, adverse clinical effects, and susceptibility.

[b] Antibody probes used to detect susceptibility to a specific chemical exposure.

[c] Antibody titers used to detect susceptibility to specific pathogenic diseases.

[d] Inherent deficiencies used to detect susceptibility to general pathogenic diseases. SCID, Severe Combined Immunodeficiency Disorder.

[e] Antibody probes used to detect dose from a specific chemical exposure.

[f] Antibody titers used to detect response to a specific chemical exposure.

[g] Selective changes in immune response coincide with certain chemical exposures.

[h] Antibody probes used to detect effects from a specific chemical exposure.

[i] Antibody IgE titers used to detect effects from a specific chemical exposure.

[j] Immunocompetency changes detect effects from a specified chemical exposure.

antibody titers to isocyanates (absent in nonexposed people) denote a prior exposure to these chemicals; if significant specific IgE antibody titers are found, the titers support the ability to attribute adverse effects (allergies) to the specific chemical exposure. The second category of immunologic biomarker is immune response in which the *function* of the immune system is evaluated for selective changes that are, in general, characteristics of a specific chemical exposure. Such immunologic profiles of selective changes in immune response usually are characterized first in animal models or by *in vitro* experiments. Many dose-related selective responses to certain chemical exposure conditions have been described. For example, ethanol selectively interferes with the interleukin 2 receptor, whereas dioxin (TCDD) primarily affects T lymphocytes, and pentachlorophenol has more generalized effects on immune responses. Finally, changes in resistance to disease also can be measured, as a result of known exposure to specified chemicals or certain other environmental immunomodulating agents that can produce potentially harmful effects, including increased susceptibility to infections or cancer and autoimmune disorders. Depending on the design of the field study, any one or a combination of these immunologic end points may be used as an epidemiologic biomarker to assess susceptibility, internal dose, early responses, or adverse effects. Susceptibility to certain environmental agents also poten-

tially can be measured through *in vitro* immunization tests to evaluate the propensity of an individual to develop antibody titers to a specific chemical, or by evaluating baseline immune responses to estimate the impact of immunomodulating agents on the ability of individuals to resist disease.

Choice of Tests

Tests for immune markers used in field studies should be selected to provide the most cost-effective information relevant to the focus of investigation. A serviceable approach to categorizing and selecting immune markers was developed by a subcommittee of the Centers for Disease Control (CDC) and the Agency for Toxic Substances and Disease Registry (ATSDR), convened to develop guidelines for the use of biomarker tests in health assessment studies conducted at Superfund sites (Centers for Disease Control, 1990).

The subcommittee identified three general categories of tests: (1) basic tests that provide a general evaluation of immune status; (2) focused/reflex tests that address particular aspects of immune function as indicated by clinical findings, suspected exposures, or results of prior tests, and (3) research tests that require evaluation in well-defined control populations (Table 16.5).

Tests in both the basic group and the focused/reflex group should have clinical interpretations for disease end points when values lie outside established reference ranges. Tests from the basic group should be included in most studies, since they provide the minimal "core" assessment of immune status. Although they may be omitted in studies addressing very specific concerns, the interpretation of other tests may suffer without the supporting data. Tests from the focused/reflex panel are suggested by particular clinical symptoms, prior laboratory findings, or specific exposures; they may be used individually or be augmented by tests from the basic group. Research tests should be used under the auspices of an investigative protocol with control populations that have known exposure or disease end points. Before a test is considered to have completed the investigative phases, the biochemical or physical abnormalities associated with changes in the marker should be identified, and the nature of any disease associations should be determined. Because of the intrinsic variability of the immune system within and between individuals, longitudinal studies are essential in evaluating research tests for immune markers.

Study Design

In addition to test selection, the overall study design must be orchestrated carefully to insure interpretability of results. The basic goal should be to identify all sources of variability in the tests: analytical (laboratory error), within individuals (over time), among individuals within each group, and among study groups. Analytic variability can be assessed by including a subset of duplicate (split) samples. Variability within individuals can be assessed only by longitudinal studies. Variability among individuals within a study

TABLE 16.5 Classification of Tests for Immune Markers

Test Category	Characteristics	Specific tests
Basic Should be included with general panels	General indicators of immune status Relatively low cost Assay methods are standardized among laboratories Results outside reference ranges are clinically interpretable	Complete blood counts Serum IgG, IgA, IgM levels Surface marker phenotypes for major lymphocyte subsets
Focused/Reflex Should be included when indicated by clinical findings, suspected exposures, or prior test results	Indicators of specific immune functions/events Cost varies Assay methods are standardized among laboratories Results outside reference ranges are clinically interpretable	Histocompatibility genotype Antibodies to infectious agents Total serum IgE Allergen-specific IgE Autoantibodies Skin tests for hypersensitivity Granulocyte oxidative burst Histopathology (tissue biopsy)
Research Should be included only with control populations and careful study design	Indicators of general or specific immune functions/events Cost varies; often expensive Assay methods are usually not standardized among laboratories Results outside reference ranges are often not clinically interpretable	*In vitro* stimulation assays Cell activation surface markers Cytokine serum concentrations Clonality assays (antibody, cellular, genetic) Cytotoxicity tests

group may be quite high, and may require a large number per group to assess properly. Identification of biologically significant variability among groups, the general goal of controlled studies, is possible only with careful selection of the populations to control for the many differences in susceptibility and the confounding variables that influence the immune system.

Once a significant difference among groups is established for one or more markers, long-term follow-up is required to determine the extent to which such differences are predictive for overt health effects. Identifying predictive immune markers in epidemiologic field studies will lead to a better understanding of immune-related disease mechanisms and enhanced measures for prevention and intervention.

Data Management

Interpretation of many immune markers requires that results be evaluated in the face of evolving analytical methods, shifting calibration values, and a plethora of confounding variables. A properly designed database will allow any result to be related to analytical method, reagents, bias relative to reference calibrators, analytic and biologic variability, and, ultimately, human health outcome. The structure of the database should be an integral part of designing the study and should precede any data collection (see Chapter 4).

Illustrations Using Immune Markers in Health Effect Studies

The following examples have been chosen to illustrate the potential of immune system components as cellular and molecular markers as well as some of the practical considerations involved with such use. Each example is given as a brief summary to point out its main features of interest. Readers should consult the original references for full information and analyses.

Immune Markers in HIV Infection

Infection by the human immunodeficiency virus (HIV) leads to the acquired immune deficiency syndrome (AIDS), usually long after the initial infection. A few years after a surface protein called CD4 was identified on a subpopulation of helper T lymphocytes, this protein was shown to be the site of viral attachment by HIV (McDougall *et al.*, 1985). The CD4 protein is present not only on helper T cells, but also on monocytes, macrophages, and dendritic cells. Consequently, these cells also can be infected by HIV. Subsequent events in the pathogenesis of AIDS are not well understood, but it appears to be more complex than the simple destruction of virus-infected cells (Margolick and Vogt, 1992). Many different immune markers have been critically important in the diagnosis, monitoring, and evaluation of HIV infection, as well as in attempts to understand and mitigate the basic disease processes.

Assays for antibodies to HIV are the mainstay of screening and diagnosing the infection. Generally a two-stage testing protocol is used; the sensitivity and specificity of the combined tests both exceed 99% if properly performed (MMWR, 1990). Antibody tests can be applied to bloodspots taken routinely for neonatal screening; these results provide the most broad-based data on the prevalence of the infection (Quinn *et al.*, 1992). Such bloodspots also can be tested for other factors potentially related to HIV infection, for example, illicit drugs (Henderson *et al.*, 1992), providing important information to help focus efforts of prevention.

Although antibody tests are valuable tools for HIV screening and diag-

nosis, they do not provide much information about the progress of the infection. Low peripheral blood counts for CD4 lymphocytes are currently the best laboratory indicator of impending opportunistic infections by *Pneumocystis carinii* (Figure 16.8A). CD4 counts also correlate with the response to therapy by AZT (Figure 16.8B); because of the slow progress of the disease and earlier therapeutic intervention, CD4 counts will become increasingly important in the evaluation of new therapeutics and vaccines. A number of technical issues concerning CD4 lymphocyte measurements are still unresolved (Kidd and Vogt, 1989), but are receiving increased attention in light of the public health importance of the test (MMWR, 1992).

Changes in other lymphocyte subset phenotypes also may provide important insight into the pathogenesis of AIDS. A subtle trend in peripheral blood T cells associated with HIV seroconversion was identified when over 1500 results from 268 subjects were compiled from a multisite study (Margolick *et al.*, 1989) (Figure 16.9). Over a 27-month period after the anti-HIV serum antibodies first became detectable, the number of T cells with neither CD4 nor CD8 surface markers increased significantly, suggesting that seroconversion is associated with an expansion of natural killer (NK) or gamma–delta T cells. Although predictive value of this change is low, it provides another clue to the immunologic responses that accompany HIV infection. Such subtle changes would be difficult or impossible to detect in the face of wide normal variation without large longitudinal multisite studies using standardized methods.

Leukocyte Counts in Benzene Exposure

The volatile organic compound benzene is toxic to bone marrow cells. Exposure at high levels can lead to aplastic anemia (a complete loss of all blood-forming stem cells), acute myelogenous leukemia, and possibly immunoproliferative malignancies including lymphomas and multiple myeloma (Young, 1989; Goldstein, 1990). This bone marrow toxicity can cause subclinical decreases in peripheral blood leukocytes in humans exposed to benzene at levels that once were common in occupational settings. Kipen *et al.* (1988) found that the average peripheral blood leukocyte counts monitored from 1940 to 1975 in a large rubber factory cohort increased linearly over an 8-year period during which the exhaust system of the plant was improved and a recommended exposure standard was implemented (Figure 16.10). The investigators used this relationship for a retrospective exposure assessment that seemed warranted, but changes in the blood counts also have been attributed to methodologic differences. Since that time benzene has been regulated to levels at which this effect is no longer detectable, but the acceptable levels for minimal health risk remain controversial (Brett *et al.*, 1989; Nicholson and Landrigan, 1989) Moreover, methodologic biases are still possible, even on very simple markers such as blood cell counts (see Chapter 4).

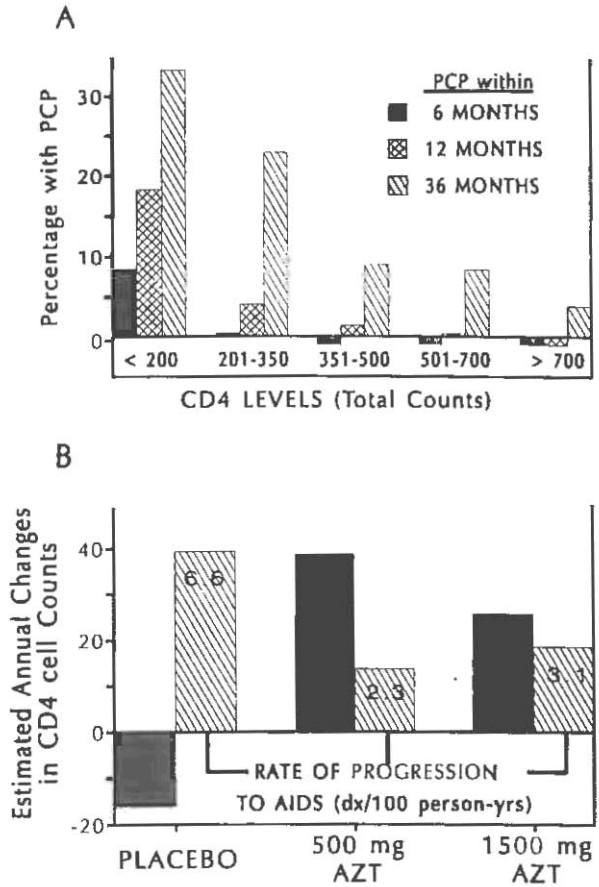

FIGURE 16.8 (A) CD4 lymphocyte counts in peripheral blood indicate susceptibility to pneumocystic carinii pneumonia (PCP) in HIV-infected individuals. When counts are above 200 cells/μl, the risk of PCP within 1 year is negligible; below 200, the risk approaches 20%. The average CD4 in normal individuals is around 1000 cells/μl with a lower limit around 400. (Adapted from Morbidity and Mortality Weekly update, 1989). Guidelines for prophylaxis against pneumocystitis carinii pneumonia for persons infected with Human Immunodeficiency Virus. 38(S-5), 6. (B) CD4 lymphocyte counts also reflect the response to treatment of HIV-infected individuals with AZT. Among those receiving placebo treatment, CD4 counts fell about 15% per year, and the rate of progression to AIDS was 6.6 diagnoses per 100 person-years. Lower-dose AZT treatment resulted in an annual increase in CD4 counts and cut the rate of progression to AIDS by two-thirds. Higher-dose AZT treatment also increased the CD4 count, but not as much as the lower-dose regimen; moreover, the rate of progression to AIDS was higher than that in the lower-dose cohort. (Adapted with permission of the New England Journal of Medicine from Volberding *et al.*, 1990.)

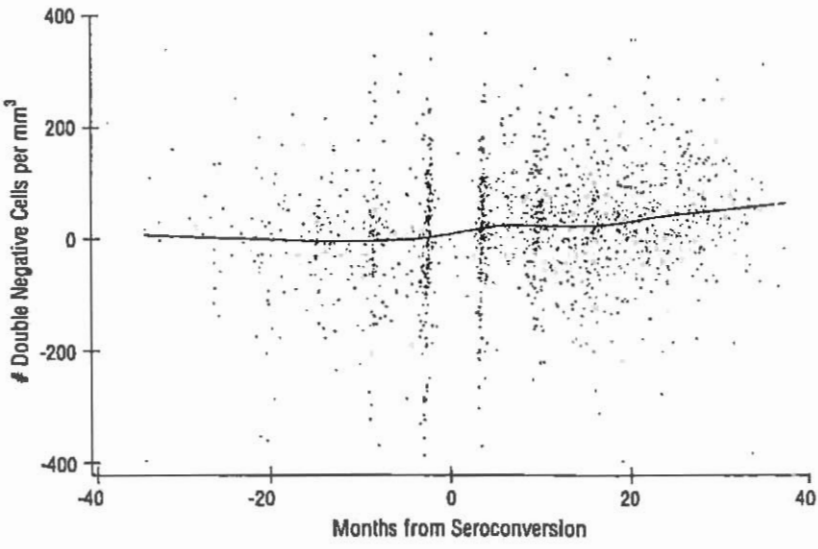

FIGURE 16.9 Increased $CD3^+CD4^-CD8^-$ cells in HIV-1 infection. This longitudinal study showed a subtle but significant increase in an unusual peripheral blood T-lymphocyte subset when participants became infected by HIV-1.

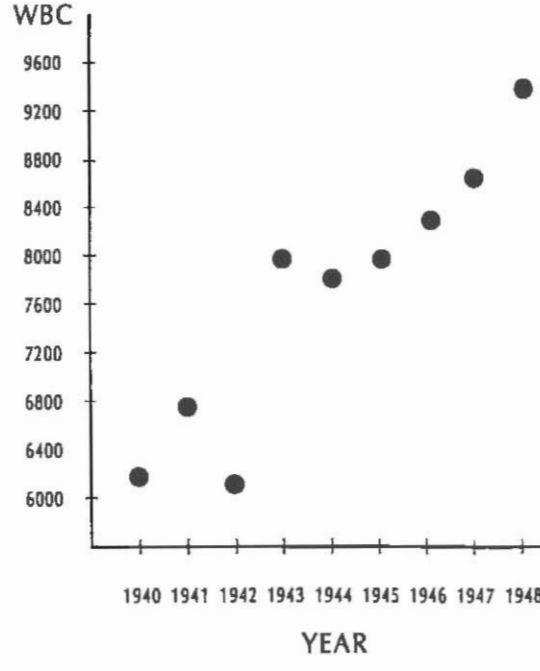

FIGURE 16.10 Annual average white blood cell count (WBC) in a cohort occupationally exposed to benzene. The results showed, on average, a striking progressive increase in WBC over the 8-year period during which benzene exposure levels declined due to revisions of the recommended exposure standard and improvements in the exhaust system of the plant. (Reprinted with permission from Kipen *et al.*, 1988.)

Air Pollution and Acute Inflammation

Complement is a series of proteins activated in a cascading sequence by a variety of nonspecific stimuli as well as by specific antibody–antigen interactions. The most prominent protein in the complement system, designated C3, is one of the acute-phase reactant serum proteins that increase in concentration with ongoing stimulation of acute inflammatory responses (Dowton and Colten, 1988; Katz *et al.*, 1990) However, if large-scale acute inflammation continues long enough, C3 levels actually can decline because depletion surpasses synthesis.

When serum C3 levels were compared in populations residing in areas with varying degrees of air pollution, the results showed small but significant increases as the pollution index rose (Stiller-Winkler *et al.*, 1989) (Figure 16.11). These findings emphasize both the inflammatory effects of air pollution and the potential impact of subtle confounding variables on immune markers.

Infiltration of respiratory mucosal surfaces by neutrophils is another indicator of acute inflammatory reactions to airborne irritants or sensitizers. Controlled exposures of normal subjects to ozone at part per million levels has been shown to cause an increase in the number of neutrophils obtained by nasal lavage (Koren, 1990), another confirmation that air pollution is associated with respiratory tract inflammation.

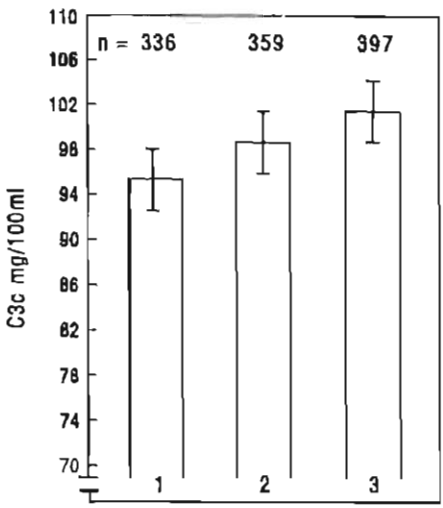

FIGURE 16.11 Serum levels of complement component C3 in males living in areas characterized by little (1), moderate (2), or heavy (3) air pollution. Complement is an acute-phase reactant that shows a nonspecific increase under a variety of proinflammatory conditions. This effect could represent a marker of exposure, a marker of effect, or a confounding variable, depending on the goal and design of the study. (Reprinted with permission from Stiller-Winkler *et al.*, 1989.)

Chronic Lymphocytic Leukemia and Cell Surface Protein Expression

B-cell chronic lymphocytic leukemia (B-CLL), a disease most prominent in older populations and that sometime runs in families, is a clonal expansion of B cells that may be undetected clinically for many years. In the leukemic form of B-CLL, blood and bone marrow lymphocytosis predominates, whereas in the lymphomatous form, lymphadenopathy is dominant. Mixtures of these forms also occur. Peripheral blood lymphocyte counts can increase more than 10-fold above normal levels before symptoms are noticeable, and the disease generally remains indolent for some time after that. Although B-CLL cells appear very similar to normal resting lymphocytes on stained blood smears, small morphologic differences often can be discerned. The cause of CLL is unknown, but a discrete molecular lesion affecting regulatory function is suspected (Marti and Fleisher, 1987). Gene probe studies in one familial cohort suggest that immunoglobulin heavy-chain genes are rearranged in CLL cells, whereas the T-cell receptor gene is not rearranged; moreover, the rearrangements were different in the affected cases (Shah *et al.*, 1992).

When immunophenotyping by flow cytometry was applied to the study of B-CLL, deviations from the normal expression of several cell-surface markers became apparent through differences in the fluorescence intensity of the stained cells (Marti *et al.*, 1989). In particular, B-CLL cells were stained more strongly than normal cells by antibodies to Class II histocompatibility proteins, and less strongly by antibodies to the B-cell lineage-specific CD20 and CD22 proteins (Figure 16.12). The differences do not appear to be caused by technical factors (Marti et al., 1992); therefore, they represent alterations in the actual amounts of surface protein receptors expressed by the malignant cells, presumably related to the molecular lesion responsible for their neoplastic behavior.

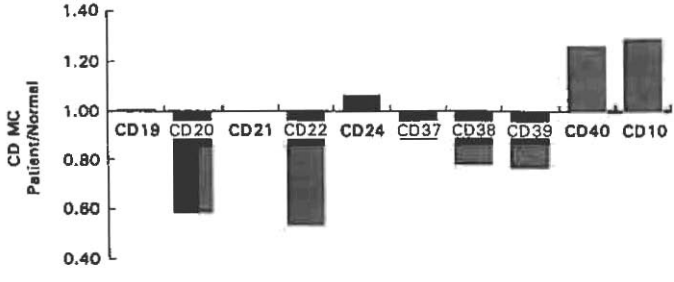

FIGURE 16.12 Relative differences in the fluorescent intensity of normal lymphocytes and chronic lymphocytic leukemia (CLL) lymphocytes when cells are stained with fluorescent probes for different surface proteins. The CD20 and CD22 surface proteins stain much less strongly in CLL cells than in normal cells, suggesting that the CLL cells express fewer of these proteins on their surfaces. In contrast, the CD40 and CD10 proteins appear to be expressed more abundantly in CLL cells.

The morphologic and surface marker differences in B-CLL cells make immunophenotyping by flow cytometry an ideal technique for detecting subclinical disease (Figure 16.13). Since these tests rarely are applied on a broad population basis, the true prevalence of B-CLL is unknown but is probably much higher than current figures suggest, especially in older populations.

Ankylosing Spondylitis and Histocompatibility Genes

Ankylosing spondylitis (AS) is an arthritis-like autoimmune disease affecting the joints of the spine and pelvis that can lead ultimately to bony fusion of the vertebra. AS afflicts about 1 in 1000 individuals in Caucasian populations; 96% of those individuals possess the HLA-B27 histocompatibility gene, compared with 7% of the general population (Benjamin and Parham, 1990). The specificity of the HLA-B27 relationship is not strong, however, since only about 2% of HLA-B27 positive individuals contract AS. Other genetic factors also influence risk, since the disease runs in families and varies among races in prevalence as well as in association with HLA-B27.

The molecular basis for the connection between AS and HLA-B27, a class I histocompatibility human leukocyte antigen (HLA), is not clear. In general, the class I HLA molecules are cell-surface proteins that restrict the cytotoxic activity of CD8 T cells by binding and presenting antigenic peptides within a molecular "groove" of their three-dimensional structure (Figure 16.14). Some regions of amino acid sequences that compose the groove display considerable variability among different HLA alleles and even between different subtypes of a particular family such as B27, whereas other regions are conserved strongly in all B27 proteins yet differ from the other HLA-B molecules. The epidemiologic observation that AS is associated with the four most common B27 subtypes may, therefore, be translated directly into molecular terms: the disease-associated parts of the molecule must be unique to HLA-B27 but shared by all common subtypes within that family. Protein structure analysis suggests as a likely candidate a five amino acid residue stretch positioned in or near the antigen-binding groove (Figure 16.13). If this association is authentic, it could help explain the tissue specificity of disease activity, because the B27 groove could bind a peptide unique to the target tissue (Benjamin and Parham, 1990). Other theories based on the molecular structure of B27 have been proposed; further epidemiologic and laboratory investigation will be necessary to find the true mechanism(s) of the pathogenic process.

In Vitro *and* in Vivo *Methods for the Evaluation of Inhalent Allergy*

Common allergies to inhalent antigens such as pollens and skin danders are caused by immediate hypersensitivity reactions. Immediate hypersensitivity occurs when IgE molecules bound to the surface of mast cells or basophils become cross-linked through contact with antigen, causing cell degranula-

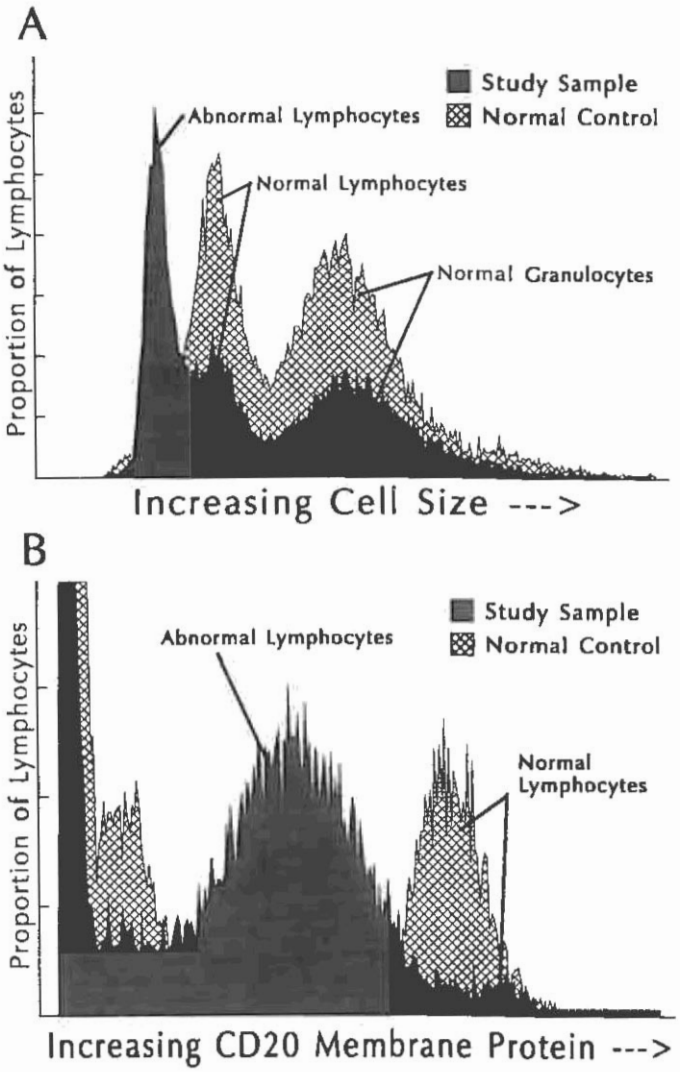

FIGURE 16.13 Fluorescent flow cytograms from an individual with subclinical B-cell chronic lymphocytic leukemia (CLL) compared to those from a normal individual. The light scatter pattern (A) from the CLL sample shows a distinct subpopulation to the lower left of the lymphocyte cluster not seen in the normal sample. The fluorescence pattern (B) shows an increase in cells staining for the B-cell marker CD20, and most of the cells are much dimmer than the CD20 staining in normal B cells. A few normal B-cells are present in the CLL sample, as shown by their brighter staining. The findings are consistent with a monoclonal expansion of B cells expressing low CD20 surface protein, or a preclinical CLL. This condition was easily recognized by flow cytometric analysis, although it was not apparent from the complete blood count.

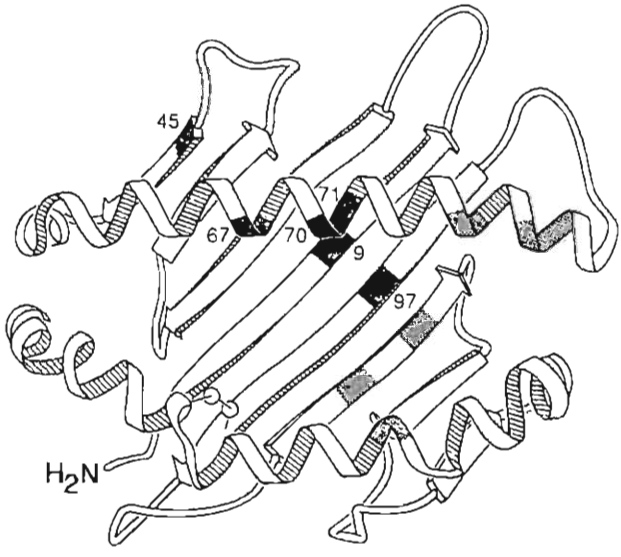

FIGURE 16.14 A model of the three-dimensional structure of the HLA protein molecule showing the conserved (solid) and polymorphic (stippled) sites in the four HLA-B27 subtypes associated with an increased risk of ankylosing spondylitis (AS). The conserved residues (numbered) that line the "groove" of the molecule are unique to the B27 type among HLA-B molecules and may be the specific sites associated with the increased risk of AS. (Reprinted with permission from Benjamin and Parham, 1990.)

tion and the release of active mediators, including histamine. Allergy skin tests detect cell-bound IgE by the rapid onset of wheal-and-flare reaction when antigen causes mast cell degranulation and immediate hypersensitivity inflammatory reaction in the area of the test site. Skin tests often are done by first applying a very small amount of antigen with a "puncture" method, then following weak or negative reactions with a larger amount through intracutaneous injection. The sensitivity and specificity of skin tests depends largely on the person who applies the antigen and interprets the reaction.

Although some IgE is present in serum, the levels are ordinarily very low (less than a million-fold below IgG levels). Serum IgE does not contribute directly to the pathogenesis of allergic reactions. However, serum IgE represents a sampling of total IgE production and therefore can serve as a marker of susceptibility to immediate hypersensitivity reactions. A number of laboratory methods to measure antigen-specific IgE are in common use; they differ in both technical and interpretative aspects.

The optimal approach to using skin tests (*in vivo* testing) and the various serum IgE assays (*in vitro* testing) in the evaluation of allergy has been a continuing source of controversy, particularly in the lower ranges of skin test reactivity and with antigen-specific IgE levels close to the limit of detection. In a careful study comparing skin tests with three *in vitro* methods for evalu-

ating inhalent allergy (Williams *et al.*, 1992), the investigators found generally good agreement among all methods, but ROC analysis (Figure 16.15; see Chapter 3) showed that skin testing by an experienced allergist was the most sensitive and specific approach. A modification of the original *in vitro* test increased sensitivity, but only with a corresponding decrease in specificity, whereas a newer *in vitro* assay showed a marginal improvement in both sensitivity and specificity.

A number of issues impact the relative sensitivity and specificity of these tests. The major biologic considerations are that (1) IgE bound to mast cells persists much longer than IgE in the serum, which has a half-life of less than 1 day (Tada *et al.*, 1975), and (2) the skin test response involves biologic amplification through the immediate hypersensitivity reaction. Both factors contribute to the better sensitivity of allergy skin tests, but the newest technologies for measuring specific IgE approach sensitivity of skin tests for many inhalent allergens. Technical considerations include the subjective nature of reading skin tests compared with objective numerical results of serum

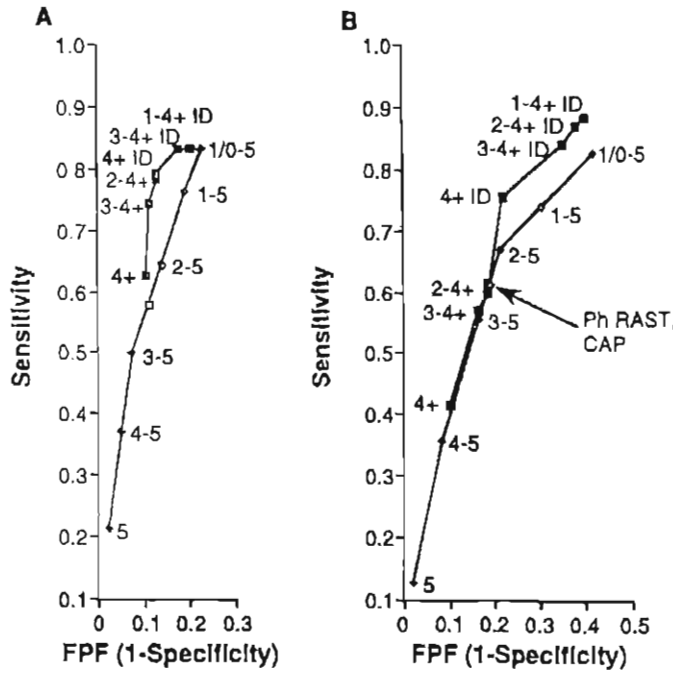

FIGURE 16.15 Receiver–operator characteristic (ROC) curves comparing skin tests and three different *in vitro* assays for the diagnosis of allergy to timothy (A) and cat dander (B). The skin test (■) curves go from a 4+ positive puncture test (the least sensitive) to a negative puncture test (P) with a positive intradermal (ID) (the most sensitive). The ROC curve for skin tests is better at all points than the multipoint curve for the modified radio-allergosorbent test (modified RAST ♦), for the single cut-off point for the original RAST (Phadebas RAST □), or for the newer CAP system (◇). (Reprinted with permission from Williams *et al.*, 1992.)

IgE assays. In less experienced hands, *in vitro* tests may well be superior to *in vivo* tests.

Summary

The discussion and examples presented in this chapter should confer a sense of cautious enthusiasm to those who would use immune cells and mediators as markers of health-related events in epidemiologic studies. The enthusiasm is warranted because of the rapid evolution of technologies that allow extensive characterization of cellular and molecular markers. It is justified further by the success of using immune markers in epidemiologic studies to elucidate health effects and environmental exposures in many infectious, autoimmune, allergic, and neoplastic diseases as the preceding examples demonstrate. However, the rapid pace of technology also should invoke a sense of caution, because it means that changing methodologies will be applied to problems that may have an uncertain biologic basis and an unclear public health significance. The interpretation of results from such studies can be highly problematic.

Methodology is the most critical aspect of immune marker use in epidemiologic field studies. In the field, suitable control groups, appropriate study designs, and proper sample handling are essential elements. In the laboratory, quality control of assay methods through comparisons within and between runs, days, methods, and laboratories is equally essential. The selection of tests and the interpretation of results are other critical issues that are closely intertwined. Investigators should select tests that have known clinical significance or can be interpreted in light of some understanding of the underlying biologic processes. Since most immune markers have not been tested in large groups of normal individuals, the extent to which outlying values or shifts in overall population distributions reflect genuine health effects is often uncertain.

In summary, the great promise of immune markers as tools for exposure and health assessment can be realized only through the application of rigorous epidemiologic and laboratory methods coupled with an understanding of the basic biology of the host defense system. Efforts to this end have already begun through multicenter activities such as the AIDS Clinical Trials Group (Margolick *et al.*, 1989; Paxton *et al.*, 1989) and the Immune Biomarkers Demonstration Project for environmental health studies (Vogt *et al.*, 1990). Future activities should strengthen the role immune markers can play in epidemiologic studies.

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References

Balkwill, F. R., and Burke, F. (1989). The cytokine network. *Immuno. Today* 10, 299–303.

Bascom, R., Naclerio, R. M., Fitzgerald, T. K., Kagey-Sobotka, A., and Proud, D. (1990). Effect of ozone inhalation on the response to nasal challenge with antigen of allergic subjects. *Am. Rev. Resp. Dis.* 142, 594–601.

Benjamin, R., and Parham, P. (1990). Guilt by association: HLA-B27 and ankylosing spondylitis. *Immun. Today* 11, 137–142.

Bias, W. B., Reveille, J. D., Beaty, T. H., Meyers, D. A., and Arnett, F. C. (1986). Evidence that autoimmunity in man is a Mendelian dominant trait. *Am. J. Hum. Genet.* 39, 584–602.

Black, C. M., Welsh, K. I., Walker, A. E., Bernstein, R. M., Catoggio, L. J., McGregor, A. R., and Jones, J. K. (1983). Genetic susceptibility to scleroderma-like syndrome induced by vinyl chloride. *Lancet* 1, 53–55.

Brett, S. M., Rodricks, J. V., and Chinchilli, V. M. (1989). Review and update of leukemia risk potentially associated with occupational exposure to benzene. *Environ. Health Perspect.* 82, 267–281.

Centers for Disease Control (1990). "Internal Report on Biomarkers of Organ Damage and Dysfunction for the Renal, Hepatobiliary, and Immune Systems." Subcommittee on Biomarkers of Organ Damage and Dysfunction of the Centers for Disease Control and Agency for Toxic Substances and Disease Registry, Atlanta, Georgia. [Available from Clinical Biochemistry Branch, Mailstop F-19, CDC, Atlanta, Georgia 30333.]

Choi, Y., Lafferty, J. A., Clements, J. R., Todd, J. K., Gelfand, E. W., Kappler, J., Marrack, P., and Kotzin, B. L. (1990). Selective expansion of T cells expressing V beta 2 in toxic shock syndrome. *J. Exp. Med.* 172(3), 981–984.

Clayton, J. P., Gammon, G. M., Ando, D. G., Kono, D. H., Hood, L., and Sercarz, E. E. (1989). Peptide-specific prevention of experimental allergic encephalomyelitis. Neonatal tolerance induced to the dominant T cell determinant of myelin basic protein. *J. Exp. Med.* 169(5), 1681–1691.

Cossman, J., Zehnbauer, B., Garrett, C. T., Smith, L. J., Williams, M., Jaffe, E. S., Hanson, L. O., and Love, J. (1991). Gene rearrangements in the diagnosis of lymphoma/leukemia. Guidelines for use based on a multiinstitutional study. *Am. J. Clin. Pathol.* 95(3), 347–354.

Davis, M. M., and Bjorkman, P. J. (1988). T-cell antigen receptor genes and T-cell recognition. *Nature (London)* 334, 395–402.

Dowton, S. B., and Colten, H. R. (1988). Acute phase reactants in inflammation and infection. *Semin. Hematol.* 25(2), 84–90.

Emtestam, L., Carlsson, B., Marcusson, J. A., Wallin, J., Moller, E. (1989). Specificity of HLA restricting elements for human nickel reactive T cell clones. *Tissue Antigens* 33(5), 531–541.

Fey, M. F., Kulozik, A. E., Hansen-Hagge, T. E., and Tobler, A. (1991). The polymerase chain reaction: A new tool for the detection of minimal residual disease in haematological malignancies. *Eur. J. Cancer* 27(1), 89–94.

Goldstein, B. D. (1990). Is exposure to benzene a cause of human multiple myeloma? *Ann. N.Y. Acad. Sci.* 609, 225–230.

Greene, R., Fowler, D., MacGlashan, D., and Wienreich, D. (1988). IgE-challenged human lung mast cells excite vagal sensory neurons *in vitro*. *J. Appl. Physiol.* 64, 2249–2253.

Haluska, F. G., Tsujimoto, Y., and Croce, C. M. (1986). Molecular genetics of B- and T-cell neoplasia. *Int. Symp. Princess Takamatsu Cancer Res. Fund* 17, 151–158.

Henderson, L. O., Powell, M. K., Hannon, W. H., Miller, B. B., Martin, M. L., Hanzlick, R. L.,

Vroon, D., and Sexon, W. R. (1992). Radioimmunoassay screening of dried blood spot materials for benzoylecgonine. *Anal. Toxicol. (in press)*

Hood, L. E., Weissman, I. L., and Wood, W. B. (1978). "Immunology." Benjamin/Cummings, Menlo Park, California

Katz, P. R., Karuza, J., Gutman, S. I., Bartholomew, W., and Richmond, G. (1990). A comparison between erythrocyte sedimentation rate (ESR) and selected acute-phase proteins in the elderly. *Am. J. Clin. Pathol.* 94(5), 634–640.

Kidd, M., and Vogt, R. F. (1989). Evaluation of T-cell subsets during HIV infection and AIDS: A workshop report. *Clin. Immunol. Immunopathol.* 52, 3–9.

Kipen, H. M., Cody, R. P., Crump, K. S., Allen, B. C., and Goldstein, B. D. (1988). Hematologic effects of benzene: A thirty-five year longitudinal study of rubber workers. *Toxicol. Ind. Health* 4, 411–430.

Koenig, J. Q. (1987). Pulmonary reaction to environmental pollutants. *J. Allergy Clin. Immunol.* 79, 833–843.

Koren, H. S., Hatch, G. E., and Graham, D. E. (1990). Nasal lavage as a tool in assessing acute inflammation in response to inhaled pollutants. *Toxicol.* 60, 15–25.

Kusnecov, A., King, M. G., and Husband, A. J. (1989). Immunomodulation by behavioural conditioning. *Biol. Psychol.* 28, 25–39.

McDougall, J. S., Mawle, A., Cort, S. P., Nicholson, J. K. A., Cross, G. D., Scheppler-Campbell, J. A. Hicks, D., and Sligh, J. (1985). Cellular tropism of the human retrovirus HTLV-III/LAV. I. Role of T cell activation and expression of the T4 antigen. *J. Immunol.* 135, 3151–3162.

MacQueen, G., Marshall, J., Perdue, M., Siegel, S., and Bienenstock, J. (1989). Pavlovian conditioning of rat mucosal mast cells to secrete rat mast cell protease II. *Science* 243, 83–85.

Maggi, E., Del Prete, G. F., Parronchi, P., Tiri, A., Macchia, D., Biswas, P., Simonelli, C., Ricci, M., and Romagnani, S. (1989). Role of T cells, IL-2 and IL-6 in the IL-4-dependent in vitro human IgE synthesis. *Immunol.* 68, 300–306.

Margolick, J. B., et al. (1989). Development of antibodies to HIV-1 is associated with an increase in circulating CD3+CD4−CD8− lymphocytes. Clin. Immunol. Immunopathol. 51(3), 348–361

Margolick, J. B., and Vogt, R. F. (1992). Environmental effects on the human immune system and the risk of cancer: Facts and fears in the era of AIDS. *Environ. Carc. Exotoxicol. Rev.* C9(2), 155–206.

Marrack, P., and Kappler, J. (1986). The T cell and its receptor. *Sci. Am.* 254, 35–45.

Marrack, P., and Kappler, J. (1987). The T cell receptor. *Science* 238, 1073–1079.

Marrack, P., and Kappler, J. W. (1990). The T cell receptors. *Chem. Immunol.* 49, 69–81.

Marti, G. E., and Fleisher, T. A. (1987). B Cell CLL Immunophenotypes. *In* "Chronic Lymphocytic Leukemia: Recent Progress, Future Directions." (R. P. Gale and K. Rai, eds.), pp. 195–204. UCLA Symposia on Molecular and Cell Biology, New Series, Volume 59. Alan R. Liss, New York.

Marti, G. E., Zenger, V., Caproaso, N. E., Brown, M., Washington, G. C., Carter, P., Schechter, G., and Noguchi, P. (1989). Antigenic expression of B-cell chronic lymphocytic leukemic lymphocytes. *Anal. Quant. Cytol. Histol.* 11(5), 315–323.

Marti, G. E., Faguet, G., Bertin, P., Agee, J., Washington, G. C., Ruiz, S., Carter, P., Zenger, V., Vogt, R., and Noguchi, P. (1992). CD20 and CD5 expression in B-chronic lymphocytic leukemia. *Ann. New York Acad. Sci.* 651, 480–483.

Mavligit, G. M., and Stuckey, S. (1983). Colorectal carcinoma. Evidence for circulating CEA–anti-CEA complexes. *Cancer* 52(1), 146–9.

Morbidity and Mortality Weekly Report (1990). Update: Serologic testing for HIV-1 antibody—United States, 1988 and 1989. 39(22), 380–383.

Morbidity and Mortality Weekly Report (1992). Guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus infection. 47(RR-8), 1–17.

Muranaka, M., Suzuki, K., Koizumi, S., and Takafuji, S. (1986). Adjuvant activity of diesel-

exhaust particulates for the production of IgE antibody in mice. *J. Allergy Clin. Immunol.* 77, 616–623.

Nicholson, W. J., and Landrigan, P. J. (1989). Quantitative assessment of lives lost due to delay in the regulation of occupational exposure to benzene. *Environ. Health Perspect.* 82, 185–188.

Nowell, P. C., and Croce, C. M. (1988). Chromosomal approaches to oncogenes and oncogenesis. *FASEB J.* 2(15), 3054–60.

NRC (National Research Council) (1992). Biologic markers in immune toxicology. Washington, D.C. National Academy Press. 206 pp.

Paul, W. (1989). "Fundamental Immunology," 2d Ed. Raven Press, New York.

Pugh-Humphries, R. G. P., Woo, J., and Thomson, A. W. (1991).

Quinn, M., Redus, M. A., Granade, T. C., Hannon, W. H., and George, J. R. (1992). HIV-1 serologic test results for one million newborn dried blood specimens: Assay performance and implications for screening. *J. AIDS (in press).*

Reidenberg, M. M., and Drayer, D. E. (1986). Procainamide, *N*-acetylprocainamide, antinuclear antibody and systemic lupus erythematosus. *Angiology* 37, 968–971.

Roitt, I. M., Brostoff, J., and Male, D. K. (1987). "Immunology." Mosby, St. Louis, Missouri.

Rothman, K. J. (1986). "Modern Epidemiology." Little, Brown, Boston.

Rystedt, I. (1985). Atopic background in patients with occupational hand eczema. *Contact Derm.* 12, 247–254.

Shah, A. R., Maeda, K., Deegan, M. J., Roth, M. S., and Schnitzer, B. (1992). A clinicopathologic study of familial chronic lymphocytic leukemia. *Am. J. Clin. Pathol.* 97, 184–188.

Sinha, A. A., Lopez, M. T., and McDevitt, H. O. (1990). Autoimmune diseases: The failure of self tolerance. *Science* 248, 1380–1388.

Sinha, A. A., Bell, R. B., Steinman, L., and McDevitt, H. O. (1991). Oligonucleotide dot-blot analysis of HLA-DQ beta alleles associated with multiple sclerosis. *J. Neuroimmunol.* 32, 61–65.

Staberg, B. and Serup, J. (1988) Allergic and irritant skin reactions evaluated by laser Doppler flowmetry. *Contact Derm.* 1, 40–45.

Stiller-Winkler, R., Kramer, U., Fiedler, E., Ewers, U., Dolgner, R. (1989). C3c concentrations in sera of persons living in areas with different levels of air pollution in Northrhine-Westphalia. (Federal Republic of Germany). *Environ. Res.* 49, 7–19.

Tada, T., Okumura, K., Platteau, B., Beckers, A., and Bazin, H. (1975). Half-lives of two types of rat homocytotropic antibodies in circulation and in the skin. *Int. Arch. Allergy Appl. Immunol.* 48, 116–131.

Vicaro, J. L., Serrano-Rios, M., Andres, F., *et al.* (1982). HLA-DR3, DR4 increase in chronic stage of Spanish Oil Disease. *Lancet* 1, 276.

Vogt, R. F. (1991). Use of laboratory tests for immune biomarkers in environmental health studies concerned with exposure to indoor air pollutants. *Environ. Health Perspect.* 95, 85–91.

Vogt, R. F., Dannenberg, A. M., Papirmeister, B., and Scofield, B. (1984). Pathogenesis of skin lesions caused by sulfur mustard. *Fund. Appl. Toxicol.* 4, S71–78.

Waldmann, T. A. (1987). The arrangement of immunoglobulin and T cell receptor genes in human lymphoproliferative disorders. *Adv. Immunol.* 40, 247–321.

Westermann, J., and Pabst, R. (1990). Lymphocyte subsets in the blood: a diagnostic window on the lymphoid system? *Immunol. Today* 11(11), 406–410.

Williams, P. B., Dolen, W. K., Koepke, J. W., and Selner, J. C. (1992). Comparison of skin testing and three in vitro assays for specific IgE in the clinical evaluation of immediate hypersensitivity. *Ann. Allergy* 68(1), 35–45.

Winfield, J. B., and Jarjour, W. N. (1991). Stress proteins, autoimmunity, and autoimmune disease. *Curr. Top. Microbiol. Immunol.* 167, 161–89.

Wordsworth, P. (1991). PCR-SSO typing in HLA-disease association studies. *Eur. J. Immunogen.* 18, 139–146.

Young, N. (1989). Benzene and lymphoma. *Am. J. Ind. Med.* 15, 495–498.

Molecular Epidemiology

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