# Immunotoxicology—Regulatory and Risk Assessment Concepts

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**This article provides a review of presentations given at the symposium on Immunotoxicology: Regulatory and Risk Assessment Concepts held at the American College of Toxicology meeting in Orlando, Florida, in November, 1998. Immune system alterations have typically been assessed by histopathology of select lymphoid tissue, clinical pathology, clinical chemistry, plaque forming cell assay for humoral immunity, and allergic contact hypersensitivity. Advances in immunology and molecular biology have led to various activities to optimize hazard identification and risk assessment processes and strategies for immunotoxicants. With such advances, regulatory agencies have been either implementing immunotoxicology guidance as part of the safety of medical devices, evaluating environmental chemicals, or considering immunotoxicologic criteria for nonclinical assessments. Reviews of the guidance document provided by the Food and Drug Administration (FDA)/Center for Device and Radiological Health and concepts being considered by the FDA/Center for Drug Evaluation and Research are presented. In addition, a review of the process for evaluation of the murine local lymph node assay by the Interagency Coordinating Committee on the Validation of Alternative Methods and the state of risk assessment for chemical-induced autoimmunity are presented.**

**Keywords** Autoimmunity, Chemical Pharmaceutic, Immunotoxicology, Medical Device, Regulatory, Risk Assessment

Advancements in our understanding of immune disease processes have brought increased attention to the effects of pharmaceutics, environmental and occupational agents, and implant devices on immune function as well as provided opportunities to improve the risk assessment process. Within the last several years, regulatory agencies have been either implementing immunotoxicology guidelines for assessing the safety of medical devices or considering immunotoxicologic criteria for nonclinical assessments. A symposium providing a regulatory perspective of immunotoxicology was held at the 1998 American College of Toxicology meeting. Included in this symposium were speakers who provided overviews on: immunotoxicity criteria for medical devices and chemical immunosuppressant evaluations, autoimmunity testing and its role in risk assessment, and the status of the local lymph node assay as an alternative to guinea pig testing for dermal sensitization. This article provides a summary of some concepts presented.

## GUIDANCE FOR IMMUNOTOXICITY TESTING IN THE FOOD AND DRUG ADMINISTRATION: CENTER FOR DEVICES AND RADIOLOGICAL HEALTH (J. Langone)

### Background

The Center for Devices and Radiological Health (CDRH) in the Food and Drug Administration (FDA) has written an *Immunotoxicity Testing Guidance* for regulatory reviewers and manufacturers of medical devices. The Guidance provides a systematic approach for determining when nonclinical immunotoxicity testing is needed, and what types of testing to consider as part of the overall evaluation of product safety. The Guidance provides an assessment of the types of immunotoxicity testing currently available, and a process for selecting appropriate test methods. The goal is to obtain adequate information to help make confident regulatory decisions, not to make claims that a device or material is not immunotoxic. From the regulatory perspective, evidence supporting nonimmunotoxicity does not establish safety, but should provide some level of assurance that serious immunotoxic reactions are unlikely to occur in the intended patient population. Like all FDA guidance documents,

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the *Immunotoxicity Testing Guidance* only provides recommendations. It does not impose requirements on either regulated industry or FDA.[1]

## Availability of the Guidance Document

The *Immunotoxicity Testing Guidance* document has been approved through the CDRH *Good Guidance Practices*, a process of rigorous review and evaluation both internal and external to the FDA. The complete document is available on the CDRH World Wide Web site: www.fda.gov/cdrh.

## Immunotoxicity

For purposes of the guidance document, immunotoxicity is defined as "Any adverse effect on the structure or function of the immune system, or on other systems as a result of immune system dysfunction." This definition incorporates the fact that biological systems (e.g., immune and neuroendocrine) are interrelated, and may produce or be affected by the same humoral or cellular mediators. In practice, laboratory testing based on the guidance focuses on measures of functional activity, mediator concentrations, or other factors that reflect changes in the immune system itself. Unlike drugs or biologics, many materials used in medical devices that contact the body are intended to have little or no biological activity. For example, materials commonly used in implants for the restoration of structural integrity and mechanical function, such as joint prostheses and heart valves, are designed to be biocompatible rather than bioactive. They generally have low risk of causing immunotoxic reactions. However, other materials are potentially immunotoxic. For example, latex proteins and certain plastics and polymers (e.g., acrylics) are known to cause type I allergic reactions, whereas thiurams in latex and bisphenol A in dental resins may cause type IV reactions. Solid materials that contact the blood potentially can activate the complement system with release of anaphylatoxins (e.g., cellulose-based and synthetic hemodialysis membranes), whereas gels and oils (e.g., silicone) may act as adjuvants. Because substantial clinical experience, mainly postmarket, suggests that many materials present low immunotoxic risk, testing will be needed mainly for new materials that have not been shown to be compatible with the immune system. Testing may be needed for established materials if body contact is different, of longer duration, or concentrations are higher.

## Format

The Guidance consists of a flow chart and three tables referred to as the framework, along with explanatory text. Framework Table 1 follows the format of International Organization for Standardization (ISO) document 10993 (i.e., G95-1), in which recommended testing is based on the type and duration of body contact.[2]

## Consensus Standards and Testing Challenges

The framework is intended to be a practical tool for selecting the best available tests, based on current knowledge in the field. It recommends certain standardized tests and other commonly used tests that are widely accepted as providing reliable information because of the added quality assurance they provide for regulatory purposes. Although other tests that may provide useful information on immunotoxicity are included, they do not have the same regulatory weight or significance as consensus standards. Additional predictive immunotoxicity tests (e.g., in the form of consensus standards) are needed for regulatory purposes. Rigorously validated and standardized tests, such as those available through voluntary standards setting organizations like the American Society for Testing and Materials (ASTM) (standards F1905 and F1906), the Association for the Advancement of Medical Instrumentation (AAMI), and ISO, help provide FDA reviewers with the added assurance needed to make sound regulatory decisions. As improved tests become available, they also will be given special recommendation. As an example, the mouse local lymph node assay (LLNA) is being considered as an alternative to guinea pig testing for identification of skin sensitizing chemicals. The LLNA may offer advantages including objective endpoint, identification of sensitizers at the induction rather than challenge phase of the process, quantitative dose-response results, and reduction or refinement in the use of animals (e.g., the animals are tested over a shorter time and without the use of adjuvant).[3]

Immunotoxicity testing of implanted medical devices offers special challenges. Implants may remain in place for many years, making it difficult to model biological effects in animals with relatively short lifetimes.[4] In addition, implants are often mixtures of several constituents, they may undergo biochemical degradation and leaching, or produce wear particles that can migrate to distant anatomical sites and cause local or systemic

[1]The guidance should be used within the broader context of the Center's General Program Memorandum G95-1. This is a FDA-modified version of International Standard ISO-10993, *Biological Evaluation of Medical Devices-Part1: Evaluation and Testing*, that provides an overview of the general types of toxicity testing to consider for a medical device or constituent materials.

[2]ISO 10993 provides a collection of several national and international standards for biological testing of medical devices and materials. Although ISO 10993 includes testing for irritation and delayed hypersensitivity, it does not include a specific category covering the broader field of immunotoxicity testing. A task force has been organized to evaluate the current status of immunotoxicity testing of medical devices and whether a more complete coverage of the field in ISO 10993 is justified at this time.

[3]On September 17, 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) convened a panel of experts for scientific review of the LLNA as an alternative to guinea pig testing (e.g., the guinea pig maximization and occluded patch tests). Regulatory agencies, including FDA, have reviewed the panel's report and assessed the acceptability of the LLNA for regulatory purposes. CDRH has accepted the panel's recommendations, with provisions added to the standard protocol applicable to medical devices/biomaterials.

[4]Tests in vitro may be used for accelerated testing of mechanical and chemical integrity.

immunotoxic effects. This complexity is a concern even with established methods like the guinea pig maximization test (GPMT) and LLNA. Although these tests are useful for identifying certain classes of chemical sensitizers, they have been developed using pure chemicals that generally bear little resemblance to materials used in medical devices. Relatively little information has appeared in the scientific literature on their applicability to testing mixtures, and they may not be highly predictive for metals and metal salts. Although additional work is needed to validate these tests for the complex materials used in medical devices, the GPMT has a long history of use and device manufacturers often provide CDRH with test results that are useful for regulatory purposes. Similar information may be forthcoming if the LLNA gains regulatory acceptance for medical devices.

## Using the Guidance

The flow chart (Figure 1) is used to determine if nonclinical immunotoxicity testing is needed. In general, testing will be appropriate for new materials, or materials already in use in medical devices that may raise concerns because of reports indicating potential immunotoxic reactions in patients. Additional immunotoxicity testing may not be needed if a material has the same type of body contact as in previously marketed devices, the dose and duration of exposure are the same, there is a history of use of the material without indications of immunotoxicity, or data in the scientific or clinical literature support a lack of immunotoxicity. Scientifically sound studies carried out according to accepted standard procedures would not need to be repeated. If the flow chart indicates that immunotoxicity testing is recommended, the tables can be used sequentially to help select appropriate types of testing. Table 1 describes the types of immunotoxic effects that might occur, based on materials commonly used in medical devices and the location and duration of body contact. Chronic inflammation and immunostimulation are included, in addition to hypersensitivity, immuosuppression, and autoimmunity. Although acute inflammation is a normal physiological response to implanted devices, chronic inflammation with permanent implants (see Table 1) may lead to loosening of implants or other undesirable effects. Immunostimulation is listed separately from hypersensitivity to account specifically for biomaterials that may be immunogenic or have adjuvant

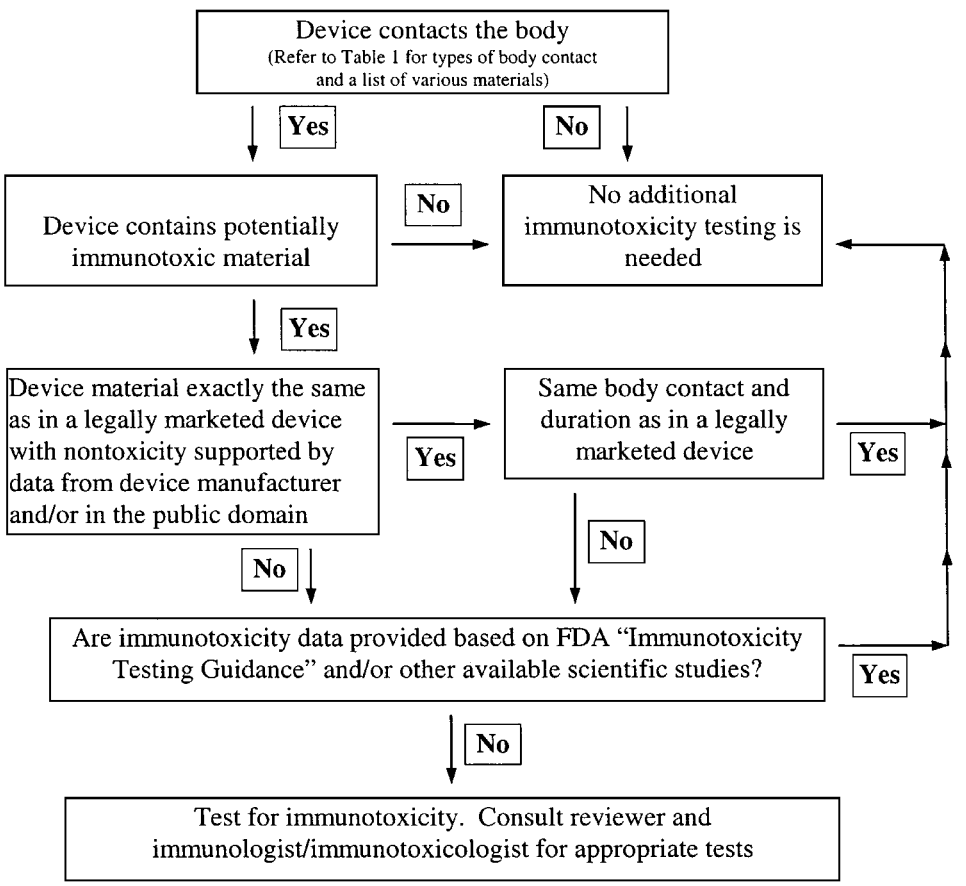


**FIGURE 1**
Flow chart for immunotoxicity testing.

**TABLE 1**
Potential immunotoxic effects of devices and constituent materials

DEVICE NAME: _____

| Body contact | | Contact duration | Immunotoxic effects 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| Surface devices | Skin | A | pmb× | | × | | |
| | | B | pmb× | × | × | × | × |
| | | C | pmb× | × | × | × | × |
| | Mucosal membranes | A | pmb× | | × | | |
| | | B | pmb× | pmb× | mb× | × | × |
| | | C | pmb× | pmb× | mb× | mb× | mb× |
| | Breached or | A | pmb× | | × | | |
| | compromised | B | pmb× | pmb× | mb× | mb× | mb× |
| | surface | C | pmb× | pmb× | mb× | mb× | mb× |
| External communicating devices | Blood path, direct | A | pmb× | | × | | |
| External devices that contact the circulating | and indirect | B | pmb× | pmb× | mb× | pmb× | mb× |
| blood (e.g., dialyzers and immunoadsorbents); | | C | pmb× | pmb× | mb× | pmb× | mb× |
| or the blood path indirectly at one point and | Tissue/bone/dentin | A | pmb× | | × | | |
| serve as a conduit for entry into the vascular | communicating | B | pmb× | cpmb× | mb× | pmb× | mb× |
| system (e.g., solution and blood administration | | C | pmb× | cpmb× | mb× | pmb× | mb× |
| sets); or tissue/bone/dentin (e.g., skin staples, laparoscopes, dental filling materials) | | | | | | | |
| Implant devices | Tissue/bone, | A | pmb× | | × | | |
| | blood, and | B | pmb× | cpmb× | mb× | pmb× | mb× |
| | other body fluids | C | pmb× | cpmb× | mb× | pmb× | mb× |

A = Limited (≤ 24 hrs)
B = Prolonged (> 24 hrs to 30 days)
C = Permanent (> 30 days)
Effects expected for various materials
Plastics and other polymers = p
Metals = m
Ceramics, glasses, composites = c
Biological materials = b
Other materials (specify) = x

1 = Hypersensitivity
2 = Chronic inflammation
3 = Immunosuppression
4 = Immunostimulation
5 = Autoimmunity

activity that could lead to immunopathological effects resulting, for example, in an autoimmune response.

Table 2 lists a set of immune indicators that may be associated with the immunotoxic effects shown in Table 1: histopathology, humoral and cellular responses, host resistance, and signs of illness in experimental animals. These responses and indicators have been prioritized as critical or noncritical. Critical indicators are of primary importance in testing for the associated immunotoxic effects, whereas testing for noncritical responses and indicators may provide supportive evidence of positive or negative immunotoxicity if critical tests are positive.

Table 3 provides representative examples of specific tests, indicators, and models that may be used to evaluate the immune responses in Table 2. Commonly used tests, indicated by an asterisk, are strongly recommended because they are standardized and/or have a long history of use that supports their validity. Table 3 only suggests some tests from which to choose. Alternative methods may be used if there is sufficient evidence supporting their validity.

Generally, tests performed in animals provide a better indication of potential immunotoxic effects than in vitro tests. This is in part because the intact immune system has alternative mechanisms that may compensate for deficiencies in a particular immune function, whereas this capability is lacking in vitro. Although in vivo testing is generally preferred, use of in vitro methods is encouraged to minimize the number of experimental animals, provided the choice is supported by a sound scientific rationale. The guidance excludes routine testing for autoimmune diseases. There are few good animal models of human autoimmune diseases, and without clinical indications to guide the selection, the choice of model to use in nonclinical testing is usually not obvious (see Autoimmunity and Immunotoxicology section). Also, for the vast majority of materials used in medical devices, there is no apparent reason to

**TABLE 2**
Classification of immune responses associated with potential immunotoxic effects

| Immunotoxic effects | Immune responses | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Cellular responses | | | | | |
| | Histopathology | Humoral response | T cells | Natural killer cells | Macrophages | Granulocytes* | Host resistance | Observe for signs of illness |
| Hypersensitivity | NC | C (IgE in type I reactions only) | C (type IV reactions only) | NA | NA | C | NA | C |
| Inflammation | C | NC | C | NA | C | C | NA | C |
| Immunosuppression | NC | C | C | C | C | C | C | C |
| Immunostimulation | NC | C | C | NA | NC | NA | NC | C |
| Autoimmunity** | C | C | C | NA | NA | NC | NA | C |

*Notes.* C = critical; NC = noncritical; NA = not applicable or not needed; *basophils, eosinophils, and/or neutrophils; **routine testing for autoimmunity is not recommended (see text).

**TABLE 3**
Examples of tests, indicators, and models for the evaluation of immune responses*

| Immune responses | Functional assays | Soluble mediators | Phenotyping | Other** |
|---|---|---|---|---|
| Histopathology | NA | NA | Cell surface markers | Morphology |
| Humoral response | Immunoassays (e.g., ELISA) for antibody response to antigen plus adjuvant* | Complement (including C3a and C5a anaphylatoxins),* immune complexes | Cell surface markers | |
| | Plaque-forming cells<br>Lymphocyte proliferation<br>Antibody-dependent cell-mediated cytotoxicity<br>Passive cutaneous anaphylaxis<br>Direct anaphylaxis | | | |
| Cellular responses | | | | |
| T cells | Guinea pig maximization test*<br>Mouse local lymph node assay*<br>Mouse ear swelling test<br>Lymphocyte proliferation<br>Mixed lymphocyte reaction | Cytokine patterns indicative of T-cell subsets (e.g., Th1 and Th2) | Cell surface markers (helper and cytotoxic T cells) | |
| Natural killer cells | Tumor cytotoxicity | NA | Cell surface markers | |
| Macrophages | Phagocytosis*<br>Antigen presentation | Cytokines (IL-1, TNF$\alpha$, IL-6, TGF$\beta$) | MHC markers | |
| Granulocytes*** | Degranulation<br>Phagocytosis | Chemokines, bioactive amines, inflammatory cytokines, enzymes | NA | Cytochemistry |
| Host resistance | Resistance to bacteria, viruses and tumors | NA | NA | |
| Signs of illness | NA | NA | NA | Allergy, skin rash, urticaria, edema, lymphadenopathy |

*Notes*. NA = not applicable or not needed.
*Most commonly used tests. Functional assays are generally more important than tests for soluble mediators or phenotyping. References at the end of this guidance provide detailed testing protocols.
**Animal models of some human autoimmune diseases are available (see references at the end of guidance). However, routine testing for induction of autoimmune disease by materials/devices is not recommended.
***Basophils, eosinophils, and/or neutrophils.

anticipate autoimmune reactions. Assuming validated tests were available, testing for an autoimmune response, indicated by the presence of autoantibodies or autoreactive T cells, may be justifiable in limited cases, e.g., for materials suspected of having adjuvant properties, or for foreign proteins that mimic the structure of their human counterparts. Studying autoimmune disease in animals would provide useful mechanistic information if long-term use raises concerns that the material causes classical autoimmune disease in humans that has a comparable animal analog.

## What if a Material is Found to be Immunotoxic

Device manufacturers are expected to use tests that have been shown to provide accurate and reproducible results, and are sufficiently sensitive and specific to yield valid data. All studies should have a sound statistical design so that differences between experimental and appropriate control groups can be determined at a desired level of statistical significance (e.g., $p < .05$). In addition, materials should be tested to mimic the intended use as closely as possible, including route of exposure/site of implantation, dose, and duration. The purpose of nonclinical immunotoxicity testing is to help assess the biocompatibility of materials as part of the overall safety evaluation of medical devices. Significant indications of immunotoxicity may suggest that studies of immune function should be included in clinical trials and postmarket studies. Indications of immunotoxicity in product-labeling may also be useful information for physicians. Two basic issues in nonclinical testing are biological versus statistical significance and clinical predictivity. Further research, particularly related to medical devices and biomaterials with

their unique properties and uses, is clearly needed to identify additional predictive tests and to establish biological relevance of changes observed in immunotoxicity testing. The *Immunotoxicity Testing Guidance* is a first step in providing reviewers and manufacturers with a coherent approach to immunotoxicity testing, based on the methodology available now.

## NONCLINICAL IMMUNOTOXICITY TESTING IN DRUG SAFETY EVALUATION: CONCEPTS UNDER CONSIDERATION (S. Wilson)

### Background

The data derived from nonclinical studies are indispensable in determining not only if a drug is safe for clinical use, but also in defining the adverse reaction profile and criterion for the safe use of that drug. As for any organ system, assessment of the potential adverse effects of an investigational new drug on the immune system is a standard component of nonclinical toxicology studies. The Center for Drug Evaluation and Research (CDER) Immunotoxicology Committee has discussed nonclinical approaches to predict the potential immunotoxicity of a new drug.

### Concepts

For nonclinical evaluation of potential drug-induced immunosuppression, a staged protocol currently under consideration is being designed to maximize the data that can be collected in standard nonclinical repeat-dose toxicology studies. This staged protocol is based on the assumption that changes in hematology, serum chemistry, organ weights, clinical observations, necropsy, and histopathology can be utilized to screen for the potential of a drug to adversely impact the immune system. These changes include, but are not limited to, blood dyscrasias and evidence of myelosuppresion, decreases in basal serum immunoglobulin (Ig) levels, increased incidence of infections and certain types of tumors, significant changes in the weight of immune organs (e.g., spleen and thymus), and hypocellularity or necrosis of immune organs such as the spleen, thymus, and lymph nodes. Data obtained from other nonclinical studies routinely conducted during drug development may provide additional support that a drug is potentially an immunotoxicant. For example, pharmacokinetic studies may indicate that a drug accumulates or persists in an immune organ or specific cell type (e.g., macrophage).

Application of this approach underscores the need to examine the totality of data obtained in repeat dose nonclinical studies; however, this approach is highly dependent on histopathological evaluation. There is still considerable controversy as to the reliability of histopathology to identify potential immunotoxicants, especially when the perturbation is subtle (ILSI 1994). It is known that for some drugs, e.g., cyclosporine A, immune function is suppressed in animals at doses lower than those required to induce histopathological changes (Schurrman, Kuper, and Vos 1994). This emphasizes the importance of evaluating doses up to toxicity limiting dose levels. It also emphasizes the fact that histopathological changes in immune organs should not be dismissed simply because they are observed only at doses that significantly exceed the anticipated human exposure for a given drug. It is acknowledged that controversy exists regarding the sensitivity of selected endpoints in standard nonclinical repeat-dose toxicology studies to screen immunosuppressant drugs. At this time and within the context of drug development, the proposed approach is considered reasonable (De Waal et al. 1995; Richter-Reichhelm et al. 1995; Va der Laan and Dean 1996). However, as additional data become available, modifications to this proposed approach may be indicated.

In the event that changes in the nonclinical studies observed are consistent with immunosuppression the question arises as to what additional studies would be appropriate to further characterize the immunosuppressive potential of the drug. One methodology, flow cytometric analysis of surface marker expression to quantify splenic lymphocyte populations, has been considered as a possible follow-on assay. The rationale for this was based on several considerations. This assay has been shown to have a high concordance in mice with known immunotoxicants in an extensive evaluation by the National Toxicology Program (NTP) (Luster et al. 1992). In addition, this assay has the benefit of being readily incorporated into standard nonclinical toxicology studies as well as clinical trials. One of the primary criticisms of this assay, as part of nonclinical drug safety evaluation, is its limitation with respect to risk assessment. In other words, for most immune cell populations, it is currently not known what magnitude of decrease translates into compromised immune function. Additionally, immune function may be perturbed without changes in immune cell number. Currently, there is a collaborative investigation between the Food and Drug Administration and the National Institute of Environmental Health Sciences that will attempt to address the correlation between changes in immune cell phenotypes and immune cell function. Despite this limitation, flow cytometric quantitation of splenic lymphocytes is one approach that many believe can provide a basis for hazard identification and suggest additional nonclinical immunotoxicity tests. The data derived from quantitation of splenic lymphocytes in animal studies may also suggest immunotoxicity endpoints that may be of value to monitor in the clinical setting. It is recognized that none of the endpoints discussed above are measures of immune function. It is also recognized that under certain circumstances, functional assays may provide useful data. The IgM response to sheep red blood cells in rodents (the plaque assay or an enzyme-linked immunosorbent assay [ELISA]) is considered to be a sensitive indicator of the status of the immune system due to its dependence on the functional integration of B lymphocytes, T helper lymphocytes, and macrophages (Holsapple et al. 1995). As noted earlier, the selection of any immune assay(s) that may be appropriate to include in the nonclinical evaluation of a drug should be driven by data obtained from the nonclinical studies (i.e., repeat-dose toxicology studies, pharmacokinetic studies) routinely conducted during the drug development process.

These considerations are relatively consistent with the European recommendations. The Organization for Economic Cooperation and Development (OECD) Guidelines 408 and 409 (90-Day Repeat Dose Toxicity Study in Rodents and Dogs, respectively) recommend incorporation of expanded histopathology with a standardization of grading criteria into standard repeat-dose nonclinical toxicology studies (OECD 1996a,b). In addition, evaluation of the primary, or IgM, response to sheep red blood cells in the rodent is being considered for incorporation in OECD Guideline 407 (28-Day Repeat Dose Toxicity Study in Rodents) if there are indications of immunotoxicity observed in the repeat dose toxicology studies (OECD 1995).

## Dermal Sensitization and ICCVAM

The sensitizing potential of a drug may not be readily detected in standard nonclinical repeat dose toxicology studies. Consequently, nonclinical methods to specifically address the ability of topically administered drugs to induce skin sensitization have also been discussed by the CDER Immunotoxicology Committee. Historically, the FDA has accepted a number of guinea pig assays for the nonclinical evaluation of the sensitizing potential of a drug, e.g., Buehler Test (Buehler 1964), Guinea Pig Maximization Test (Magnusson and Kligman 1969), Draize Test (Draize 1959), and Split Adjuvant Technique (Maguire 1972). These assays rely on a qualitative evaluation of the elicitation phase. This is also the endpoint measured in the clinical setting. Other assays, such as the murine LLNA (Kimber, Hilton, and Weisenberger 1989), have been developed which measure the induction phase of the hypersensitivity reaction. This assay measures incorporation of $^{3}$H-thymidine into proliferating lymphocytes in draining lymph nodes from animals topically administered a drug or chemical. While exhibiting concordance with guinea pig assays (NIEHS 1999), the LLNA offers several advantages over these assays. The LLNA is more conducive to determination of a dose-dependent relationship, can more readily detect the sensitizing potential of colored compounds, has an endpoint that is quantitative rather than qualitative, and has a mechanistic basis. It has been suggested that because elicitation of an allergic response is dependent on a number of factors and may not always be manifested in an exposed, sensitized individual, assessment of the induction phase may be a more relevant indicator of sensitization potential (NIEHS 1999). Currently, OECD Guideline No. 406 (OECD 1993) recommends use of the LLNA as a screening assay. The results are acceptable if the assay is positive. If the assay is negative, then OECD guidelines indicate that a guinea pig maximization test should be conducted. Recently, the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) has considered the LLNA as an alternative to the guinea pig assays within the regulatory setting. This is the first assay that has been evaluated under this process.

ICCVAM was established by the National Institute of Environmental Health Sciences (NIEHS) in response to Public Law 103-43 which mandated that a process be developed which would not only facilitate the validation of alternative toxicological methods, but also facilitate regulatory acceptance of these newly validated methods. Furthermore, it was determined that these new methodologies should promote what is referred to as the 3 Rs: (1) Refinement—methods that reduce or eliminate the distress to animals; (2) Reduction—methods that reduce the number of animals used; and (3) Replacement—methods that use nonanimal systems or a phylogenetically lower animal. ICCVAM, which is comprised of representatives from 14 U.S. regulatory and research agencies, established validation and regulatory acceptance criteria to attain these goals (NIH 1997).

In 1997, Drs. Frank Gerberick (Proctor and Gamble), Ian Kimber (Zeneca, UK), and David Basketeer (Unilever, UK) sponsored the LLNA as a stand-alone alternative to guinea pig assays for determination of the potential of a drug or chemical to induce skin sensitization (NIEHS 1999). Subsequently, ICCVAM established an Immunotoxicity Working Group (IWG) consisting of representatives from the various member regulatory and research agencies. The IWG established a Peer Review Panel composed of scientists from U.S. industry, academia, and government as well as representatives from Europe and Japan. The role of the Peer Review Panel was to arrive at a scientific consensus as to the utility of the LLNA as a stand-alone alternative to guinea pig assays and to determine whether the LLNA is consistent with the 3 Rs. To achieve this end, the IWG formulated questions, consistent with ICCVAM guidelines, with respect to quality of test method description and data; the performance, transferability, and reliability of the LLNA method; and limitations of this assay (NIEHS 1999).

In September, 1998, a public meeting of the Peer Review Panel was held at which the Panel unanimously endorsed acceptance of the LLNA as a stand-alone assay for the nonclinical assessment of the potential of a test article to induce skin sensitization in humans. The Panel recommended that certain modifications be incorporated into the protocol for this assay. A sample protocol has been provided in NIH Publication 99-4494 (NIEHS 1999). As already noted, the LLNA offers several advantages over the standard guinea pig assays. The Peer Review Panel, however, indicated that weak sensitizers and strong irritants might not be accurately predicted. In terms of predictivity, both positive and negative, the Panel concluded that the LLNA was comparable to the guinea pig assays. The guinea pig assays and LLNA were also comparable in performance based on human data provided. It is recognized that there are certain compounds, such as metals, for which the standard guinea pig assays may be more appropriate. Evaluation of mixtures and pharmaceuticals is also limited (NIEHS 1999). Because the LLNA is mechanistically based and a large number of test articles representing a number of chemical classes have been evaluated, it is anticipated that the LLNA will exhibit comparable predictivity for pharmaceuticals and mixtures (Memorandum; January 11, 1999). Furthermore, in a preliminary analysis, the LLNA was shown to be predictive for several drugs (Kimber et al. 1998). It should be emphasized that this assay is intended solely for

the detection of the skin sensitizing potential of a test article. It is not considered appropriate to use this assay to assess the potential of a drug or chemical to induce either respiratory or systemic hypersensitivity.

The conclusions and recommendations of both the Peer Review Panel and the IWG have been forwarded to the various regulatory agencies. The agencies will decide whether the LLNA is acceptable for regulatory purposes and under what conditions it is acceptable. In addition, the agencies will implement steps to notify those impacted by this decision, e.g., the pharmaceutical and chemical industry and the regulatory community.

### Conclusion

The CDER Immunotoxicology Committee has discussed what may constitute appropriate and scientifically founded nonclinical approaches to predict the immunotoxic potential of a new drug. Consideration has focussed on maximizing data collected in standard nonclinical repeat-dose toxicology studies to provide an initial assessment. If the repeat-dose studies indicate an immunotoxic potential for a given drug, flow cytometric analysis of surface marker expression to quantify splenic lymphocyte populations has been considered as one possible follow-on assay. Inclusion of functional assays (e.g., primary antibody response to sheep red blood cells in rodents) in the nonclinical drug evaluation process should be data-driven.

The nonclinical evaluation of the contact sensitization potential of a new topical drug has historically been assessed by guinea pig assays. A Peer Review Panel and an Immunotoxicity Working Group under the auspices of ICCVAM have rigorously evaluated the murine LLNA. The Panel concluded that the LLNA meets the criteria established by ICCVAM for validation of an alternative method and recommended the assay as a standalone alternative to guinea pig assays for assessment of the skin sensitizing potential of a drug. CDER is evaluating the acceptability of this assay for use in safety assessment for meeting its regulatory responsibilities, as are other centers within the FDA.

## AUTOIMMUNITY AND IMMUNOTOXICOLOGY (M. Luster)

### Background

Autoimmunity comprises two processes. Initially, an immune response occurs to normal components of the host and secondly, a pathological condition may ensue in which the response causes structural or functional damage. As often noted by our colleagues in immunology, this immune response is not a correlate for disease, but rather is a prerequisite. The autoimmune response can be cellular in nature, mediated by $CD4^+$ and/or $CD8^+$ T cells. More often, however, it arises from antibody mediated by specific B cells that are driven by cytokines derived from $CD4^+$ cells. Autoimmune disease is complicated by the fact that it is not a single disease, but rather represents more than 25 different diseases which are either systemic or organ-specific in nature. The most common autoimmune diseases are rheumatoid arthritis and those associated with the thyroid, such as Graves' disease (Jacobsen et al. 1997). In total, they represent a significant and chronic morbidity problem; recent estimates indicating that 1 in 31 individuals in the United States are affected, with women at 2.7 times greater risk than men (Jacobsen et al. 1997).

The mechanism(s) responsible for autoimmune diseases are not clear. It is believed that the failure of any one of several immune processes can result in their development. However, a key event involves the loss of self-tolerance, such as the missed deletion or activation of autoreactive lymphocyte precursors. This process may be exacerbated by altered immunoregulation, such as overexpression of the immunoregulatory cytokine interleukin-1 (IL-1) or underexpression of interferon gamma ($INF\gamma$). Autoimmunity may also occur in the absence of an aberration in the immune system, such as when microbial agents express cryptic determinants (Sercarz, Lehmann, and Ametani 1993). Although autoimmunity is a disease of the immune system, nonimmunological genetic and epigenetic factors play a major role in disease development. For example, autoimmunity is influenced strongly by infectious agents, stress, and diet (epigenetic) as well as polymorphisms in the T-cell receptor and drug-metabolizing phenotypes (genetic). The association of autoimmune diseases with certain haplotypes of the major histocompatibility complex (MHC), such as HLA-DR3 in systemic lupus, is striking. A detailed description of the potential mechanisms and the factors that influence autoimmune disease development is beyond the scope of this section, and the reader is referred to recent reviews (Sercarz, Lehmann, and Ametani 1993; Liblan, Singer, and McDevitt 1995; Theofilopoulus 1995).

### Autoimmunity and Xenobiotic Exposure

Autoimmune diseases have been associated with exposure to several chemicals and certain drugs. These diseases differ somewhat from their idiopathic counterparts in terms of their clinical spectrum or their specific immunological response (Rose and Caturegli 1997). As such, chemical/drug-induced autoimmune diseases normally remit when the agent is removed. In contrast to most agents, some biologics, such as $INF\gamma$, are not themselves etiological agents for autoimmune disease, but are believed to exacerbate pre-existing disease (Biagazzi 1995). This occurs through their immunomodulatory properties rather than their ability to unmask novel antigenic determinants or affect tolerance. The most common examples of drugs which produce autoimmune disease are those that cause hematological disorders such as neutropenia, thrombocytopenia, and immune hemolysis and include a variety of antibiotics as well as anticonvulsants such as phenytoin (Table 4). Another autoimmune disease commonly associated with drug exposure is systemic lupus erythematosus (SLE). Approximately 10 to 20% of patients receiving procainamide and 5 to 20% receiving hydralazine develop drug-induced SLE (Biagazzi 1995). Although not as well documented as with drugs, considerable evidence

**TABLE 4**
Example of drugs and chemicals implicated in autoimmune disease

| Pathology | Agent | |
|---|---|---|
| Systemic lupus erythematosus/immune complex glomerulonephritis | Hydralazine | Heavy metals |
| | Penicillamine | Isoniazid |
| | Chlorpromazine | Organic solvents |
| | Anticonvulsants | Procainamide |
| | Alfalfa sprouts (L-canavanine) | |
| Hemolytic anemia | Methyldopa | Diphenylhydantoin |
| | Penicillin | Interferon $\alpha$ |
| | Mefenamic acid | Sulfa |
| Thrombocytopenia | Acetazolamide | *p*-Aminosalicylic acid |
| | Chlorothiazide | Rifampin |
| | Gold salts | Quinidine |
| Scleroderma-like disease | Vinyl chloride | L-tryptophan |
| | Silica | |
| Pemphigus | Penicillamine | |
| Thyroiditis | PCBs | Lithium |
| | Iodine | Interleukin-2 |

exists that autoimmune diseases can also be induced by substances found in food or the environment. Regarding food consumption, strong associations have been found to exist between the consumption of iodine and autoimmune thyroiditis, L-5-hydroxytryptophan and scleroderma, and alfalfa seeds and SLE. Exposure to occupational agents has also been linked to autoimmune diseases. Scleroderma-like skin diseases can result from exposure to vinyl chloride, silica, or aniline derivatives, the latter presumably the active agent causing the 'toxic oil syndrome' (Kamuller, Bloksma, and Seinen 1988). Heavy metals, nitrofurantoins, and organic solvents such as trichloroethylene are associated with SLE or glomerulonephritis. Like their idiopathic counterparts, xenobiotic-induced autoimmune disease is associated with a favorable genetic background. Thus, individuals who are low acetylators are at increased risk of developing drug-induced SLE and individuals who possess the HLA DR3 allele have a 32-fold relative increased risk for developing autoimmunity from gold salts (for review see Cooper, Miller, and Pandey 1999). Experimental studies of mercury-induced autoimmunity in the Brown-Norway rat and B.10 mice suggest the same genetic influences apply in animals (Pelletier, Ramanathan, and Druet 1997).

## Assessment of Xenobiotic-Induced Autoimmunity

Although there is general consensus within the toxicology community that there is a major need to screen drugs or chemical agents for their potential to induce autoimmunity (Dean, Hincks, and Remandet 1998), suitable validated models do not as yet exist. This is despite the fact that a large number of experimental animal and in vitro models are available to study the mechanisms of autoimmune disease. The primary reason for the lack of validated assays probably stems from the complexity of the disease. First, and as mentioned earlier, autoimmune disease is not one disease but a group of over 25 diseases effecting distinct organs, often through different mechanisms. Unless a common early process is identified, a single test would be unlikely to provide an adequate degree of concordance to be useful for predictive risk assessment. Secondly, it is difficult to develop tests that adequately control all of the genetic and epigenetic factors that influence autoimmunity. Lastly, when using animal models, there is some uncertainty regarding what actually constitutes autoimmunity. This is reflected by a lack of well-defined diagnostic tests for identifying autoimmune disease in humans. Despite these challenges, attempts to develop predictive screening assays for detecting xenobiotic-induced autoimmunity have been undertaken in several laboratories. Currently, four screening approaches, which are clearly different from those used for mechanistic studies, have been suggested. Each has received varying levels of attention and include (1) monitoring changes in the frequency or rate of autoimmune disease using autoimmune prone rodents (Lai and Forster 1991), (2) identifying immunoglobulin complexes or immunoglobulin deposits using immunohistological procedures, (3) monitoring for increased levels of serum autoantibodies, and (4) the use of the popliteal lymph node assay (PLNA) with reporter antigens. The successful use of exacerbating disease in already autoimmune-prone rodent species has been illustrated by injection of streptozotocin in diabetic mice (Leiter 1982) or injections of $HgCl_2$ in glomerulonephritis-prone mice and rats (Hultman et al. 1996). Less studied has been the monitoring of autoantibody production following chemical exposure (Kilburn and Warshaw 1992)

or immunohistological techniques. The approach that has received the most attention is the PLNA with reporter antigens (Albers et al. 1997). In this model, the 'autoimmunogenecity' of a chemical, like graft versus host (GVH) reactions, is determined by its ability to stimulate specific IgG responses to trinitrophenol (TNP)-Ficoll and TNP-ovalbumin from cells in the popliteal lymph node. Although not an indicator for disease, it may prove useful as a 'first-tier' screen, as it is independent of the nature of the neo-antigens and eliminates many of the potential genetic confounders. In this assay the test compound is co-injected with TNP-ficoll or TNP-ovalbumin subcutaneously into the right hind paw of mice. The amount of test substance injected can be equimolar to a related compound or, if known, a concentration demonstrated to be stimulatory in the PLNA. Seven days following treatments, the thickness of the paw is measured using a micrometer and the draining PLN isolated. Specific antibody-forming cells from the PLN are quantified by any one of several methods such as the ELISPOT. As there is a test for 'adjuvancy,' further validation is clearly warranted before being recommended as a predictive method to assess autoimmune potential of agents.

### Risk Assessment Considerations

To improve the risk assessment process, screening models for autoimmunity, as described above, will need to be developed and validated which not only incorporate mechanistic information into the assessment process, but allow for the consideration of the genetic, physiological, and environmental influences that lead to the loss of self-tolerance and autoimmune disease. Despite the challenges in developing such screening tests, the considerable amount of data generated by immunologists and pharmacologists pertaining to basic mechanisms of chemical-induced autoimmune diseases have provided a conceptual framework that allows the establishment of potential structure-activity relationships. For example, estrogens are known to be a major factor in classical autoimmune diseases due presumably to their ability to stimulate certain components of the immune system (Homo-Delarche et al. 1991), and, as such, agents with estrogenic activity may be of concern. Laboratory studies have also shown that thymolytic chemicals, such as cyclophosphamide and cyclosporin A, can induce autoimmunity when given neonatally by altering normal patterns of autoreactive T-cell deletion (Sakaguchi and Sakaguchi 1989). In this respect, the thymus has been shown to be a target for many toxic chemicals. As in the case of halothane, chemicals that form protein adducts or damage tissue in such a way to allow expression of cryptic determinants would provide novel host antigens which could now be recognized by T cells. Agents that have 'adjuvant' activity or biologicals which stimulate certain cytokines may shift the balance of Th1 and Th2 cells and allow exacerbation of pre-existing autoimmune disease (Chazerain, Meyer, and Kahn 1992). Common features associated with many drugs that induce autoimmune diseases are that they serve as myeloperoxidase substrates and/or cause changed in methylation (Greim, Gleichmann, and Shaw 1997). The explanation for the latter association is less clear, but may require identification of the specific antigenic epitopes responsible for the autoimmune response. In the case of the association with myeloperoxidase substrates, it has been suggested that many of the chemicals require metabolism in proximity to immune cells in order to be antigenic and immune cells, such as monocytes that contain high levels of myeloperoxidase. Nonetheless, structure activity relationships (SARs) will not substitute for validated screening assays.

## SUMMARY

Outcomes of immunotoxicity may be characterized as pathologies causing immunosuppression, leading to increased host susceptibility or a heightened, inappropriate immune response resulting in hypersensitivity/allergy or autoimmunity. Currently accepted modes for assessing immune alterations include histopathology of select lymphoid tissue, clinical pathology, clinical chemistry, plaque-forming cell assay for humoral immunity, and allergic contact hypersensitivity. Advances in immunology and molecular biology over the years have led to various activities to optimize risk assessment processes and strategies for immunotoxicants.

The CDRH has prepared guidance that is recommended for assessing immunotoxic potential of medical devices or medical device components. Many of the assays outlined in the CDRH document have been developed under the NTP and undergone an interlaboratory validation (Luster et al. 1988). The Immunotoxicology Working Group of CDER is discussing means to optimize risk assessment strategies. Advances in allergic contact hypersensitivity have led to the development of the murine local lymph node assay. This model has been approved recently by the ICCVAM as an alternative to the guinea pig model. While the FDA is considering the LLNA as an alternative test for regulatory use, OECD recommends that a negative finding in the murine LLNA be confirmed by the guinea pig assay.

Chemical-induced autoimmunity, as well as systemic hypersensitivity, are areas in which nonclinical assessments are likely to continue to improve. These areas are difficult to assess due to variables such as genetic makeup, age, and sex that contribute to the low frequency observed. A recent survey of the pharmaceutical industry indicated that systemic hypersensitivity reactions comprise a large percentage of the new drugs producing immunotoxicity that are terminated from clinical development (Dean, Hincks, and Remandet 1998). Continual improvement in nonclinical tests may lead to increased success of candidate drugs clinically. As different types of autoimmune diseases with multiple causes exist, it will be difficult to establish a model or battery of procedures to detect induction of these diseases without first obtaining a better understanding of underlying mechanisms and interspecies differences in responses.

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