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Epidemiology studies in immunotoxicity evaluations

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Abstract

Studies in humans designed to detect immunomodulation from exposure to xenobiotics present challenging problems to epidemiologists and immunotoxicologists. Exposed and control groups must be carefully selected, exposure to the xenobiotic must be sufficiently high and well-documented, and the referent group should be as similar as possible to the exposed. Immune markers/functional tests in an individual may be influenced by sunlight exposure, medication, illness and use of recreational drugs; all of these potential confounding factors must be addressed. Sample acquisition is usually performed at sites geographically distant from the controlled environment of an investigator's laboratory, yielding an assortment of new problems that would not occur in clinical or hospital situations. Regulations and guidelines concerning the transport of biological samples and potential hazards of HIV and HBV exposures to personnel must be adapted to field conditions. Since the application of immunotoxicological techniques to populations exposed to xenobiotics is relatively new, and the ability to measure an increasing number of immune biomarkers of activation, suppression, autoimmunity or hypersensitivity is rapidly expanding, there are difficulties in the interpretation of statistically positive results (sometimes within the normal range) and their potential health significance. Finally, both biological and methodological factors complicate the assessment of dose–response/concentration–effect relationships in human immunotoxicity studies, and traditional dose–response relationships may not always be present. Published by Elsevier Science Ireland Ltd.

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1. Introduction

Field studies in humans designed to detect immunomodulation from exposure to xenobiotics present some of the most challenging problems to epidemiologists and immunotoxicologists. Investigators must choose exposed populations with an adequate dose to detect an effect if one is present, quantify exposure on an individual basis if possible, and rule out concurrent exposure of the population to other potential immunotoxicants. A control group must be identified that is similar to the exposed group in all characteristics except for

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exposure to the xenobiotic under study. In some cases this may not be possible, e.g. air pollution studies, where large differences in the exposure concentrations may be the only design possible. Many exposures and circumstances can affect immune function in an individual, including sunlight, stress, medication use and illness; some of these factors may produce immune alterations as great or greater than those predicted from occupational or environmental exposure to xenobiotics (Baadsgaard et al., 1990; Plotnikoff et al., 1991; Clement-Lacroix and Dubertret, 1992; NRC, 1992; Hersey et al., 1993). Such exposures can be evaluated by administering validated questionnaires to subjects, which must necessarily include sensitive topics such as recreational drug use (Weiss et al., 1998) and HIV infection. Sample acquisition (usually of peripheral blood and/or saliva) is performed at sites geographically and temporally distant from the controlled environment of an investigator's laboratory, yielding an assortment of new problems that would not occur in a clinical or hospital situation. Some assays, such as lymphocyte transformation tests, which require almost immediate processing of blood samples may not be possible in field studies where blood samples must be transported to the laboratory over large distances or from remote locales. Some immunological and clinical tests, which might yield important data in some studies (examples are vaccination of study subjects to measure primary antibody response, bronchial provocation testing with workplace antigens), are rarely if ever used in immunotoxicity studies because of concerns about the risk/benefit ratio to study subjects. Subjects involved in immunotoxicity field studies must be briefed about the nature and purpose of the study, provided informed consent and notified of their individual test results and their possible clinical significance as soon as feasible after testing is complete. Since the application of immunotoxicological techniques to populations exposed to xenobiotics is relatively new, there are difficulties in the interpretation of statistically positive results and their potential health significance.

2. A review of the human immune system

2.1. Effect of xenobiotics

A number of textbooks and reviews (Hood et al., 1978; Roitt et al., 1987; Samter, 1988; Paul, 1989; Vogt, 1991; NRC, 1992; Rose and Margolick, 1992) address the general structure of the host defense system, which consists of an integrated network of cells and mediators with recognition and response functions throughout most tissues. Primary functions of the host defense system include tissue repair, the identification and removal of foreign substances, destruction or containment of infectious agents, and the removal of neoplastic cells. These functions are performed both through non-specific mechanisms (innate or natural immunity) and through specific mechanisms (acquired immunity).

Much current literature on immune assessment concerns severe immune deficiency, the first clinical evidence of which is usually frequent or prolonged infections. Such deficiencies can be acquired or congenital, and excellent reviews of the assessment of each type are available (Hood et al., 1978; Roitt et al., 1987; Samter, 1988; Paul, 1989; Stiehm, 1989). Current efforts in the area of immunotoxicology concerns the possibility of subtle xenobiotic-induced defects that may exert their adverse effects over a long period of time, such as in the development of cancer, hypersensitivity reactions or clinically relevant autoimmunities.

Table 1 presents a summary of the major molecular and cellular constituents of the host defense system. Many of the defense and regulatory functions of the immune system are carried out by peptide and non-peptide chemical mediators released from cells. Antibodies (which constitute the humoral branch of the immune response) are antigen-specific. They are secreted by stimulated B-lymphocytes and are comprised of several major classes with different functional capacities. IgM and IgG antibodies facilitate phagocytosis, antigen clearance, and the destruction of parasites. IgA antibodies are secreted at mucous membranes, where they help to prevent attachment and invasion by microbes and parasites that come in contact with the surface tissues. IgE antibodies,

Table 1
Major components of the host defense system[a]

Component	Function
Molecular mediators	
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	
Immunoglobulins; T-cell antigen receptor	Specific antigen recognition on Iymphocytes
Immunoglobulin E	Specific antigen recognition on mast cells and basophils
Class I histocompatibility proteins	
Class II histocompatibility proteins	
	Cell–cell interactions
Immunoglobulin-related protems (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	
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

[a] Modified from Hood et al. (1978).

bound to the outer membrane of mast cells and basophils, help initiate immune responses and are involved with immunity against worms and mites and are also responsible for classical allergic reactions such as hay fever and asthma.

Cytokines are extremely potent peptide molecules that activate or suppress target cell populations supporting appropriate receptors. Numerous interleukins (as of this printing, IL-18 has been described, its major activity is in the induction of IFN-gamma (γ-IFN) production from anti-CD3-activated Th1 cells in the presence of IL-12; Yoshimoto et al., 1997) have been identified as participants in the complicated network of immune regulation. Complement is one of several plasma proteins involved in acute non-specific responses to tissue injury and invasion. Complement is actually a 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 non-peptide 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 an allergy. Several other chemical mediators impact the activity of cells in the immune system, although they are not central to its function. Such mediators include catecholamines (such as adrenalin), endorphins, and insulin. Most of the several different types of cells that constitute the cellular branch of the immune response 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- 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 antibodies (B-cells), killing virus-infected cells or regulating immune activities (T-cells).

An important component of the T-cell is the T-cell receptor (TCR)/CD3 complex, which is a multichain glycoprotein assembly on the surface of T-cells, responsible for peptide–major histocompatibility complex (MHC) recognition and subsequent signal transduction. The TCR α and β (and γ and δ) chains have variable (V) and constant (C) domains analogous to those of antibodies. Hypervariable segments of polypeptides in the TCR V domains are probably functionally equivalent to the complementary determining regions (CDRs) of antibodies, in that they mediate antigen recognition (peptide–MHC in the case of TCRs) (Fields and Mariuzza, 1996).

T-helper cells (CD4+) have been further dichotomized based on their individual patterns of cytokine secretion (Del Prete et al., 1991). In mice, T-helper 1 (Th1) cells produce interleukin 2 (IL2), gamma interferon (γ-IFN) and lymphotoxicin (LT), whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Human Th1 and Th2 cells produce similar patterns, although the synthesis of IL-2, IL-6, IL-10 and IL-13 is not as tightly restricted to a single subset as in mouse T-cells. Several other proteins are secreted both by Th1 and Th2 cells, including IL-3, tumor necrosis factor a (TNF-a), granulocyte–macrophage colony-stimulating factor (GM–CSF), etc. (Mossman and Sad, 1996). In general, Th1 responses are involved in cell-mediated inflammation and delayed-type hypersensitivity (γ-IFN, etc.). Th2 cells are generally involved in humoral antibody production, especially IgE in allergic responses (IL-4, etc.).

Monocytes are immature cells that differentiate into macrophages after emigration 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 non-specific, 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 also primary participants in the allergic responses to pollens, foods, and other substances. In addition, they appear to be involved in inflammatory reactions to certain toxic and sensitizing chemical exposures (Vogt et al., 1984; Vogt and Schulte, 1993).

Immunotoxic agents can exert effects on either the cellular, the humoral, or both branches of the immune response. Classes and examples of chemicals causing immunologic changes are shown in Table 2. Coincident hematological changes (deficiencies involving erythrocytes, platelets, or leukocytes) give evidence for toxic effects at the level of the stem cell. Chronic benzene exposure may induce pancytopenia by a direct effect on bone marrow (Snyder, 1984). Reduction in the immune response may result in decreased host resistance to infection and malignancy. If the deficiency occurs in the T-cell lineage, it characteristically presents clinically as repeated infections by intracellular pathogens (including some protozoan parasites, pathogenic fungi, viruses and certain bacteria). Because the T-cell also seems to play a

Table 2
Classes and examples of chemicals causing immunological changes[a,b]

Class	Examples
Polyhalogenated aromatic	TCDD, PBBs, PCDF, PCBs, hexachlorobenzene hydrocarbons
Metals	Lead, calcium, arsenic, methyl mercury
Aromatic hydrocarbons (solvents)	Benzene, toluene
Polycyclic aromatic hydrocarbons	DMBA, B[*a*]P, MCA
Pesticides	Trimethyl phosphorothioate, carbofuran, chlordane, malathion
Organotins	TBTO
Aromatic amines	Benzidene, acetyl aminofluorene
Oxidant gases	Nitrogen dioxide, ozone, sulfur, dioxide
Particles	Silica, asbestos
Natural products	Selected vitamins, antibiotics, vinca alkaloids, estrogen, plant alkaloids, mycotoxins
Drugs of abuse	Ethanol, cannabinoids, cocaine, opioids
Therapeutic drugs	Diphenylhydantoin, lithium
Others	Nitrosamine, BHA

[a] TCDD, 2,3,7,8-tetrachlorodlbenzo-*p*-dioxin; PBBs, polybrominated biphenyls; PCDF, polychlorinated dibenzofuran; PCBs, polychlorinated biphenyls; DMBA, dimethylbenzanthracene; B[*a*]P, benzo[*a*]pyrene; MCA, methylcholanthrene; TBTO, bis(tris-*n*-butylin)oxide; BHA, butylated hydroxyanisole.

[b] Adapted from Stiehm (1989).

role in resistance to certain malignancies, either directly through the action of cytotoxic T-cells or indirectly through the production of lymphokines, an increased incidence of malignancy may also be a manifestation of cellular immune deficiency. However, it should be noted that no environmental exposure has yet been shown to have caused a cellular immune deficiency severe enough to manifest itself as opportunistic infection or neoplasm. In addition, the neoplasms that occur with increased frequency in immunocompromised humans tend to be derived from cells of the immune system, making it difficult to determine if the immune defect is responsible for the neoplasms or whether some other factor is causing both the neoplasms and the immune deficiency. B-cell deficiency produces a distinct clinical syndrome characterized by increased susceptibility to acute infections with pyogenic bacteria, such as recurrent pneumonia, meningitis, or abscesses, against which antibody is the main protective mechanism. Complement deficiencies show many similarities with B-cell deficiencies. Complement deficiencies involving C6 and C8 characteristically predispose to infections with gram-negative cocci. The manifestations of defects in phagocytes can also mimic B-cell deficiency because of the role of opsonins in facilitating phagocytosis. However, neutrophils from persons with chronic granulomatous disease phagocytize normally but are incapable of producing an oxidative burst in metabolism following phagocytosis. These individuals are prone to chronic infections because the phagocytes can ingest the common organisms but cannot digest and kill them. Although the importance of NK cells for human health is not as well-established as that of B-cells and T-cells, they are believed to be important in host defense against viruses and tumors (Penn, 1981). There is a case report that describes a patient with recurrent herpesvirus infections who was shown to have a selective absence of NK cells (Biron et al., 1990).

Autoimmune diseases are immune reactive disorders in which the immune system reacts against the host's tissues. Autoimmune reactions are often associated with antibodies that react to self proteins, in particular tissues or cell components. Auto-immune 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 (Vogt and Schulte, 1993). Systemic lupus erythematosus (SLE, evaluated by titer of antinuclear auto-antibodies) has been shown to be associated with exposure to trichloroethylene and other chemicals in contaminated well water (Kilburn and Warshaw, 1992) and by occupational exposure to hydralazine (Reidenberg et al., 1983).

3. Consequences of immunosuppression

The study of human immunodeficiency disease

Table 3
Consequences of immunosuppression-deficiencies and diseases[a]

Syndrome	Cell type affected	Result
DiGeorge syndrome	T-cell	Increased bacterial, viral and yeast infections
Nezeof's syndrome	T-cell	Increased bacterial, viral and protozoan infections
Common variable immunodeficiency (CVD)	B-cell (T-cell)	Increased bacterial infections
Bruton's disease X-linked infantile hypogammaglobulinemia	B-cell	Increased bacterial infections
Selective IgA deficiency	B-cell	Increased bacterial infections
Waistcoat-Aldrich syndrome	B- and T-cells; monocytes	Increased bacterial and viral infections
Ataxia telangiectasia (A-T)	B- and T-cells	Increased bacterial and viral infections
Severe combined immunodeficiency disease (SCID)	B- and T-cells	Increased bacterial and viral infections
Reticular dysgenesis	Leukocytes	Increased bacterial and viral infections
Adenosine deaminase (ADA) deficiency	Th-cells (direct); B-cells (indirect)	Increased bacterial and viral infections
Chediak- Hagashi syndrome	Phagocytes, NKs, and Tc-cells	Increased bacterial infections
Chronic granulomatous disease	Phagocytes (primarily neutrophils)	Increased bacterial infections
Complement deficiency C1-C8	–	Increased bacterial infection

[a] Adapted from Stiehm (1989).

syndromes reveals a clear association between the suppression or absence of an immunological function and an increased incidence of infectious or neoplastic disease. Numerous examples of such deficiency diseases have been reported and are well-characterized in humans (Table 3). A deficiency in one or more immunological function can lead to severe, recurrent infections throughout life. These infections can be bacterial, viral, fungal, or protozoan, and the predominant type of infection depends on the associated immunological lesion. Some infections can be treated with antibiotics or gamma-globulin, and in some cases the immunological defect can be restored by bone marrow transplantation. However, other immunodeficiency diseases are much more severe. For example, children born with reticular dysgenesis have no white blood cells and usually die from infectious disease in the first year of life; children born with ataxia telangiectasia rarely survive past puberty. These diseases of genetic deficiency are more severe than those caused by environmental toxicants, because they are the result of the absence of part of the immune system. They demonstrate well-characterized consequences of immunosuppression. These same diseases would be expected to be associated with specific immunosuppression, whether the causes were genetic or environmental (NRC, 1992).

3.1. Xenobiotic effects on the immune system

Unintentional exposure to environmental toxicants has also been shown to have consequences on the immune system. Exposure to contaminated rice oil containing halogenated biphenyls (Yusho) in Japanese populations resulted in alterations in immune status and the respiratory system. Respiratory distress occurring in these individuals, although improving over time, still exist many years after exposure. Changes in the immune status of Yusho patients exposed to polychlorinated biphenyl (PCB) and polychlorinated dibenzofuran (PCDF) include decreased serum IgA and IgM at early stages of the disease. Long-term suppression of cellular immunity has been reported in Taiwanese patients (Nakanishi et al., 1985).

More recently, occupational exposures to ethical narcotics (Biagini et al., 1990, 1992, 1995) and macrocyclic tricothecenes from *Stachybotrys char-*

tarum (Johanning et al., 1996) have also been shown to be toxic to the immune system. *Stachybotrys chartarum* exposure lowered the percentage of CD3+ T-cells in exposed employees when compared with controls. Regression analyses showed significantly lower percentages of CD3+ among those reporting a history of upper respiratory infections.

Occupational ethical narcotic exposures have been shown to significantly increase the percent of T-helper-inducer (CD3+/CD4+) cells with no effect on absolute numbers of T-helper-inducer cells; significant increases in cells expressing the HLA-DR phenotype and a significant decrease in lymphocyte transformation stimulated by pokeweed mitogen. Other immune abnormalities were also noted (significantly elevated specific antimorphine IgG levels, lower epicutaneous threshold concentrations [ETC] for positive skin tests and changes in pulmonary function).

4. Non-xenobiotic immunomodulators

Stress (from psycho-social or occupational stressors) or malnutrition (Zaman et al., 1997) may result in immune suppression, which may in turn, lead to reduced disease resistance (Cohen and Williamson, 1991). In animal models, lowered disease resistance has resulted in infections, cancer or auto-immunity, while clinical case studies have shown severe stress (i.e. bereavement) to be associated with increased mortality, altered immunity including suppression of lymphocyte responses to mitogens, reduced natural killer (NK) cell activity and changes in T-cell subpopulations (Plotnikoff et al., 1991). Stress also can affect the normal homeostatic relationships between the immune, nervous, and endocrine systems (Jankovic et al., 1987; NRC, 1989).

Psychometric instruments (questionnaires) for measuring job stress have been developed and applied to the workplace; however, biological indicators of stress (biomarkers) would be a valuable objective measure to complement these questionnaires (Hurrell and McLaney, 1988). Several biological indices have been studied in the past to ascertain their value in detecting physiological and health effects of various types of stress. Levels of cortisol in saliva, urine and serum have been studied most often (Fibiger et al., 1985), while other endpoints examined include blood pressure, heart rate, visual accommodation, ACTH, catecholamines, blood counts, immunoglobulins, cytokines, immunocompetency, etc. (Jemmott and McClelland, 1989; Plotnikoff et al., 1991). Due to problems with methods/design, these studies have had varying degrees of success. Changes in cortisol, catecholamines, and other hormones are usually only transient responses to acute stressors, and have not proven to be appropriate measures of chronic stress. Many of the transient responses have relatively large natural fluctuations due to biological rhythms (Jemmott and McClelland, 1989), and are also quite variable in heterogeneous human populations and environments.

Salivary IgA (sIgA) is another potential biomarker for stress-induced immunologic effects in workers (Kugler, 1991; Henningsen et al., 1992). sIgA has particular appeal as a potential biomarker because (a) it can be obtained non-invasively, easily and frequently compared with blood; (b) it is biologically relevant as a functional immune endpoint; (c) it can be quantified; and (d) it is more stable with a longer biological half-life than cortisol and catecholamine. Controversy remains concerning the best methodology to measure sIgA (Stone et al., 1987; Jemmott and McClelland, 1989; Mouton et al., 1989); therefore, total IgA levels, specific IgA titers, or both, and total salivary protein concentration and/or salivary flow rates have been measured in previous studies (O'Reilly, 1989). Some important confounding factors that may affect sIgA levels include disease, nutrition, age, hormonal activity, certain medications, trauma or exertion, and biorhythms (Vogt and Schulte, 1993).

5. Effects of radiation

It is well-known that exposure to ionizing radiation damages the immune, hematopoietic, and gastrointestinal components of the host defense system. This may lead to serious endogenous or

exogenous infections. When radiation injury is combined with other physical trauma, e.g. burns or wounds, the resulting damage to these systems is synergistic (Ledney et al., 1992).

Exposure to non-ionizing ultraviolet radiation (sunlight, UV) is the most common non-xenobiotic exposure with effects on the immune system. Ultraviolet exposure can lead to suppression of the normal immune response, which may play an important part in the development of skin cancers, infectious diseases, and auto-immune responses (Clement-Lacroix and Dubertret, 1992). NK cell activity has been shown to be suppressed in volunteer subjects exposed to ultraviolet radiation from solarium lamps (Hersey et al., 1993). In studies using xenon arc lamp sources, it also appears that UV-A may have equivalent or greater direct immunosuppressive effects than UV-B (Hersey et al., 1993). Epidermal cells from UV-exposed skin, in contrast to epidermal cells from normal skin, potently activate autologous CD4+ T-cells, and, in particular, the CD45RA+ (2H4+) (suppressor-inducer) subset, suggesting that UV-exposure in humans leads to a T-cell response in which suppression dominates (Baadsgaard et al., 1990).

6. Design of field studies

Clinical assessment of individuals usually starts with evidence of an immunological deficit or dysfunction. This immunological deficit or dysfunction is then investigated in order to associate the effect to exposure to a particular drug, toxic agent or exposure. This chapter will focus on studies of groups of individuals with occupational or environmental exposure to a potentially toxic agent to detect (usually) subclinical changes in immune function. General issues in epidemiological study design and analysis are discussed in several texts (Kelsey et al., 1986; Rothman, 1998). The most common epidemiological study design used in immunotoxicity research is the cross-sectional study. In such a study, exposure status and immunological function are measured at one point in time or over a short period of time in study subjects (Colton, 1974). The immune function of exposed subjects is compared with the immune function of a comparable group of non-exposed individuals.

The first challenge in conducting an immune assessment study is to identify the exposed group. In studies designed to evaluate the immunotoxicity of a chemical (as opposed to studies where immune function evaluation is prompted by a public health concern), the study should include populations at the upper end of human exposure, unless previous studies have already established an immunotoxic effect in that range. Where possible, the study should incorporate individual estimates or measurement of dose, and utilize biological monitoring to estimate internal dose. Once the exposed group has been identified, a clear definition is needed of who is eligible to participate in the study. For example, in an occupational study, all individuals who have worked in a particular department might be considered eligible exposed subjects; in an environmental study, eligible persons might include all residents of a community or a sample of households in a community. It is important to enumerate the number of potentially eligible subjects, as well as the number who eventually participated, in order to assess the likelihood that selection bias has influenced the study findings. Selection bias may occur when an individual's willingness to participate varies with characteristics related to exposure status or health status of the individual. Although it is difficult to avoid or detect selection bias in a voluntary study, a high degree of participation makes it less likely that selection bias has influenced the results.

In many field situations, the potential immune effects of other chemicals present in the industrial or residential environment needs to be considered. The investigator should assess all chemicals present in the exposed and control environments and whether any of these other chemicals have either known or suspected effects on the immune system. Exposures of individual study subjects to chemicals outside the study environment should also be evaluated. For example, in an occupationally-based study, subjects could be questioned about chemical exposures in hobbies or second jobs. In a study of community residents exposed to an immunotoxin as a result of environmental

contamination from a nearby factory, an assessment should by made of other contaminants in the local environment, as well as potential occupational chemical exposures of study subjects.

Known risk factors that might influence the outcome of immune function tests (such as age, gender, cigarette smoking, sunlight exposure, stress, use of certain medications and recreational drugs) should be matched in the design of the study or controlled in the analysis. However, there is limited qualitative or quantitative data on the influence of these factors on immune function in the general population, and it is impossible for an epidemiological study to match or analyze all potential factors. For example, differences in dietary habits, exercise levels, or community specific exposures to particular viruses might conceivably influence comparisons between an exposed and non-exposed population. Yet it is often not practical to collect information on all such factors. It is therefore, desirable to select the comparison group to be as similar as possible to the exposed group in order to (hopefully) match factors that cannot be measured. For example, in an occupationally-based study, the comparison group should be selected so that the community of residence, socio-economic status and ethnicity is similar to the exposed group. In an environmental study, the comparison group should be selected from a geographical area whose residents have a similar ethnic distribution, socio-economic status and employment pattern as residents in the exposed area.

The study should account for medical factors that might have a major impact on immune function. For example, individuals who are immunosuppressed as a result of chemotherapy or steroid treatment should be excluded from the study. Other medications and medical exposures (immunizations, medications, radiation) in the recent time period should be inquired about and evaluated in the statistical analyses.

Because many of the immune function tests have potential variability between laboratories or within a laboratory over time, it is desirable to have each test run by the same laboratory for all exposed and unexposed subjects in a particular study (or if that is not possible, to at least ensure that equal proportions of exposed and unexposed subjects are tested by each laboratory). The laboratory conducting the tests should validate each test procedure to assess measurement variability and variability within subjects before the analysis of study samples begins. In addition, an intra-laboratory study, where identical tests are conducted by numerous laboratories and their results compared, is sometimes useful. It is also desirable to recruit exposed and unexposed subjects into the study during the same time period, so that samples analyzed on a particular day include both types of subjects. This will minimize the effect on the study findings of any undetected changes in laboratory conditions that shift test results upwards or downwards on a particular day. Finally, samples should be acquired at near the same time of day from all subjects to minimize the effects of circadian rhythm.

In addition to the characteristics that should be considered in evaluating the methodology of study, there are some issues that are particularly important in the interpretation of positive studies, and others that are particularly important in the interpretation of negative studies.

In interpreting studies that show significant differences between the exposed and nonexposed populations, it is important to recognize that large cross-sectional studies (i.e. studies with 100–200 exposed and control subjects, will have adequate statistical power to detect relatively small differences (on the order of 10%) in immunological endpoints, such as proportion of CD4+ and CD8+ cells. Although such studies might be interpreted as positive, particularly if there is evidence of a dose–response relationship, there will be considerable overlap between the values of unexposed and exposed individuals, and it is possible that none of the individual values will fall outside the clinically normal range. Interpretation of such findings is complicated because there is little quantitative data on the degree to which such parameters need to be modified in a population before the population experiences an increased risk of disease (Trizio et al., 1988).

In addition, because statistically significant differences measured between the two populations may be small in magnitude and of unknown

clinical significance, the possibility that they reflect methodological errors rather than a true biological effect is of concern. Methodological problems that might spuriously produce such a finding may include selection bias, laboratory variability or lack of control for confounding variables. Careful design and analysis of studies examining changes in immune function tests as the primary outcome is therefore of critical importance.

Evidence of a dose–response relationship is usually an important criterion in the assessment of a toxic exposure. However, both biological and methodological factors complicate the assessment of dose–response in human immunotoxicity studies. Traditional dose–response relationships may not always be present for immunologically-mediated effects. For example, experimental models suggest that higher doses might be tolerogenic, while moderate doses might be immunogenic (Biagini et al., 1983). For some hypersensitivity phenomena, the question of individual susceptibility may complicate the assessment of dose–response in the population. Idiosyncratic immune responses may also be observed with treatment by immunosuppressive agents (Hanly et al., 1995).

In evaluating a positive study that does not demonstrate a dose–response relationship, a general issue is whether the dose estimate employed takes into account data on the absorption, metabolism and distribution of the chemical. Frequently, epidemiological studies assume that the quantitative dose measurements available (i.e. concentration of the chemical in air, blood or urine) are proportional to potential dose at the target organ of concern (i.e. bone marrow, primary or secondary lymphatic organs). This is not always the case. For example, air concentration may be a poor surrogate for internal dose if the compound can be absorbed through the skin as well as inhaled, or if respiratory protection has been used by some workers and not others. For chemicals with a long half-life, such as lead, an identical urinary lead concentration may reflect substantially different tissue-specific concentrations in long- versus short-term workers. In addition to inferring dose level estimates for individual subjects from quantitative measurements taken at the time of the study (or from using available historical measurements), the dose-estimate used in the model should be restricted to what is considered the most biologically relevant time period. For example, in a cross-sectional immunotoxicity study, the relevant time period may be the previous 6 months rather than the cumulative lifetime exposure. Because the biologically relevant time interval is not known, and may differ for different immunological outcomes, the assessment of dose response is even more complex than in epidemiological studies of other outcomes. The healthy worker effect (HWE, apparent decreased mortality and morbidity in workers when compared with the general population) is also a potential confounder in the interpretation of the immunotoxic outcomes from exposure to xenobiotics, especially when exposed workers are compared with normal reference values for a particular outcome (Choi, 1992).

In evaluating a negative study, one important issue is whether the study size was adequate to detect a difference of a specified size in the immune function parameters of interests (statistical power). The statistical power of a study to detect a difference between two populations in the mean of a continuous variable (such as serum IgG level, proportion of CD4+ lymphocytes) depends on the size of the study groups, the mean and variance of the outcome in the study groups, the specified type I difference, and the size of the difference to be detected (Colton, 1974). Power calculations are usually done a priori in planning an epidemiological study. However, there are occasions when they may be useful in interpreting a negative study result. In comparing the results of contradictory studies, one issue that can be considered is the precision of the differences in point estimate (i.e. the confidence interval for the estimated difference between the two groups). Of equal importance in evaluating a negative study is whether there is evidence that the exposed population actually had substantial (well-documented biomonitoring or environmental sampling indicating exposure) to the xenobiotic of interest.

Other types of data on the immunotoxic effects of chemicals in humans are potentially useful. Case reports may arise from clinical identification

of individuals with a particular exposure who have immune function changes or a disease of the immune system. Such reports are particularly valuable in generating hypotheses for well-designed epidemiological studies, and may provide support for other toxicological or epidemiological data.

Aside from the cross-sectional study design, two alternative epidemiological study designs may be utilized in immunotoxicity studies. In longitudinal studies, one or more groups of people that are free of disease and that differ according to extent of exposure to a potential cause of the disease are compared with respect to incidence of the disease in each of the groups (Rothman, 1998). A variant of this design that might be utilized in immunotoxicity studies would compare immune function test results within individuals before and after a defined exposure. Another variant would define exposed and unexposed groups cross-sectionally, administer immune function tests and follow the subjects prospectively to assess relationships between immune function test results and development of clinical disease.

In case–control studies, persons with a given disease (the cases) and persons without a given disease (the controls) are selected: the proportion of cases and controls who have certain background characteristics or have been exposed to possible risk factors are then determined and compared (Kelsey et al., 1986). It should be considered here that case definition may include individuals with a continuum of immunological changes from exposure, ranging from homeostatic immunological responses to frank immunologically-mediated disease. Case–control studies of the etiology of immunologically-mediated diseases might identify increased risk of previous exposure to particular chemicals among the cases; such a finding would be particularly relevant if supported by toxicological studies or evidence of immune function changes among humans exposed to the chemical.

Similar methodological considerations apply to the evaluation of findings from cohort studies and case–control studies as were discussed for cross-sectional studies. These are discussed in textbooks of epidemiology (Kelsey et al., 1986; Rothman, 1998).

7. Logistics of sample acquisition and analysis

Pre-sample acquisition planning is the hallmark of a successful and safe field study designed to investigate immunological endpoints. Human field studies are often limited to sampling peripheral blood, which does provide a convenient source of cells and mediators and in some cases, saliva. However, it should be kept in mind that 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 interstitial tissue at local sites of injury and infection. Cell traffic and recirculation through the blood is controlled carefully 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 (Vogt and Schulte, 1993).

Before actual acquisition of the first sample, numerous technical and safety details must be considered. Recognition of the possibility of transmission of the human immunodeficiency virus (HIV) and with it the acquired immunodeficiency syndrome (AIDS) to healthcare workers is a contemporary reality (Wicher, 1993). Other infectious agents, such as hepatitis B virus (HBV) and tuberculosis (Hellman and Gram, 1993) pose a risk to healthcare workers (Jackson and Pugliese, 1992). A thorough understanding of the appropriate procedures, responsibilities, and risks inherent in the collection and handling of patient specimens should be acquired by everyone with potential exposure. Methods for decontaminating surfaces, disposal of broken glass, contaminated equipment, etc., in the event of a spill or accident at the field site also need to be planned in advance of a study. The Agent Summary Statement for Human Immunodeficiency Virus (HIV) (MMWR, 1988) is a valuable resource document. The Occupational Safety and Health Administration (OSHA) has also published a guide for OSHA personnel and employers: *Enforcement Procedures for Occupational Exposure to Hepatitis B Virus and Human Immunodeficiency Virus (HIV)* (OSHA, 1988). Universal Precautions as appropriate practices for laboratory personnel exposed to human samples

has been addressed by the CDC (MMWR, 1989). Glove use by health care workers is a major aspect of Universal Precautions. However, recent research indicates the possibility of allergic reaction to latex in some individuals, and this should be taken into account (NIOSH, 1997).

The collection of blood in a field situation requires planning for emergencies, such as cardiovascular responses to phlebotomy in middle-aged and elderly subjects (Kuchel et al., 1992). Phlebotomy technicians should have recent certification in cardiopulmonary resuscitation (CPR) techniques. The location of the nearest hospital with emergency services, and the telephone number to summon emergency medical assistance should be immediately available. A private site without through traffic, with appropriate chairs and space for phlebotomy should be utilized.

Additional planning is needed to ensure that samples collected in the field are accurately labeled and transported to the laboratory quickly and safely. A labeling scheme and system that is designed to unequivocally identify samples and be rugged enough to withstand the rigors of transport has to be designed (i.e. labels or identifiers don't come off during shipment). All state and federal regulations concerning the transport of blood or blood products should be adhered to (NCCLS, 1985; CFR, 1987). Cardboard boxes with styrofoam liners are the transport system of choice for blood samples. Whole blood for immunological analyses is best transported and maintained at room temperature (18–22 C). Temperatures less than 10 C and/or greater than 37 C should be avoided (Shield et al., 1983; McCoy et al., 1990). This can be accomplished (depending on the season or the expected temperatures to which the shipment may be subjected) by adding a cooling pack to the transport box with the addition of an insulating material (such as newspaper). Obviously, in cooler weather, insulation only will suffice. A maximum–minimum recording thermometer should also be added to the shipment container to document temperature transients experienced.

The time between phlebotomy and sample preparation/measurement at the investigator's laboratory should be kept to a minimum. The choice of anticoagulants is also important. The age of a blood specimen can influence the results of some determinations, often in a method-dependent fashion. Although stable differential counts were reported over 24 h of storage by one automated method (Warner and Reardon, 1991), inspection of the data reveals significant bias in results by 18 h, as reported by others (Vineis et al., 1993). For immunophenotyping, acid, citrate, dextrose (ACD) or heparin are the anticoagulants of choice, and samples may be processed for up to 30 h post-draw (MMWR, 1997). Sera for biochemical and immunological analyses can be either separated, frozen on-site, and shipped frozen or transported in serum-separator tubes. As a design control for transport, blood from exposed subjects should be included in the same shipment as blood from non-exposed individuals. In this way, significant shipment effects, if present, can be identified by statistical analysis.

8. 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. Infectious or constitutive 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 auto-immunogenic 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 (Vogt et al., 1984). Immune markers and clinical chemical 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. An 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

Table 4
Classification of tests for immune markers[a]

Test category	Characteristics	Specific tests
Basic-General; should be included with general panels	Indicators of general health and organ system status	BUN, blood glucose, SALT, etc.
Basic-Immune; 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; auto-antibodies; 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

[a] Modified from Hood et al. (1978).

guidelines for the use of biomarker tests in health assessment studies conducted at Superfund sites (CDC, 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 4).

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. 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 group may be quite high, and may require a large number of subjects 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.

9. Other immunological and clinical tests

Many xenobiotics produce clinically significant sensitization reactions in a proportion of subjects. For example, in a study of opiate production workers, NIOSH investigators found both evidence of immunosuppression (significantly decreased percentages of T-helper-inducer [$CD4^+$] cells) (Henningsen et al., 1990) and evidence of sensitization (lowered epicutaneous thresholds to dihydrocodeine, hydrocodone, codeine, and morphine) (Biagini et al., 1992), elevated serum antimorphine IgG (Biagini et al., 1990), and an elevated prevalence of asthma (Biagini et al., 1992). In designing studies of immunomodulation by exposure to mycotoxins, potential immunologic outcomes include sensitization by fungi or their products (leading to clinical disorders such as asthma and hypersensitivity pneumonitis), as well as direct toxicity of the mycotoxin leading to immunosuppression. Frequently therefore, some clinical assessment must be incorporated into the field study. Tests that fall under the auspices of clinical review should have a degree of standardization sufficient to preclude misdiagnosis of clinical disease. However, even these tests are subject to significant methodological biases that must be monitored to insure comparability of data. This is especially true for multisite and longitudinal studies, in which the same tests will be done at different laboratories or times. Techniques readily incorporated into field studies include assessment of respiratory tract symptoms by questionnaire, pre- and post-shift and waking hour peak flow testing (Neukirch et al., 1992; Cote et al., 1993). Tests that are valuable in clinical assessment but are less readily administered in field situations include skin testing (Van-Metre et al., 1990; Brand et al., 1991) and bronchial provocation

testing (Irwin and Pratter, 1990) and nasal lavage testing to enumerate and identify nasal cells (Pinkerton et al., 1997). Such tests require greater medical expertise on the part of the field team and some tests (i.e. bronchial provocation testing) require transportation of the study subjects to a clinical facility. Although skin testing to detect sensitization to specific allergens is a well-accepted clinical procedure when FDA-approved test batteries are used (Adinoff et al., 1990), antigens of concern in the occupational or environmental setting may not be available in FDA-approved form.

One of the limitations of the immunotoxicity test battery in which components of the immune system are quantified (i.e. serum immunoglobulin levels, lymphocyte subset analysis) or specific functional activities measured (i.e. NK cell activity) in peripheral blood samples, is that it does not measure the ability of the immune system to respond in an integrated way to an antigenic challenge. For some immunotoxicants, a decreased primary antibody response is one of the main effects observed in animals (Descotes, 1988). In theory, the primary antibody response could be measured in pediatric populations, who receive many routine immunizations, although the difficulties of venipuncture in young children is an important consideration. In adults, the primary antibody response could be measured by administering vaccines that have a low frequency of side effects and some potential benefits to the general population (Gardner and Schaffner, 1993). For example, although public health guidelines limit Hepatitis B vaccination in the adult population to certain risk groups, including workers at risk of occupational contact with blood, approximately 30% of cases occur in individuals with no known risk factors (Gardner and Schaffner, 1993). It could be argued that studies utilizing Hepatitis B vaccination as an investigational tool to measure the primary antibody response would be of minimal risk, and some potential benefit to study participants who receive the vaccine. However, to the knowledge of the author, administration of vaccines to measure primary antibody response has not been done in field studies to assess the immunotoxicity of occupational or environmental exposures.

10. Clinical Laboratory Improvement Amendments (CLIA '88)

The Clinical Laboratory Improvement Amendments of 1988 (CLIA '88) legislation grew out of a series of media reports of patient harm and missed diagnoses from poor clinical laboratory performance. Congress responded to these reports by passing the CLIA '88 law. CLIA '88 is actually an amendment and technical revision to the Clinical Laboratory Improvement Act of 1967, which was intended to update laboratory requirements and impose new quality assurance standards applicable to laboratories participating in Medicare and Medicaid programs (Federal Register, 1988). In 1990, the Department of Health and Human Services issued a set of their own regulations implementing the legislation (Federal Register, 1990). The new law and the regulations that support it, expand the application of laboratory registration, personnel and performance requirements. In 1991, rules were published that set forth sanctions that could be imposed on laboratories that do not meet Federal requirements (Federal Register, 1991). Some, but not all evaluations of immunological endpoints used in immunotoxicology studies fall under either the moderate- or highly-complex CLIA test categories. A recent review of HHS categorization of CLIA test systems in laboratory immunology is available (Abbot and Homburger, 1993). Specific requirements for proficiency testing, subject test management, quality assurance, personnel and inspections are outlined in the Amendment. A valuable resource document for implementing the CLIA amendments is available (MMWR, 1992).

11. Human subjects issues

The immune function tests routinely used in immunotoxicity studies by the Centers for Disease Control and Prevention and the National Institutes for Occupational Safety and Health currently require only 40–50 ml of blood, and/or 1–5 ml of saliva, which presents minimal risk to study subjects (Turkeltaub and Gergen, 1989). Immunotoxicity protocols, informed consent

documents, and letters notifying subjects of their individual test results are reviewed by each Agency's Institutional Review Board to ensure that subjects are being adequately informed of the nature and purpose of the study, their rights as study subjects, circumstances under which Federal regulations allow release of individual identifying data, that adequate emergency plans (including the availability of staff trained in CPR) are in place, and that they will be informed of both their own tests results and the overall results of the study. One difficulty in immunotoxicity studies and other studies where a large number of tests are run, is to the utility of the various tests in a succinct but understandable way to the study participants. Because in rare circumstances, the results of a clinical or immunological test may suggest a serious clinical abnormality, it is important that test results outside the reference range be flagged by the laboratory and reviewed by a qualified physician so that the participant may be informed promptly that he or she should seek medical attention.

The rapidity of development of new knowledge concerning the immune system and the ability of making measurements without known reference ranges leads to the situation where a measurement can be made that is uninterpretable at the present time. Apparent abnormalities in these quasi-uninterpretable tests should be flagged for the physician as being of concern with a caveat that the tests are of a research nature and should be interpreted for the patient in the context of all the immunological tests performed.

The use of computerized data acquisition and analyses, relational databasing of individual results with an embedded link to an expert system or neural network to help flag individuals with potentially severe clinical immune abnormalities is a future goal of NIOSH studies designed to evaluate the effect of workplace chemicals on the immune system.

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