V D. Immunotoxicity Studies

Exposure to various chemicals has been associated with toxicity of the immune system in animals; these include environmental contaminants, chemicals in the occupational environment, and direct and indirect food additives. Regulatory agencies, including FDA, [1] have recognized the importance of these types of effects for assessing the safety of chemicals to which humans may be exposed. Because of the rapid emergence of the field of immunotoxicology during the past two decades and the abundance of information that has accumulated with regard to the immune system as a target organ, various federal agencies and international organizations are preparing guidelines for the conduct of immunotoxicity studies. [2-6] In addition, various testing approaches have been proposed by researchers in the field. [7-13]

1. Immunity: A Brief Review

The immune system has been described in detail in a number of excellent reviews. [14-18] Thus, only those aspects of immunity which are particularly relevant to immunotoxicity testing will be reviewed in this section. Immunological function encompasses a complex array of participating cell types and organ systems. Immunity may be defined in relation to the function of the various cellular components.

a. Humoral Immunity

Humoral immunity is defined in terms of the B-lymphocytes (B-cells), the antibody producing cells of the immune system. The B-cells, named because of their functional similarity to antibody-producing cells derived from the Bursa of Fabricius in birds, are found primarily in the spleen, lymph nodes, Peyer's patches in the gut, peripheral blood and bone marrow. The bone marrow is also the site of origination of B-cell precursors, the stem progenitor cells.

Immunoglobulins (Igs), the class of proteins that is comprised of the antibodies, are further classified with regard to particular peptide regions found on the light and heavy chains. At least five major classes of immunoglobulins have been defined for man and animals: IgA, IgD, IgE, IgG, and IgM. Antibodies function in concert with complement proteins that are produced in the liver and by macrophages to provide protection against bacterial and viral infections. Antibodies also help protect man and animals from agents that cause tumors and from some spontaneously occurring tumor cells.

Humoral immunity can be further classified with regard to the dependence of antibody production on T-lymphocyte help: T-cell dependent and T-cell independent immunities.

b. Cell-Mediated Immunity (CMI)

CMI derives its name from classical studies that demonstrated adaptive cell transfer of immunological function, graft v. host reactivity, etc. CMI is associated with the T-lymphocytes or T-cells (thymus-derived). Various classes of T-cells have been described, such as suppressors, helpers, inducers, and cytotoxic cells. Some of these T-cell types are involved in B-cell immunoregulation. T-cells secrete various peptide factors, referred to as lymphokines or cytokines, that modulate the activity of B- and T-cells. Cytotoxic T-cells participate in direct killing of invading microorganisms and tumor cells. T-cells are now commonly defined in terms of various membrane "antigens", such as T-4 (or CD4) for helper/cytotoxic cells and T-8 (or CD8) for suppressor/cytotoxic cells.

c. **Non-Specific Immunity**

Non-specific immunity is derived from other cell types that participate in the immune process. Natural killer (NK) cells are a group of cells that share certain properties with T-cells, but probably arise from different stem progenitor cells. [19] These cells are known to play an important role in immune surveillance against spontaneous tumor formation. They also serve as a first line of defense, in cooperation with other phagocytic leukocytes (phagocytes or granulocytes), in the destruction of invading viruses and bacteria. Macrophages (activated monocytes) play a key role in antigen processing and presentation to lymphocytes; they interact with the T- and B-cells to facilitate antibody production. These cells also secrete cytokines, such as interleukin-1, which modulate certain T-cell functions.

Modulation of host resistance to infectious organisms can be the result of either direct or indirect effects on various cell components. Reduction in host resistance is referred to as immunosuppression. Severe or prolonged immunosuppression, as manifested in acquired immunodeficiency syndrome (AIDS), can result in an overwhelming number of infections, tumor formation, and death. Immune enhancement or hyperactivity of the immune system can result in hypersensitivities, such as allergic disorders and autoimmune diseases. The mechanisms of these disorders and diseases are complex and are dependent on factors such as genetic predisposition, age, medical condition, and environment. The development of autoimmunity, which has been associated with the use of various drugs, [20] can have a pronounced toxic effect on a number of organ systems.

True allergic reactions, which are mediated mainly by IgE in man and certain animals, can result in a life-threatening condition known as anaphylactic shock. Certain food additives, such as sulfites, have been restricted in use because of their high sensitizing potential. [21] Other food chemicals have been associated with hypersensitivity-like conditions such as the toxic oil syndrome [22] and tryptophan-induced eosinophilia myalgia.[23]

2. Key Concepts in Immunotoxicity Testing

These guidelines relate to the safety assessment of direct food additives and color additives used in food; such assessments are done on a case-by-case basis. The recommendations for immunotoxicity testing of food and color additives used in food presented in this section may or may not be relevant to those of other agencies and organizations. However, certain concepts from which these recommendations derive are shared by various others[10,11,12,24] including the World Health Organization. [6] Other concepts may be unique to FDA, since these guidelines have been developed within the toxicity testing framework set forth in this book. These concepts are:

- Two types of immunotoxicity tests/procedures are defined: Type 1 Tests are those that do not require any perturbation of the test animal, such as immunization and challenge with an infectious agent.

  i) Primary indicators of immune toxicity are derived from Basic Type 1 Tests, such as hematology and serum chemistry profiles, routine histopathology examinations, and organ and body weight measurements from standard toxicity studies described in other sections of this book. Additional procedures, such as measurements of thymus weights and performance of more definitive histopathological evaluations of immune-associated organs and tissues, have been added.

  ii) Indicators of immune toxicity can also come from Expanded Type 1 Tests. These tests are logical extensions of Basic Type 1 tests; for example, Expanded Type 1 tests may extend the hematology, serum chemistry, and histopathology evaluations of standard toxicity studies. Many of these expanded tests can be performed with the same blood and tissue samples collected for the Basic Type 1 tests; in addition, many of the expanded tests can be performed retrospectively.

- Type 2 Tests include injections or exposure to test antigens, vaccines, infectious agents or tumor cells. If Type 2 tests are to be performed concurrently with a standard toxicity study, a satellite group of animals should be added to the recommended number of test animals in the study. Protocol designs for standard toxicity studies that include a satellite group of animals for Type 2 immunotoxicity tests will be

recommended when available information indicates that a test compound may present an immunotoxic risk.

◙ Sets of Basic and Expanded Type 1 Tests are defined as Level I Immunotoxicity Tests. Some Level I tests screen for immunotoxic effects in test animals; others focus on defining an immunotoxic effect more specifically, such as determining the mechanism or cell types involved. Analogously, sets of Type 2 tests are defined as Level II Immunotoxicity Tests; Level II tests also can be used to screen for, or more specifically define, immunotoxic effects of food and color additives used in food.

3. Indicators of Possible Immune Toxicity

Basic Type 1 Tests: Primary Indicators

The primary indicators of possible immune toxicity are derived from routine measurements and examinations performed in toxicity studies recommended in other sections of this publication (Basic Type 1 tests). Indicators derived from short-term and subchronic toxicity studies, and developmental toxicity studies with rodents are listed below. If a substance produces one or more of these primary indicators of immune toxicity, more definitive immunotoxicity tests (Expanded Type 1 tests or Type 2 tests) may be recommended; such decisions will be made on a case-by-case basis.

a. Indicators from Short-Term and Subchronic Toxicity Studies

◙ Hematology Indicators: Elevation or depression in white blood cell (WBC) counts; altered differential WBC counts; lymphocytosis and lymphopenia; and eosinophilia.

◙ Clinical Chemistry Indicators: Elevated or reduced total serum protein in combination with an abnormal albumin-to-globulin (A/G) ratio. Other indicators often associated with immunologic dysfunction include abnormal levels of liver proteins and enzymes, such as albumin and the transaminases.

◙ Histopathology Indicators: Abnormalities found during gross and routine histological evaluation of the lymphoid tissues, *e.g.* spleen, lymph nodes, thymus, gut-associated lymphoid tissue (GALT, in particular Peyer's patches), and bone marrow. Morphologic abnormalities such as scattered, focal mononuclear cell infiltrates in non-lymphoid organs (*e.g.* kidney and liver) may be relevant to autoimmune disease. If differences are seen in any lymphoid tissue, attention should be given to "cellularity" and prevalence of activated macrophages. The description could include *in situ* descriptions of the types of cells, density of the cell populations, lymphocyte distribution relative to distinguishing structures or defined areas of the organ. (In these instances, the effect does not need to be defined rigorously for each animal. The number of animals observed, however, should be a statistically significant sample size.) The histopathological analysis of routinely stained (hematoxylin and eosin) samples of the spleen should include descriptions of lymphocyte distribution and proliferation in known T- and B-cell areas, such as the germinal centers (for B-cells) and the periarteriolar lymphocyte sheath (PALS) for T-cells if abnormalities are observed. The histopathologic analysis of the lymph nodes and Peyer's patches should include a description of the immune activation (*i.e.* the relative number of follicles and germinal centers) when abnormalities or lesions are observed in these organs. When abnormalities of the thymus are observed, histopathologic analysis should be descriptive and quantitative as possible with regard to atrophy and necrosis and other observations. If the test compound is shown to either stimulate cell proliferation, or to cause atrophy and cell depletion in any lymphoid organ, the effect is likely to be viewed as a potentially immunotoxic effect requiring more definitive testing.

◙ Organ and Body Weight Indicators: Elevated or depressed spleen and thymus weights; elevated or depressed organ-to-body-weight ratios for the spleen and thymus (statistical treatment of the organ-to-body-weight ratios should include an analysis of co-variance, with body weight as the co-variant).

Elevated or depressed body weights, although primarily an indicator of endocrine function, may also indicate indirect immunotoxic effects, since endocrine function can significantly effect the immune system.

b. Indicators from Developmental Toxicity Studies

◘ Morbidity and Mortality Indicators: Unusual incidence of maternal infections.

◘ Histopathology Indicators: Abnormalities found during gross evaluation of the fetal liver, spleen, and thymus.

◘ For animals in the F_1 and F_2 generations:

i) Hematology Indicators: Elevation or depression in white blood cell (WBC) counts; altered differential WBC counts; lymphopenia and lymphocytosis; and eosinophilia.

ii) Clinical Chemistry Indicators: Elevated or reduced total serum protein in combination with an abnormal albumin-to-globulin (A/G) ratio.

iii) Histopathology Indicators: Abnormalities found during gross and routine histological evaluation of the lymphoid tissues, *e.g.* spleen, lymph nodes, thymus, gut-associated lymphoid tissue (GALT, in particular Peyer's patches), and bone marrow. Morphologic abnormalities such as scattered, focal mono-nuclear cell infiltrates in non-lymphoid organs (*e.g.* kidney and liver) may be relevant to autoimmune disease. If differences are seen in any lymphoid tissue, attention should be given to "cellularity" and prevalence of activated macrophages. The description could include *in situ* descriptions of the types of cells, density of the cell populations, lymphocyte distribution relative to distinguishing structures or defined areas of the organ. (In these instances, the effect does not need to be defined rigorously for each animal. The number of animals observed, however, should be a statistically significant sample size.) The histopathological analysis of routinely stained (hematoxylin and eosin) samples of the spleen should include descriptions of lymphocyte distribution and proliferation in known T- and B-cell areas, such as the germinal centers (for B-cells) and the periarteriolar lymphocyte sheath (PALS) for T-cells if abnormalities are observed. The histopathologic analysis of the lymph nodes and Peyer's patches should include a description of the immune activation (*i.e.* the relative number of follicles and germinal centers) when abnormalities or lesions are observed in these organs. When abnormalities of the thymus are observed, histopathologic analysis should be descriptive and quantitative as possible with regard to atrophy and necrosis and other observations. If the test compound is shown to either stimulate cell proliferation, or to cause atrophy and cell depletion in any lymphoid organ, the effect is likely to be viewed as a potentially immunotoxic effect requiring more definitive testing.

iv) Organ and Body Weight Indicators: Elevated or depressed spleen and thymus weights; elevated or depressed organ-to-body-weight ratios for the spleen and thymus (statistical treatment of the organ-to-body-weight ratios should include an analysis of co-variance, with body weight as the co-variant). Elevated or depressed body weights, although primarily an indicator of endocrine function, may also indicate indirect immunotoxic effects, since endocrine function can significantly effect the immune system.

4. Expanded Type 1 Immunotoxicity Tests

Assessing the safety of food and color additives used in food usually requires the completion of various

toxicity studies. In addition to the screen of primary indicators of possible immune toxicity provided by these toxicity studies and summarized above, additional tests for further evaluation of the immunotoxic potential of a test substance may be recommended by the Agency. The additional tests can be Expanded Type 1 Tests, discussed in this section, or Type 2 Tests, discussed in the next section. The Agency's recommendation that specific immunotoxicity tests be performed on test substances will be made on a case-by-case basis. Expanded Type 1 immunotoxicity tests include:

◙ Hematology Tests: Flow cytometric analysis of B-lymphocytes, T-lymphocytes, and T-lymphocyte subsets (TH + TS or CD4 and CD8); immunostaining (immunoperoxidase or immunofluorescence) of B-lymphocytes, T-lymphocytes and T-lymphocyte subsets from peripheral blood or single cell suspensions from the spleen. [25-29]

i) Hematology Indicators: Decreased or elevated percentages of any of the various lymphocytes relative to controls and abnormalities in the B-cell/T-cell and the TH/TS (CD4/CD8) cell ratios; these should be determined from differential counts of the immunostained preparations or from cytometric analysis.

◙ Serum Chemistry Tests: Electrophoretic analysis of serum proteins to permit separation and quantification of the relative percentages of albumin and the α-, β-, and τ-globulin fractions; quantification of τ-globulin fractions (IgG, IgM, IgA, and IgE); analysis of total serum complement and components of complement (such as C3) from CH-50 determinations; immunochemical assay of serum cytokines, such as IL-2, IL-1, and τ-interferon; quantification of serum auto-antibodies, such as anti-nuclear, anti-mitochondrial, and anti-parietal cell antibodies.

i) Serum Chemistry Indicators: Statistically significant variations between experimental and control groups of animals for any of the parameters listed above.

◙ Histopathology Tests: Immunostaining of B-lymphocytes in the spleen and lymph nodes, using polyclonal antibodies to IgG of the test animals; [30,31] immunostaining of T-lymphocytes and T-lymphocyte subsets in the spleen, using monoclonal or polyclonal antibodies to various cell markers; micro-metric measurements of germinal centers and PALS of the spleen and the follicles and germinal centers of lymph nodes; morphometric analysis of the relative areas of the cortex and medulla of the thymus, using routinely stained histopathology sections.

i) Histopathology Indicators: Statistically significant variations between experimental and control groups of animals for any of the parameters listed above, using both analysis of variance (ANOVA) and a multiple comparison T-test, such as Dunnett's.[32]

◙ Tests for *In Vitro* Analysis of the Functional Capacity of Specific Cell Types:

i) Activity of Natural Killer (NK) Cells: The functional capacity of NK cells can be measured using the classical ^{51}Cr chromium release assay; [19] this assay is well standardized and has been used successfully with both mice and rats in various immunotoxicity studies. [33-35] Of particular concern is reduced NK cell activity, which may be correlated with increased tumorigenesis and infectivity.

ii) Mitogenic Stimulation Assays for B- and T-Lymphocytes: Certain plant lectins stimulate blastogenesis and DNA synthesis of T- and B-lymphocytes: concanavalin-A (Con-A) and phytohemagglutinin (PHA) are known to preferentially stimulate T-lymphocytes, and an extract from pokeweed (PWM) as well as certain bacterial lipopolysaccharides (LPS) and protein extracts are known to preferentially stimulate B-lymphocytes *in vitro*. Since these assays are carried out *ex vivo*, they can be performed on preparations of peripheral blood. The assays are well characterized for use in various animals species (including man[36]), can be performed on either peripheral blood or spleen-cell suspensions, and have been used in a number of immunotoxicity studies. [2,8,9,10,12,13,35,37] Both reduced and elevated levels of blastogenesis or ^{3}H

incorporation into DNA are of interest in the evaluation of the immunotoxic potential of food and color additives used in food.

iii) Phagocytotic Index of the Macrophage: Various assays to determine the phagocytotic ability of macrophages have been described. [24,38,39] These assays measure the ability of a macrophage to ingest particulate substances, such as plastic beads or iron filings, and can be performed on peripheral blood or single cell suspensions of lymphoid organs, such as the spleen. Other assays measure the capacity of the macrophage to destroy live bacteria through lysosomal enzyme activity. [40]

iv) Stem Cell Assays: Bone marrow preparations can be used to investigate the pluripotent population or specific progenitor populations. [41] Although these assays have not been used extensively in immunotoxicity evaluations, they may be recommended when histopathological evaluation indicates that the test substance may have caused changes in bone marrow.

5. Type 2 Immunotoxicity Tests

Evaluating the functional capacity of the immune system requires injecting a substance that elicits immunological reactivity in a test animal. Various antigens provide information about the types of immunity or cells that may be involved in an immune response. For example, protein antigens usually elicit T-dependent immune responses with subsequent production of antibodies to the protein. Polysaccharides elicit T-independent immune responses. Some antigens elicit cell-mediated immune responses, while immunogens such as complex bacteria and viruses may elicit humoral and cell-mediated responses. All of the antigens listed below have been tested in rodents; when an antigen has been used preferentially with a particular rodent species, this is noted.

- T-Dependent Test Antigens: One of the most widely used antigens for rodents [2,7,8,9,10,24,30,42,43] and non-rodents is sheep red blood cells (SRBC). [1,4-9,30,34,35,42,44] For example, SRBCs have been widely used in mice in the Plaque-Forming Cell Assay: [45,46] antibody-producing spleen cell suspensions are mixed with SRBCs, placed on covered slides, and incubated; each antibody-producing cell causes a small, clear area (plaque) to form on the slide; the plaques are then counted. Other T-dependent test antigens that have been widely used include keyhole limpet hemocyanin [10] and bovine serum albumin.

- T-Independent Test Antigens: Ficoll, a branched chain polysaccharide, haptenated ficoll, polyvinylpyrrolidone, and bacterial lipopolysaccharides have been used as T-independent test antigens with mice and rats. [47]

- Human Vaccines: Human T-dependent vaccines, such as tetanus toxoid, and the T-independent vaccine containing pneumococcal polysaccharide antigens have been used in both rats and mice. [48-50] It is possible to compare responses of the test species to the vaccines with human responses, because standard human sera are available from FDA's Center for Biologics. [51]

- Test Antigens for Cell-Mediated Immune (CMI) Reactivity: Contact sensitizers such as dinitrochlorobenzene (DNCB) have been used to elicit delayed hypersensitivity (DTH) responses as a measure of CMI in animals. These assays can be performed in rodent [52] as well as non-rodent species. The DTH assays are economical and correlate well with decreased CMI and host resistance to infectious agents in humans, [53] as well as animals. [54] The mixed-lymphocyte response (MLR) assay, which uses lymphocytes from a different strain, has been successfully used to evaluate CMI in mice. [2]

- Host Resistance Assays with Infectious Agents: A number of bacterial strains have been used to measure host resistance, including *Listeria monocytogenes*, various strains of *Streptococcus*, and *Escherichia coli*. [54] Useful viral models [55,56] include influenza, herpes, and cytomegalovirus. [57] A yeast infectivity model using *Candida albicans* has been described, as well as parasitic infectivity models using *Trichinella spiralis* and *Plasmodium yoelli*.[55,58]

◙ Host Resistance Assays Using Syngeneic Tumor Cells: Various assays of host resistance have been described using a number of cultured tumor cell lines. [58,59] These assays, unlike those involving the infectious agents discussed above, do not require special barrier facilities to prevent infections from spreading throughout an animal colony. Two mouse assays have been validated: the PYB6 sarcoma assay and the B16F10 melanoma assay.[60] An assay using a lung tumor model and the MADB106 tumor cell line also has been validated for use in immunotoxicity studies. [61]

6. Relevance of Primary Indicators of Immune Toxicity to Health

a. Hematological Indicators

Hematologic screens recommended for toxicity studies are basically the same as those performed clinically as human health screens. Depressed or elevated WBC counts may be indicative of direct or indirect effects of the test substance on cellular proliferation and distribution. Total WBC counts are used clinically as a presumptive test for infection; they are also used to evaluate the severity of an inflammatory or allergic process. Routine differential WBC counts are used to differentiate among some types of infections and inflammatory responses; they also are used as a screen for toxicologic or pharmacologic effects: for example, immunosuppressive drugs may cause lymphopenia.

Altered lymphocyte counts may be relevant to immunodeficiency. Increased numbers of polymorphonuclear leukocytes can result from pathogenic infections and from pyrogenic and inflammatory processes. Eosinophilia is often associated with allergenic processes. It may also indicate an infectious, reactive, or neoplastic process. Altered red blood cell counts and platelet counts can be associated with autoimmune processes.

b. Serum Protein Indicators

Estimates of total serum proteins and the albumin/globulin ratio may give useful information about liver and lymphocyte function. The α- and β-globulins (*i.e.* α- and β-G) are primarily produced in the liver; τ-G are a product of the B-lymphocytes. Depressed β-G levels could lead to decreases in complement proteins that are required for phagocytosis; this could produce decreased resistance to bacterial infections. Reduced levels of τ-G also could mean reduced levels of antibodies necessary for humoral immunity to infectious agents. Altered levels of τ-G may indicate an effect on B-lymphocytes, T-lymphocytes, or simultaneous effects on both types of cells.

However, total globulin levels do not give specific information about which immunoglobulin classes are affected. Thus, when globulin levels are reduced, specific quantitative assays for the τ-G subclasses may be recommended. Electrophoretic and immunoelectrophoretic analyses of the serum τ-G subclasses or quantitative assays such as Enzyme-Linked Immunosorbent Assay (ELISA), Radioimmunoassay (RIA), or radial immunodiffusion may be recommended. This information may be important because reductions in τ-G and τ-M may be relevant to infection by opportunistic and pathogenic organisms, and changes in τ-A may indicate effects of the test substance on secretory immunity, such as gut-mediated immunity.

c. Histopathology Indicators

Abnormal results from gross and histological evaluation of the lymphoid organs (usually the spleen, thymus, and lymph nodes) are important indicators of various immunotoxic effects; histological evaluation of Peyer's patches and bone marrow also is recommended in screening for effects of a test substance on the immune system. Atrophy of the thymus gland with associated depletion of cortical thymocytes could be an indication of immunosuppression. Concomitantly, a similar effect on the lymphocytes in the periarterial lymphocyte sheath of the spleen (PALS) would indicate an effect of the test substance on T-cells: both cell-mediated and humoral

immunity can be affected. In the spleen and lymph nodes, defined regions are more densely populated with B-lymphocytes, with activated, antibody-producing B-cells, or with plasma cells. Effects on B-cell regions of these organs could be an indication of immunosuppression or immunoenhancement, depending on the result obtained.

d. Body and Organ Weights

Body and organ weights are generally recorded during toxicity studies. Spleen weights are usually recorded in all toxicity studies, but thymus weights may not be recorded in long-term studies. The thymus gland grows rapidly in young animals but begins to involute as the animals reach sexual maturity. In old animals, the thymus may be difficult to detect and measure because of the degree of involution.

Organ weights by themselves or in relation to body weights can be sensitive measures of organ atrophy or hypertrophy, but yield little information about immunotoxic effects. Reduced organ weights can result from direct effects on lymphocyte proliferation and differentiation and may be relevant for assessing immunosuppression. Hypertrophy of the lymphoid organs is usually associated with increased proliferation of cells (hyperplasia). Increased proliferation of lymphocytes can result from infections, stimulation by xenobiotics, altered metabolic processes, and certain forms of trauma, reactive, or autoimmune processes. In practice, however, changes in organ weights or organ-to-body-weight ratios are more relevant to immune toxicity when they are associated with appropriate histopathology findings.

7. Adequacy and Reliability of Primary Indicators of Immune Toxicity

If all primary indicators of possible immunotoxicity from toxicity studies are negative for a test substance, would this effectively rule out the possibility that the test substance produces significant immunotoxic effects? The answer to this question is complex; some of this complexity derives from the fact that the primary indicators of possible immune toxicity listed above are not sufficiently specific or sensitive to provide unambiguous answers. For example, it is not possible to differentiate B-lymphocytes from T-lymphocytes in routinely stained sections of lymphoid tissues, and standard hematology tests cannot distinguish among subcategories of T-lymphocytes. Special immunochemical stains, however, permit B- and T-cells to be visualized in tissue sections and blood smears, making available more information about the immunotoxic effects of the test substance.

If only short-term toxicity studies are performed on a particular test substance, concern about the adequacy and reliability of the immunotoxic indicators from these studies may be high. Subtle immunotoxic effects or immune toxicities that develop only after prolonged administration of the test substance may not be detected in short-term toxicity studies. Conditions of the longer-term toxicity studies, however, may make it difficult to detect some immune toxicities: the use of barrier facilities is common in carcinogenicity studies; because barrier facilities limit exposure of test animals to exogenous infectious microorganisms, detecting possible immunotoxic effects of a test substance in carcinogenicity studies may be compromised because spontaneous infection rates and mortality are evaluated as primary indicators of possible immunotoxicity in such studies.

Even with this disadvantage, many investigators and regulatory authorities recommend specific tests to identify and characterize immune system toxicities only when screening tests or indicators are positive. [6,11] Additional rationale for this approach comes from the fact that most short-term toxicity studies incorporate at least one dose in the potentially highly toxic dose range. Additional tests for immunotoxicity should be performed to verify positive immunotoxic effects noted during screening studies or to determine if the positive result obtained for a primary indicator was a false positive indication of immunotoxicity. For example, certain test substances may cause increased or decreased food intake; nutritional deprivation from significantly decreased food intake has been shown to cause thymic and splenic atrophy. [62] Effects on the endocrine system, such as stimulation of the production of growth hormone [63] or prolactin [64] and decreased levels of adrenocorticosteroids, [65] can stimulate

growth of the thymus. In response to such stimuli, involution of the thymus may proceed at a different rate in animals exposed to the test substance than in control animals. Therefore, measuring thymic weights at one specified time in a short-term toxicity study could give false positive or false negative indications of the test compound's immunotoxic potential. For this reason, the Agency recommends that a study of the effects of a test substance on thymic growth and involution be conducted at two or more time points during the study (such as midterm and final sacrifice). Because sex differences have been demonstrated for various immunologic studies,[30,66] both sexes should be included in immunotoxicity evaluations.

There are data which suggest that the primary indicators do not evaluate toxic effects on all types of immune-related cells. Recent studies have shown that NK cell function may be affected without concomitant effects on either B- or T-lymphocytes.[61] Other studies have shown that functional defects of specific lymphocytes can occur without apparent changes in the proliferation or morphology of the cells as observed in standard histopathology preparations:[11,67,68] the morphology of the cells is normal and a false negative result would be obtained in these instances.

8. Recommendations for Further Immunotoxicity Testing when Primary Indicators of Immunotoxicity are Positive

Assessing the safety of food and color additives used in food usually requires the completion of various toxicity studies. In addition to the screen of primary indicators of possible immunotoxicity provided by these toxicity studies and summarized above, additional tests for further evaluating the immunotoxic potential of a test substance may be recommended by the Agency. In the sections that follow, the adequacy of primary indicators of immunotoxicity for test substances that have been assigned to each Concern Level will be discussed. The Agency's recommendation that specific immunotoxicity tests be performed on test substances that have been assigned to Concern Levels I, II, and III will be made on a case-by-case basis.

a. Immunotoxicity Studies for Compounds that have been Assigned to Concern Level III

Test compounds that have been classified as Concern Level III substances present the highest level of concern about their safe use as direct food additives and color additives used in food. When these substances undergo toxicity testing, primary immunotoxicity indicators may be negative, marginal, or positive. Immunotoxicity tests suitable for each of these situations will be discussed below.

i. Immunological Tests when Primary Indicators of Immunotoxicity are Negative or Marginal

If the primary immunotoxicity indicators from recommended toxicity tests are not positive, then no additional tests for the immunotoxic potential of the Concern Level III test compound would be recommended unless there were special circumstances. Such circumstances may include: 1) the rodent strains employed in toxicity testing were highly inbred and are know to be resistant to immunotoxic effects; 2) barrier or other facilities were used for long-term and short-term toxicity studies, which may have precluded exposure of the test animals to normal infectious agents present in the environment; and 3) omissions from the recommended guideline for standard toxicity tests, such as not measuring thymus weights during the growth phases of the test animals or omitting histopathological analysis of certain lymphoid organs. In these situations, some Type 1 immunotoxicity tests and a Type 2 immunotoxicity study of host resistance may be recommended, particularly if specific tests for immune toxicity had not been incorporated into subchronic toxicity studies.

ii. Immunological Tests when Primary Indicators of Immunotoxicity are Positive

When any of the primary indicators suggests that a Concern Level III test substance has an immunotoxic effect, additional testing will be recommended in order to assess the extent of risk to the immune system. In addition, positive effects on other target organs may indicate the need to assess the autoimmune potential of the

compound.

Certain indicators may derive from effects on either B-cells or T-cells, or may be derived from effects on both types of cells. However, most of the primary indicators of immune toxicity are nondiscriminating with respect to specific lymphocytes involved and specific immune functions affected. Standard histopathology evaluation may provide some clues if there is an effect on the thymus or if areas in the spleen or lymph nodes are associated with specific types of lymphocytes. The objectives of expanded Levels I and II immunotoxicity tests are to delineate the specific cells type(s) which are affected, to evaluate the extent to which specific immune functions are impaired, and to relate these effects to risks such as infection, hypersensitivity, and carcinogenicity.

The immunotoxicity tests described in the following sections are for use with rats, and all tests should be conducted on each test animal. However, tests have been, or can be, adapted for use with mice or non-rodent species. When mice are used as test animals, serum from animals in each experimental group may need to be pooled if there is an insufficient quantity of serum from each animal to perform recommended hematology tests.

a) Retrospective Level I Tests: No additional animals are needed for Retrospective Level I immunotoxicity tests when at least 10 animals of each sex are used in a standard toxicity study and appropriate samples of blood and tissues are properly treated and preserved. After removing blood cells, serum samples should be prepared by high-speed centrifugation, sterilized by filtration, and stored at 4-5°C in sealed containers. At least half of each lymphoid tissue/organ should be fixed briefly in Bouin's fixative (or other fixative shown to be appropriate) and stored in alcohol; sections from the tissue/organ can be processed for histopathological analysis by routine staining or by immunostaining.

If the standard toxicity study was a subchronic or chronic study (with exposure to the test substance for 90 to 120 days), and primary indicators suggested that the test material may be immunotoxic, the following Retrospective Level I tests should be performed on serum samples from the study:

i) Electrophoresis of serum proteins.

ii) Quantification of serum immunoglobulins (IgG, IgM, IgA, IgE).

iii) Immunostaining for B- and T-lymphocytes in spleen and lymph nodes and micrometric analysis of the number of stained cells in specific regions of these organs.

iv) Screening for serum autoantibodies to DNA, mitochondria, and other cell components in one or more tissues, such as liver and smooth muscle.[69] These tests should be performed when there is an indication that the test substance may affect B- or T-lymphocytes.

v) Immunostaining for bound IgG may be recommended to determine if non-lymphoid organ toxicities noted during the standard toxicity study (particularly a long-term toxicity study) are due to an autoimmune reaction.

If the results of these Retrospective Level 1 tests demonstrate that the primary indicators of immune toxicity were false positives, then no further immunotoxicity testing would be recommended. However, if the results of these tests are inconclusive or confirm an immunotoxic effect of the test substance, additional testing would be recommended. The additional testing may include Type 1 and Type 2 immunotoxicity tests.

b) Additional Level 1 Tests: Additional Level 1 immunotoxicity tests cannot be performed retrospectively, but must be incorporated into the protocol of standard toxicity studies. However, all of the tests described in this section can be performed on the same animals that are used in the standard toxicity study, provided that samples are processed appropriately. For example, half of the spleen can be used to make a cell suspension for cellular analysis immediately following sacrifice of the test animal; the remaining half can be processed for histopathology evaluation. Additional (non-retrospective) Level 1 tests that may be recommended include:

i) Quantitative analysis of the B-cell to T-cell ratio (B/T) using either whole blood cells and spleen preparations or spleen preparations only.

ii) Determination of spleen cellularity (the total number of white blood cells and lymphocytes per gram of wet tissue) and the total number of viable cells per gram of wet tissue or per million white blood cells.

iii) Assay of mitogenic stimulation for B- and T-lymphocytes:

iv) Analysis of NK cells using a suspension of spleen cells:

v) Determination of the phagocytotic index of macrophages:

vi) Electrophoresis of serum proteins: Although this test can be performed retrospectively, it is listed here because it is particularly useful for evaluating toxic immune effects on liver, macrophages, and lymphocytes.

c) Level II Tests: If primary indicators of immunotoxicity from standard toxicity studies suggest that a test compound may be immunotoxic, Level II tests may be recommended to identify specific functional immune defects. These tests may be performed on satellite groups of test animals in conjunction with a standard toxicity study or they may be performed on test animals in a separate immunotoxicity study. In the latter case, Level II tests should be performed with the same species, strain and age of test animals and the same doses of test substance used in the standard toxicity study of comparable duration. In addition, separate Level II immunotoxicity studies should be 3 to 6 weeks in duration so that test animals will be exposed long enough to enable primary and secondary immune effects to be identified. An additional period of time at the end of the study during which the test substance is not administered would permit evaluation of the reversibility of observed immune effects.

The following Level II tests may be recommended:

i) Kinetic evaluation of primary and secondary immune responses of test animals to a T-dependent antigen, such as SRBC, tetanus toxoid, or KLH; serum antibody titers should be measured following initial and secondary injections of the antigen.

ii) Evaluation of the primary humoral response to a T-independent antigen, such as pneumococcal polysaccharides; choice of the optimum challenge dose should be justified.

iii) Evaluation of the delayed hypersensitivity response to a contact sensitizer during the second half of the study. Alternatively, evaluation of the mixed lymphocyte response can substitute for measurement of the DTH response as long as the assay has been validated with the particular rat strain used.

d) Enhanced Level II Tests: These tests are designed to determine if a test substance that produces immune toxicity in Level I or Level II tests also affects host resistance to challenge with infectious agents or tumor cells. Enhanced Level II tests may be performed with either rats or mice, because many host resistance tests have been validated in mice. These tests would be recommended in a variety of circumstances; for example:

i) If primary indicators of immunotoxicity from standard long-term toxicity studies showed increased mortality associated with administration of the test substance and effects on humoral immunity were identified from Level I and Level II tests, then bacterial (*e.g. Listeria monocytogenes*)[58] or viral (*e.g. Influenza*)[55] challenge tests associated with humoral immune protection would be recommended for evaluation of host resistance.

ii) If there are indications that consumption of the test substance is associated with increased tumorigenesis and effects on phagocytosis, tumor challenge tests with PYB6 sarcoma, which tests cytolytic activity of T-cells and NK cells in mice,[60] would be appropriate; a similar test for rats uses the MADB106 tumor line.[61]

iii) Finally, for test materials that have demonstrated T-cell or cell-mediated immune effects, challenge tests that use certain strains of *Streptococcus* [57] or *Plasmodium yoelli* [58] would be appropriate.

b. Immunotoxicity Studies for Compounds that have been Assigned to Concern Level II

Specific immunotoxicity tests generally are not recommended for test compounds that have been assigned to Concern Level II. However, if primary indicators of possible immunotoxicity from toxicity studies conducted on Concern Level II test substances are positive, additional Level I and Level II immunotoxicity tests may be recommended; such recommendations will be made on a case-by-case basis.

c. Immunotoxicity Studies for Compounds that have been Assigned to Concern Level I

Usually, short-term acute exposure studies (up to 30 days) are performed to assess the safety of Concern Level I compounds. Although guidelines for these studies (see **Chapter IV C 3**) do not recommend specific immunotoxicity tests, if primary indicators of possible immunotoxicity from short-term toxicity studies are positive, additional Level I and Level II immunotoxicity tests may be recommended. Such recommendations will be made on a case-by-case basis. One immunotoxicity test which measures the primary humoral response to the T-dependent antigen SRBC has been described for use with both rats [34] and mice [38] and has been recommended for use in short-term screening studies.[2]

9. Animal Models for Immunotoxicity Tests

a. Rodent Models

These guidelines have focused on tests designed to assess immune toxicity in the rat. Specific strains have been used and validated by the Agency, including Sprague-Dawley, Spartan,[30] and Osborne Mendel; [69] the Fisher 344 rat has been recommended by others [61] for studies with environmental compounds. Other strains of rat, such as the Buffalo strain, have been used in special studies to evaluate autoimmune disease potentiation.[70-72] In addition, several mouse strains (mainly inbred strains) have been used to assess immune toxicity.

b. Non-rodent Models

Use of the dog for various immunopharmacologic studies has been described in the scientific literature.[73] Level I immunotoxicity tests described in these guidelines can be performed on most large animal species; Level II immunotoxicity tests in other non-rodent models also may be acceptable, if validated: use of primates has been described. [74] Also, miniature swine have been shown to be an excellent non-rodent species for evaluation of various immune functions. [75-79] The Agency has validated a number of immune function assays for use with this

model.

Immunomodulation of porcine as well as other food animals have been reviewed.[75] Other perspectives on animals selection have been reviewed. [76]

10. Recommended Strategy for Assessing the Immunotoxic Potential of Direct Food Additives and Color Additives Used in Food

- Primary indicators of immunotoxicity should be evaluated for short-term (28-day) toxicity studies, subchronic (90-day) toxicity studies, and developmental toxicity studies. Results of these evaluations should be incorporated into an integrated assessment of the potential for a test chemical to adversely affect the immune system. Based on this assessment, an explicit statement should be made as to whether or not the test chemical represents a potential immunotoxic hazard which requires further testing.

- Additional studies to assess the immune toxicity of food and color additives used in food will depend on the results of the evaluation of primary indicators of immune toxicity, the Concern Level to which the additive has been assigned, and other information available concerning the immunotoxicity of the additive.

11. Conclusion

The hierarchical grouping of recommended immunotoxicity tests by specificity and mechanics (*e.g.* tests that use injectable substances) can facilitate including immunotoxicity testing in standard toxicity studies. Expanded testing on existing samples is possible, and allows for a more definitive identification of potential immunotoxic effects. Such expanded testing may be necessary when additional information about a possible immunotoxic effect is important for the safety assessment of a direct food additive or color additive used in food. Immunotoxicity tests recommended in this section are summarized in **Table 1** below.

Table 1

Summary of Immunotoxicity Testing Recommendations for Direct Food Additives

Basic Testing (Rat Model)

° CBC, WBC differential
° Total serum protein, albumin-to-globulin ratio (A/G)
° Histopathology, gross and microscopic (spleen, thymus, lymph nodes, Peyer's patches, and bone marrow)
° Lymphoid organ and body weights

Retrospective Level I Testing: Included as a Possible Requirement in Standard Toxicity Study

° Electrophoretic analysis of serum proteins* (when positive or marginal effect noted in basic testing)
° Immunostaining of spleen and lymph nodes for B and T cells* (quantification of total Ig)
° Serum autoantibody screen and deposition of Ig (micrometry for semi-quantitation of the proliferative response)

Enhanced Level I Testing: Included as a Possible Requirement for More Complete Screening in the Standard Toxicity Study Core Group, with a Satellite Animal Group, or in a Follow-Up Study

° Cellularity of spleen (lymph nodes, thymus when indicated)
- Quantification of total B and T cells (blood and/or spleen)
- Mitogen stimulation assays for B and T cells (spleen)
- NK functional analysis (spleen)
- Macrophage quantification and functional analysis (spleen)
- IL-2 functional analysis (spleen)

° When indicated or for more complete analysis, other endpoints such as total hemolytic complement activity or CH-50 assay with serum

Level II Testing: Includes a Satellite Group or Follow-Up Study for Screening of Functional Immune Effects

° Kinetic evaluation of the humoral response to a T-dependent AG (primary and secondary responses with either SRBC, TT, or other)
° Kinetic evaluation of the primary humoral response to a T-independent AG such as Pvax, TNP-LPS, or other recognized AG
° DTH response to known sensitizer of known T-cell affecter
° Reversibility evaluation

Enhanced Level II Testing: Includes a Satellite Group or Follow-Up Study For Evaluation of Potential Immunotoxic Risk

° Tumor challenge (MADB106 or other with the rat; PYB6 sarcoma with a mouse model)
° Infectivity challenge (*Trichinella*, *Candida* or other with the rat; *Listeria* or other with the mouse)

Abbreviations: CBC = complete blood count; WBC = white blood count; Ig = immunoglobulin; NK = natural killer; IL-2 = interleukin-2; SRBC = sheep red blood cells; and TNP-LPS = trinitrophenol lipopolysaccharide.
* Recommended for inclusion in basic testing.

Chapter V D: Immunotoxicity Studies - References

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