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Immunotoxicity

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As evidenced by recent documents prepared by the Office of Technology Assessment (9) and the National Research Council in 1992 (8) focusing on immunotoxicology, there has been growing interest and concern within the scientific and public communities on the capacity of environmental agents to perturb normal immune processes. The types of effects that may occur are often agent-specific as well as species-specific and include immuno-suppression in which either systemic or local immunity is targeted (e.g., lung or skin), hypersensitivity disease, manifested as respiratory tract allergies or contact allergic dermatitis, and in certain instances autoimmunity or increased autoantibodies without evidence of disease. In addition to environmental pollutants, other agents of concern have included certain therapeutics, consumer products and biological (e.g., the therapeutic use of recombinant materials). More recently, interest has also focused on potential immunological effects by such diverse agents as excessive UV-B light, electromagnetic fields and pollutants found in the indoor environment. Common indoor pollutants can include not only chemical agents but also bioaerosols such as viruses, bacteria, fungi, algae and protozoa which have the potential to act as either sensitizing agents or mediators of infectious disease.

IMMUNOSUPPRESSION - EXPERIMENTAL TESTING

The sensitivity of the immune system to suppression by exogenous agents is due as much to the general properties of the agent as to the complex nature of the immune system. Because of this complexity, the initial strategies devised by immunologists working in toxicology and safety assessment have been to select and apply a tiered panel of assays to identify immuno-suppressive or, in rare instances, immuno-stimulatory agents in laboratory animals. Although the configurations of these testing panels vary depending on the laboratory conducting the test and the animal species employed, they usually include measures for one or more of the following:

- altered lymphoid organ weights and histology;
- quantitative changes in cellularity of lymphoid tissue, peripheral blood leukocytes and/or bone marrow;
- impairment of cell function at the effecter or regulatory level; and/or
- increased susceptibility to infectious agents or transplantable tumors.

Some of the test panels that have been proposed for evaluating the immune system in experimental animals by various government agencies are shown in tables 5-1 and 5-2. Additional test panel proposals (3, 5, 10, 13) are described in the IPCS Environmental Health Criteria "Principles and Methods for Assessing Direct Immunotoxicity Associated with Exposure to Chemicals", which is in the final stages of publication. The tier testing approaches employed by these agencies are similar in design in that the first tier is a screen for immunotoxicity

This **chapter was adapted,** in part, from the International Programme on Chemical Safety, "Principles and **Methods** for **Assessing Direct Immunotoxicity Associated with Exposure to Chemicals," UNEP, ILO, WHO, September 1994.**

Table 5-1: Panel for Detecting Immune Alterations Following Chemical or Drug Exposure in Rodents[a] Adopted by NTP

Parameters	Procedures
Screen (Tier 1)	
Immunopathology	• Hematology: complete blood count and differential • Weights: body, spleen, thymus, kidney, liver • Cellularity: spleen • Histology: spleen, thymus, lymph node
Humoral immunity	• Enumerate IgM antibody plaque-forming cells to T-dependent antigen (sRBC) • LPS mitogen response
Cell-mediated immunity	• Lymphocyte blastogenesis to mitogens (Con A) • Mixed leukocyte response against allogeneic leukocytes (MLR)
Nonspecific immunity	• Natural killer (NK) cell activity
Comprehensive (Tier 2)	
Immunopathology	• Quantitation of splenic B and T cell numbers
Humoral-mediated immunity	• Enumeration of IgG antibody responses to sRBCs
Cell-mediated immunity	• Cytotoxic T lymphocyte (CTL) cytolysis • Delayed hypersensitivity response (DHR)
Nonspecific immunity	• Microphage function quantitation of resident peritoneal cells and phagocytic ability (basal and activated by MAF)
Host resistance challenge models (endpoints)[b]	• Syngeneic tumor cells • PYB6 sarcoma (tumor incidence) • B16F1O melanoma (lung burden) • *Bacterial models: Listeria monocytogenes (mortality); Streptococcus species (mortality)* • Viral models: Influenza (mortality) • Parasite models: Plasmodium yoelii (Parasitaemia)

[a]The test panel was developed using B6C3F1 female mice.

[b]For any particular chemical tested only two or three host resistance models are selected for examination.

SOURCE: Adapted from International Programme on Chemical Safety," Principles and Methods for Assessing Direct Immunotoxicity Associated with Exposure to Chemicals," UNEP, ILO, WHO, September 1994.

with the second tier consisting of more specific or confirmatory studies, host resistance studies, or in-depth mechanistic studies. At present, most information regarding these models comes from the U.S. National Institute of Environmental Health Sciences, National Toxicology Program (NIEHS/NTP) followed by the model developed at the National Institute of Public Health and Environmental Protection (RIVM) in Bilthoven, The Netherlands. These models are described in more detail, while the others are not since little, if any, data have been published on their performance. The first-tier screening at RIVM consists only of test for general parameters of specific and nonspecific immunity. In contrast, Tier I of the NIEHS-NTP panel includes "functional" tests in which an immune response is measured following *in vivo* antigenic challenge. These are considered the most sensitive indicator of immune integrity but not routinely conducted as part of subchronic toxicology studies as there is concern that immunization may compromise toxicity interpretation. At present, when the NIEHS-NTP protocol is used, functional tests are

performed in a separate groups of animals. In the RIVM screening battery (see table 5-l), histopathology of lymphoid organs is pivotal. Routine histopathology of lymphoid organs has been shown to be useful in assessing the potential immunotoxicity of a chemical, in particular when these results are combined with the effects observed on the weight of the lymphoid organs and sufficiently high doses of the chemical are tested. In the RIVM panel, if the results in tier I suggest immunotoxicity, tier II function studies can be performed to confirm and further investigate the nature of the immunotoxic effect. Information on structure-activity relationships of immunotoxic chemicals can also lead to the decision to initiate function testing. The choice for further studies depends on the type of immune abnormality observed. The second tier consists of a panel of *in vivo* and *ex vivo/in vitro* assays including cell-mediated immunity, humoral immunity, microphage and natural killer (NK) cell function, as well as host resistance assays. Recently, it was suggested that the NK cell assay be added to RIVM/s tier I since it does not require animal sensitization.

The RIVM approach is based on the Organization for Economic Cooperation and Development (OECD) proposed guidelines for testing of chemicals - ##407, Repeated Dose Oral Toxicity - Rodent: 28-day or 14-day Study - which suggests the maximum tolerated dose (MTD), to be used as the high dose level for studies. The standard exposure period is 28 days and the animal species routinely used is the rat. These tests can be performed in the context of studies aimed at determining the toxicologic profile of the compound. Testing is conducted on at least three dose levels, the highest dose being the MTD and the lowest producing no evidence of toxicity.

The most employed screening battery and presumably more sensitive than RIVM since it includes function tests in Tier I is that developed by the NIEHS-NTP (5; see table 5-2). Recently, the database generated from these studies, which consists of over 50 compounds, has been collected and analyzed in an attempt to improve the accuracy and efficiency of screening chemicals for immunosuppression and to better identify those tests that predict immune-mediated diseases (6, 7). While a number of limitations exist in the analyses, several conclusions were drawn:

1) Examination of only two or three immune parameters may be used to successfully predict immunotoxicants in mice. In particular, lymphocyte enumeration and quantitation of the T-cell dependent antibody response appear particularly beneficial. Furthermore, commonly employed apical measures (e.g., leukocyte counts, lymphoid organ weights) appear fairly insensitive;
2) A good correlation existed between changes in the immune tests and altered host resistance in that there were no instances when host resistance was altered without affecting an immune test. However, in many instances immune changes were observed in the absence of detectable changes in host resistance. This can be interpreted to reflect that immune tests are, in general, more sensitive than the host resistance assays;
3) No single immune test was identified which could be considered highly predictive for altered host resistance. However, several assays such as the PFC response, surface markers, thymic weights and DHRs, were good indicators and others, such as proliferative response to LPS and leukocyte counts, were relatively poor indicators for host resistance changes. Combining several immune tests increased the ability to predict host resistance deficits, in some cases to about 80%;
4) Considering that there exists a “background” level of infectious diseases in the population, it is possible that subtle changes in immune function may translate into a significant change in host resistance given that the population exposed is large enough. This can be demonstrated experimentally, but would be difficult to establish in a clinical study where neither the virulence nor dose of infectious agent can be controlled;

Table 5-2: Methods for Detecting Immunotoxic Alterations in the Rat Currently Being Evaluated at RIVM, Bilthoven, The Netherlands

Parameters	Procedures
Tier 1	
Non-functional	• Routine hematology, including differential cell counting • Serum IgM, G, A, and E determination; lymphoid organ weights (spleen, thymus, local and distant lymph nodes) • Histopathology of lymphoid tissue • Bone marrow cellularity • Analysis of lymphocyte subpopulations in spleen by flow cytometry
Tier 2	
Cell-mediated immunity	• Sensitization to T-cell dependent antigens (e.g., ovalbumin, tuberculin, *Listeria),* and skin test challenge • Lymphoproliferative responses to specific antigens *(Listeria)* • Mitogen responses (Con-A, PHA)
Humoral immunity	• Serum titration of IgM, IgG, IgA, IgE responses to T-dependent antigens (ovalbumin, tetanus toxoid, *Trichinella spiralis,* sheep red blood cells) by ELISA • Serum titration of T-cell independent IgM response to LPS by ELISA • Mitogen response to LPS
Microphage function	• *In vitro* phagocytosis and killing of Listeria *monocytogenes* by adherent spleen and peritoneal cells • Cytolysis of YAC-1 lymphoma cells by adherent spleen and peritoneal cells
Natural killer function	• Cytolysis of YAC-1 lymphoma cells by non-adherent spleen and peritoneal cells
Host resistance	• *Trichinella spiralis* challenge (muscle larvae counts and worm expulsion) • *Listeria monocytogenes* challenge (spleen and lung clearance) • Rat cytomegalovirus challenge (clearance from salivary gland) • Endotoxin hypersensitivity • Autoimmune models (Adjuvant arthritis, experimental allergic encephalomyelitis)

SOURCE: Adapted from International Programme on Chemical Safety,'' Principles and Methods for Assessing Direct Immunotoxicity Associated with Exposure to Chemicals," UNEP, ILO, WHO, September 1994.

5) Logistic and standard modeling, using a single large dataset indicated most immune function-host resistance relationships follow a linear rather than linear-quadratic (threshold) models suggesting that even the smallest change in immune function translates into some change in host resistance (table 5-3). However, because of the variability in the responses, it was not possible to establish linear or threshold models for most of the chemicals studied when the datasets were combined;
6) Finally, using one dataset methods were developed for modeling quantitative relationships between changes in selected immune assays and host resistance tests. It is impossible to determine at present how applicable these values will be for immunotoxic compounds with different immune profiles. However, as more analyses become available, our ability to estimate accurately potential clinical effects from immunological tests should increase.

There are, of course, a number of limitations in using such test panels. For example, some endpoints are currently not included (e.g., PMN activity, cytokine production). Furthermore, such test panels seldom examine the effects of chronic exposure, or whether tolerance or reversibility can result from the treatment. In humans, assays that involve *in vivo* antigenic challenge, which are

Table 5-3: Most Appropriate Relationships Describing the Host Resistance and Immune Test[a]

Host Resistance Test	Immune Test									
	PFC	CTL	MLR	Con A	LPS	sig+	Thy 1.2+	CD4+	CD8+	Thy/ BW
L. monocytogenes	L	L-Q	L-Q	L	L	L-Q	L-Q	L-Q	L	L-Q
PYBC Tumor	L	L	L	L	L	L-Q	L	L	L	L
S. pneumonia	L-Q	L	L-Q	L	L	L-Q	L	L	L	N

[a]L = linear; L-Q = linear-quadratic; N = neither linear nor linear-quadratic

SOURCE: Luster, et al., 1993.

usually accepted as the most sensitive and informative of immune tests, are considered "invasive" procedures since they involve immunization and, as such, are not usually feasible or practical for inclusion in human studies.

A variety of factors need to be considered when evaluating the potential of an environmental agent or drug to adversely influence the immune system of experimental animals. These include appropriate selection of animal models and exposure variables, inclusion of general toxicological parameters, and an understanding of the biologic relevance of the endpoints to be measured. Treatment conditions should take into account the potential route and level of human exposure, biophysical properties of the agent such as half-life and any available information on the mechanism of action. Dose levels should be selected which attempt to establish clear dose-response curves as well as a no-observable-effect-level (NOEL). Although in some instances it is beneficial to include a dose level which induces overt toxicity, any immune change observed at such a dose level should not be considered biologically significant since severe stress and malnutrition are known to impair immune responsiveness. If studies are being designed specifically to establish reference doses for toxic chemicals, additional exposure levels are advisable. In addition, inclusion of a positive control group with an agent that shares characteristics of the test compound may be advantageous under certain circumstances when experimental and fiscal constraints permit.

The selection of the exposure route should parallel the most probable route of human exposure, which is most frequently oral, respiratory or dermal. A requirement for accurate delivered dose may require the use of a parenteral exposure route. However, this may significantly alter the metabolism or distribution of the agent from that which would occur following natural exposure and prevents any evaluation of effects on local immune responses at the site of entry.

The selection of the most appropriate animal model for immunotoxicology studies has been a matter of great concern. Ideally, toxicity testing should be performed with a species that will respond to a test chemical in a pharmacologic and toxicologic manner similar to that anticipated in humans (i.e., the test animals and humans will metabolize the chemical similarly and will have identical target organ responses and toxicity). Toxicologic studies are often conducted in several animal species, since it is assumed that the more species showing a specific toxic response, the more likely that the response will occur in humans. For most immunosuppressive therapeutics, rodent data on target organ toxicities and the comparability of immunosuppressive doses have generally been predictive of later observations in the clinic. Exceptions to the predictive value of rodent toxicological data are infrequent but have occurred, such as in studies of glucocorticoids, which are lympholytic in rodents, but not in primates. Although certain compounds may exhibit different pharmacokinetic properties in rodents than in humans, rodents still appear to be

the most appropriate animal model for examining the immunotoxicity of non-species-specific compounds, based on established similarities of toxicological profiles as well as the relative ease of generating host resistance and immune function data. Comparative toxicologic studies should be continued and expanded, particularly for novel recombinant biological compounds and natural products, since their safety assessment will likely present species-specific host interactions and toxicological profiles.

In summary, it is clear that the current OECD guideline #407 or "standard" subchronic toxicological studies are not suited to adequately assess potential adverse effects of exposure to the test chemical on the immune system, since, with respect to immunologic parameters, it is restricted to total and differential leukocyte counting and histopathology of the spleen. An evaluation of this test scheme (12, 13) indicated that in a series of almost 20 chemicals over 50°/0 of the immunotoxic chemicals would not have been identified as such if the tests would have strictly adhered to the guideline. In fact, it is even doubtful if those chemicals that were indicated to be immunotoxic only on the basis of guideline #407 would have been identified as such. For example, in a toxicological experiment a small, but significant, change in the percentage of basophilic leukocytes, without any other parameter to suggest that an effect on the immune system might have been present, would of itself probably not be considered biologically relevant.

▮ IMMUNOSUPPRESSION / HUMANS

Establishing immune changes in humans is considerably more complex than in animals considering non-invasive tests are limited, exposure levels to the agent (i.e., dose) are difficult to establish and responses in the population are extremely heterogeneous. With respect to the latter, the variation in immune responses (genetic or environmental) can exceed a coefficient of variation greater than 20 to 30%. Because many immune changes in humans following chemical exposure may be sporadic and subtle, it is essential that recently exposed populations be studied and sensitive tests for assessing the immune system be performed. Since many of the immune tests performed in humans have a certain degree of overlap (redundancy), it is also important that a positive diagnosis be based not on a change in one test, but only if a profile (pattern) of changes occur, similar to that observed in primary or secondary immunodeficiency diseases. For example, low CD4:CD8 ratios are often accompanied by changes in skin tests to recall antigens. The World Health Organization (WHO) has recently prepared a monograph (4) which provides testing methods and pitfalls for examining immune system changes in humans. However, it should be noted that the selection of most of these tests were derived from observations in patients with primary immunodeficiency diseases. Such individuals suffer from severe recurring infections and the degree of immunosuppression would likely be considerably more severe than that induced by chemicals. Thus, the document may be of limited value for examining potential chemical-induced immuno-suppression, although it should provide a focus for further methods evaluation.

In lieu of the difficulties that exist in identifying chemical-induced immunosuppression in humans, establishment of exposure levels (e.g., blood or tissue levels) of the suspected chemical(s) would not only be useful but in many instances essential to determine a cause-effect relationship. It should not be necessary to observe clinical diseases in order for immunosuppression to be meaningful for several reasons. First, uncertainties exist regarding whether the relationship between immune function and clinical disease follows linear or threshold responses. For instance, in a linear relationship even minor changes in immune function would relate to increased disease, given that the population examined is large enough. While the relationship at the low end of the dose-response curve is unclear, obviously, at the high end of the curve (i.e., severe immunosuppression), clinical disease is readily apparent. This is exemplified

by increases in opportunistic infections that occur in AIDS patients. Secondly, clinical disease may be difficult to establish considering neoplastic diseases may involve a 10-20 year latency before tumor detection and increases in infections are difficult to ascertain in epidemiological surveys (e.g., increased numbers or severity of colds).

The Agency for Toxic Substances and Disease Registry with the CDC (ATSDR/CDC) and National Research Council's subcommittee on "Biologic Markers in Immunotoxicology" have proposed testing batteries which attempts to address many of the above described problems and pit-falls by implementing a comprehensive state-of-the-art immunological evaluation in conjunction with more traditional tests (8, 11). Many of these tests are similar to those used to identify chemical-induced immunosuppression in lab-oratory animals and should help to predict the probability of developing suppressed host resistance or clinical disease in humans. These tests are also recommended in a tiered approach.

HYPERSENSITIVITY

Chemicals that induce hypersensitivity response are often small, highly reactive molecules (haptens) or protein products and produce an antigen-specific immune response. The clinical characteristic that sets these responses apart from immune mechanisms involved in host defense is that the reaction is excessive and often leads to tissue damage. Clinical differentiation of allergic responses from non-immune irritant responses is their persistence and severity. Chemical-induced hypersensitivities fall into two categories distinguished not only mechanistically but temporally; 1) delayed-type hypersensitivity which is a cell-mediated response that occurs within 24-48 hours after challenge; and 2) immediate hypersensitivity which is mediated by immunoglobulin, most commonly lgE, and manifests within minutes following exposure to an allergen. The type of immediate hypersensitivity response elicited (i.e., anaphylactic, cytotocix, Arthus or immune complex) depends upon the interaction of the sensitizing antigen or structurally related compound with antibody. In contrast, delayed-type hypersensitivity responses are characterized by T lymphocytes, bearing antigen-specific receptors which, on contact with cell-associated antigen, respond by secreting cytokines. Hypersensitivity responses usually occur at potential xenobiotic portals of entry, such as the skin and respiratory tract. Mononuclear phagocytic cells (e.g., alveolar macrophages in the lung, Kupffer cells in the liver, and Langerhans cells in the skin) have a major role in mediating local responses initially via antigen processing and later via the release of reactive oxygen species and cytokines that modulate the recruitment and activation of additional cell types including PMNs and lymphocytes. In addition to leukocytes, other cell types are involved including keratinocytes in the skin, epithelial cells and fibroblasts.

Historically, the guinea pig has been used to test for potential sensitizers. In the primary exposure (induction phase), the guinea pigs are treated with the test agent intradermally and/or topically, followed by re-exposure(s) (challenge phase) to the same test compound, normally after a period of 10-14 days. Redness and swelling are measured at the site of the challenge exposure with a non-irritant concentration of test compound. Because guinea pigs are large, several graded doses of antigen may be tested and an entire dose-response curve can be generated by comparing skin reactions in individual animals. However, it is expensive to purchase as well as maintain guinea pigs, there are few inbred strains and immunological reagents are not widely available.

Many variations in procedures for guinea pig hypersensitivity assays exist (e.g., Buehler occluded, guinea pig maximization, split adjuvant); details of which can be found elsewhere (l). These guinea pig models are very sensitive and it has been suggested "too sensitive" in that false positives may occur. This argument may not be valid, however, as there are "sensitive" human populations which need to be considered.

Efforts are presently underway to replace the guinea pig assays with mouse models. Gad *et al.*

(3) have proposed a mouse ear swelling test (MEST). This procedure is similar technically to the guinea pig assay in that both induction and challenge phases are required but the response is quantitated by measuring an increase in ear thickness when the material for challenge is applied. At present, the most promising new assay is the local lymph node assay (LLNA) (2), In this procedure, the test material is applied topically in three successive daily applications to both ears. Control mice are treated with the vehicle alone. After 5 days of exposure, mice are injected with radioisotopically labelled DNA precursors (e.g., ^{3}H-thymidine), and single-cell suspensions are prepared from the lymph nodes draining the ears. At least one concentration of the test chemical must produce a three-fold increase or greater in lymphocyte proliferation in the draining lymph nodes of test animals compared with vehicle-treated control mice to be considered a positive. The primary advantage of this assay is that it minimizes the manipulation of animals. There is some question regarding its sensitivity which can be approved by pretreating the animals with vitamin A. The LLNA would represent a distinct improvement over conventional guinea pig tests. Presently, interlaboratory validation using the LLNA are underway.

SUMMARY

Adverse effects on the immune system that may occur from exposure to chemical agents include autoimmunity, hypersensitivity and immuno-suppression. The diverse pathogenesis of these diseases necessitates that different testing strategies be employed for their assessment. For autoimmunity, there are no models presently available for rapid screening. Autoimmune-prone and hyperimmune rodent models have been used to establish that certain compounds (e.g., lead) contribute to the etiology of autoimmune diseases but their utility in screening is unknown. For hypersensitivity, guinea pig models have been historically used. More recently, a mouse assay has been developed which appear to have similar sensitivity to the guinea pig but is neither more rapid nor reduces the number of animals required. The local lymph node assay (LLNA), which is undergoing extensive validation, should represent a marked improvement for screening purposes. Rapid tests for immunosuppression are currently available. The "gold-standard" test is quantitation of the antibody response following immunization with a T-dependent antigen such as sheep erythrocytes in rodents. Antibody responses can be determined in sera by ELISA or by the plaque forming cell response. In studies where groups of animals are not available for immunization, "non-functional" tests can be used such as described by the RIVM although sensitivity will be lost. Because of the complexities of the immune system, at present *in vitro* test models are not suited for screening. Screening tests need to take into account potential sensitive populations such as the developing immune system as well as wild-life. Regarding the latter, such studies are often hampered by a lack of suitable test reagents.

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