CHAPTER 15

Methods for Assessing the Effects of Chemicals on the Immune System

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15.1 INTRODUCTION

In this review, methods to assess chemically-induced lesions of acquired cellular and humoral immunity as well as natural resistance will be discussed. Not included are tests for the assessment of the allergic potential of chemicals.

15.1.1 Relevant human and other mammalian health considerations

Exposure of rodents to certain chemicals at doses that do not cause overt toxicity can produce immune alterations sufficient to result in altered host resistance to infectious agents (e.g. bacteria, viruses and parasites) and neoplastic cells (see reviews by Golstein et al., 1976; Vos, 1977; Vos et al., 1980; Faith et al., 1980; Dean et al., 1982, 1986a). Exposure to chemicals of environmental concern has likewise been shown to adversely affect the immune system of man. For example, accidental exposure of Michigan dairy farmers and factory workers to polychlorinated biphenyls (Bekesi et al., 1978) and exposure of Chinese to polychlorinated biphenyl contaminated with polychlorinated dibenzofurans (Chang et al., 1982) resulted in demonstrable immune alterations. In 1981, an outbreak of pneumonitis occurred in Spain which was linked to the ingestion of chemically-altered cooking oil (Centers for Disease Control, 1981b). It has been suggested that this 'toxic oil syndrome' was a chemically-induced graft versus host disease caused by the presence of isothiocyanate-derived, imidazolidinethione compounds in adulterated rapeseed oil (Kammuller et al., 1984). Additionally, an increased incidence of pulmonary infections in humans has been associated with exposure to noxious gases and airborne particulates (Lunn, et al., 1967; French et al., 1973), similar to effects seen in rodents exposed by inhalation to these same airborne contaminants (see reviews by Ehrlich, 1966; Gardner, 1984; Dean and Adams, 1985).

During the past decade, substantial evidence has accumulated indicating that
there is an association between the therapeutic use of immunosuppressive drugs or congenital immune deficiency diseases and an increased incidence of infectious and neoplastic diseases in humans (see reviews by Gatti and Good, 1971; Koers et al., 1975; Allen, 1976; Penn, 1978, 1985). For example, an increased incidence of lymphomas and leukaemias has been observed in patients with congenital immunodeficiency disorders (Gatti and Good, 1971). Allograft recipients chronically receiving immunosuppressive agents (mainly corticosteroids and anti-metabolites such as azathioprine) have a remarkable frequency of secondary cancer (26 per cent at 1 year and 47 per cent at 10 years); the types of tumours found included non-Hodgkin's lymphoma, Kaposi's sarcoma, carcinoma of the cervix, and skin and lip cancer (Penn, 1985). Likewise, the prevalence of opportunistic infections and Kaposi's sarcoma among individuals with acquired immune deficiency syndrome (AIDS) (Centre for Disease Control, 1981a) also suggests a central role for T-cell mediated immunity in host resistance to tumours and infectious agents.

In animal models, a variety of immunosuppressive treatments including x-irradiation, neonatal thymectomy, or lymphocytotoxic drugs have been shown to result in enhanced tumour incidence, growth rate and/or metastases. Furthermore, support for an immune-based component in the regulation of infectious disease or tumour growth is provided by in vitro and in vivo observations indicating that specific cells of the host (e.g. lymphocytes and macrophages) can recognize and destroy tumour cells and infectious agents (see Section 15.2).

Toxicological manifestations in the immune system following xenobiotic exposure in experimental animals may appear as: changes in lymphoid organ weights and/or histology; quantitative or qualitative changes in cellularity of lymphoid tissue, bone marrow or numbers of peripheral leukocytes; impairment of immune cell function; and increased susceptibility to infectious agents or transplantable tumours.

The use of the immune system as a sensitive parameter for detecting subclinical toxic injury is justified for several reasons: functionally immunocompetent cells are required for host resistance to opportunistic infectious agents or neoplasia; immunocompetent cells require continued proliferation and differentiation for self-renewal and are thus susceptible to agents which affect cell proliferation or differentiation; and the immune system is a tightly regulated organization of lymphoid cells which are interdependent in function.

Immunocompetent cells communicate through soluble mediators or cell–cell interactions and any agent altering this delicate regulatory balance, affecting a particular cell type or altering intercellular communications, can lead to an immune alteration. An imbalance of the immune system resulting from cellular injury might be expressed as either immune enhancement (e.g. possibly leading to autoimmunity or hypersensitivity) or immune suppression (e.g. immune dysfunction or altered host resistance). Some investigators are of the opinion that any immune alteration observed in rodents following xenobiotic exposure is of potential consequence for man. An alternative opinion is that only those immune alterations in rodents which
are associated with hypersensitivity or altered host resistance to infectious agents or neoplastic cells are of major concern. In either case, the interpretation of immune alterations observed in toxicity studies in terms of risk for man deserves special and continued consideration. The incorporation of reliable methods for assessing immune parameters into routine toxicity testing will provide useful and necessary information for a rational approach to the safety assessment of chemicals.

15.1.2 General comments on successes and failures using routine in vivo toxicity testing

Procedures currently used to detect immune alterations in routine in vivo toxicity studies include: weight and histology of thymus, spleen, mesenteric or popliteal lymph nodes; peripheral lymphocyte and monocyte counts; and serum IgG and IgM levels (Vos, 1977). Using this abbreviated approach, a total of seventeen pesticides were screened at three dose levels for possible immunotoxicity during subacute toxicity studies in weaned male rats receiving the test compounds through the diet (Vos et al., 1983b). More recently, the pesticide tributyltin oxide was studied using this protocol (Krajnc et al., 1984). From these experiments, it appeared that seven chemicals (benomyl, chlorfenson, pp'-DDT, diuron, dinitro-o-cresol, endosulphan and lead acetate) did not cause, or caused only marginal, effects on the immune system. Six compounds (azinphosmethy1, chlor IPC, quintozene, 2,4,5-trichlorophenoxyacetic acid, zineb and hexachlorobenzene) affected both immunological and general toxicological parameters. Finally, five chemicals (atrazine, captan, lead arsenate, triphenyltin hydroxide and tributyltin oxide) significantly altered one or more immune parameters which appeared to be the most sensitive criterion of their toxicity.

Immune function tests comprising cell-mediated immunity, humoral immunity and non-specific resistance (Vos et al., 1983b; 1984a, b) were performed in rats exposed pre- and post-natally and after weaning to atrazine, captan, lead arsenate, triphenyltin hydroxide and hexachlorobenzene (HCB). Functional immune effects were virtually absent in rats exposed to captan and lead arsenate. Of the different parameters of cell-mediated immunity (CMI) studied, the main effect exhibited by triphenyltin hydroxide was a suppression of delayed-type hypersensitivity (DTH). Tributyltin oxide caused a pronounced suppression of different parameters of CMI; of thymus-dependent antibody responses; and of natural resistance (e.g. macrophage phagocytosis and tumoricidal activity, and natural killer cell activity). In contrast, HCB markedly enhanced the antibody response to tetanus toxoid. Combined pre-and post-natal exposure to HCB, at a dose that did not alter liver weight or morphology, also enhanced the DTH response to antigen. From the results of this screening study, it was concluded that eleven of eighteen chemicals tested affected the immune system. Results of immune function assays indicated that in the rat model, alterations in lymphoid organ weights, histology, or cellularity of lymphoid organs
did not necessarily equate with functional immune alterations. Likewise in mice, House et al. (1985) recently reported thymus atrophy in mice following exposure to ethylene glycol monomethyl ether and its metabolite methoxyacetic acid without a functional immune defect.

It is too early to draw definite conclusions on the absolute reliability of this battery of screening procedures to predict immune dysfunction. For example, pronounced thymic atrophy produced in the rat by diethylstilboestrol exposure did not cause suppression of thymus-dependent immunity (Vos, unpublished data) while, in the mouse, such treatment resulted in severe immunosuppression (reviewed by Dean et al., 1982). Species differences are the most likely explanation of this discrepancy.

Differences in the immunotoxic effects of chemicals may also be linked to different modes of action. For example, both 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and organotin compounds produce thymus atrophy and immunotoxicity in the rat. However, pre- and post-natal exposure (i.e. during immune ontogenesis in the rat) may be a prerequisite for pronounced immunosuppression by TCDD (reviewed by Vos et al., 1980; Dean and Lauer, 1984), whereas organotin compounds also appear to depress thymus-dependent immunity in young adult rats (Seinen, 1981; Vos et al., 1984a, b). Thus, exposure during immune ontogenesis is not prerequisite for the organotins to produce immune dysfunction. In contrast to the direct cytotoxic effects of the organotins for thymic lymphocytes, recent studies (Greenlee et al., 1984; Nagarkatti et al., 1984) suggest that thymic epithelial cells, which promote thymocyte proliferation and differentiation, are a possible target for TCDD-induced immunotoxicity. Impaired production of thymic hormones or inappropriate cell–cell interaction might explain the TCDD-induced thymic atrophy and immune dysfunction.

15.1.3 Consideration of experimental parameters

In designing protocols for immunotoxicity assessment of chemicals, special attention should be given to the choice of species and strain, age of animals, duration and level of exposure, as well as the route of exposure.

For practical reasons, it is desirable to use the same species and strain of animal for immune function studies that is being used in the routine toxicity study. This allows immune studies to be evaluated against the background of other standard toxicological parameters, thus eliminating the need for dose–response comparisons between different species or strains. The rationale for selecting the mouse for immunotoxicity studies is based on the fact that the immune system of the mouse is better characterized, and functional assays are better defined. However, the rat is the rodent most frequently used in routine toxicity assessment. Most of the immunological methods developed in the mouse can, with minor modifications, be adapted for use in the rat (see Section 15.2).
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It is well established that the most profound effects of compounds that interfere with the immune response occur when the animal is exposed during ontogenesis of the lymphoid system (as with TCDD for example (discussed by Vos, 1977)). Thus, in utero and neonatal exposure appear to be the most sensitive method especially for chemicals that affect the thymus by impairing the proliferation and differentiation of the thymocytes. The second best choice is to use weaning animals.

The exposure interval required for a chemical to produce immune dysfunction differs and depends on a number of variables such as the type of immunological injury, chemical threshold, and the toxicokinetics of the compound. Few systematic studies have been done in this respect; in general, a subacute exposure regimen of 14–30 days is employed prior to assessment of immune parameters.

Dose selection is likewise critical. High doses producing overt toxicity should be avoided since severe stress and malnutrition are known to impair immune responses. For proper dose selection, information on the effect of the chemical on general toxicological parameters (e.g. LD50, LD10 and type of acute or subchronic toxicity associated with exposure) is important. To establish dose–effect relationships, two or three exposure levels are recommended. The highest dose selected for exposure should be less than the LD10, and ideally have no associated mortality.

The route of exposure should be the same as the natural route of exposure in man whenever possible. For the majority of environmental chemicals, the oral route of exposure (i.e. feeding or gavage) is preferred. In the case of airborne agents, inhalation exposure is commonly utilized.

15.2 IN VIVO STUDIES

15.2.1 Clinical observation

It is unlikely that immunotoxicity will be manifested by changes that can be easily observed clinically with the exception of an increased incidence of infectious disease or neoplasia. In studies involving animals that are not specified pathogen-free, immune suppression might result in the appearance of spontaneous infections. For example, Hansen et al. (1971) reported the development of fungus-like skin lesions in fish after PCB exposure.

If severe growth depression is observed in animals exposed to the test compound during the perinatal period, an immunologically-based wasting syndrome might be suspected. This wasting syndrome was first described by Miller (1962) in mice that were thymectomized at birth. In these mice, thymus-dependent immunological responses were severely impaired whereas adult thymectomy had only slight effects and did not produce wasting. Examples of compounds that can produce an immune-based wasting syndrome are cortisone (Ioachim, 1971), busulphan (Pinto-Machado, 1970) and TCDD (Vos and Moore, 1974).
15.2.2 Morphology

In many studies, it has been shown that routine histopathology of lymphoid organs is useful in assessing the immunotoxicity of a chemical (in particular, when these data are combined with the effects observed on the weight of thymus, spleen and peripheral lymph nodes). Because of the structural division of the spleen and lymph nodes into thymus-dependent and thymus-independent areas, a qualitative assessment can be made regarding the relative effects of the chemical for T or B cell compartments. It is also sometimes necessary to examine bronchus- or gut-associated lymphoid tissue (BALT and GALT) depending on the route of chemical exposure. Atrophy of the thymus and thymus-dependent areas of the spleen and lymph nodes were observed after di-n-octyltindichloride exposure (Seinen and Willems, 1976). In contrast, exposure to certain chemicals may lead to immune stimulation and lymphoid tissue hypertrophy. For example, pre- and post-natal treatment of rats with hexachlorobenzene caused proliferation of the high-endothelial venules in thymus-dependent areas of lymph nodes and in thymus-dependent areas of the Peyer’s patches of the small intestine. These changes correlated with an enhanced immune response upon immune functional assessment (Vos et al., 1983a).

Since the bone marrow is an integral part of the immune system (containing multipotent stem cells capable of differentiating along haemopoietic lines to B and T lymphocytes and macrophages), morphological analysis of this tissue is essential in immunotoxicity assessment. Morphological analysis can be done on tissue sections, marrow smears or on cytospin preparations of cells collected from the bone marrow. The latter technique has the advantage that the cell number and viability can be determined. In studies with TCDD, bone marrow cellularity proved to be a sensitive indicator of toxicity (Luster et al., 1980). Similarly, enumeration and characterization of free alveolar macrophages obtained by bronchoalveolar lavage appears to be a good quantitative method to determine the effect of inhalation exposure to a compound (Bingham et al., 1972). Macrophage quantitation and functional assessment has yielded insights into the function of immune and inflammatory processes in the human lung (Hunninghake et al., 1979; Dean et al., 1986b; for a review see Dean and Adams, 1985).

In routine toxicity studies, histopathologic examination depends mostly on haemotoxylin and eosin staining. Immunofluorescence methods, enzyme immune histochemistry and electron microscopy are techniques that are of great value to better understand the nature of chemically-induced lesions of the immune system. For example, enzyme histochemistry appears to be a useful method for the identification and enumeration of macrophages by staining for non-specific esterase activity (Koski et al., 1976). However, the requirement of specially prepared tissue, expensive instrumentation, and experienced personnel has prevented the widespread use of fluorescence, enzyme histochemistry or ultrastructural methods.

Recent progress in immunohistochemistry, namely, the development of im-
munoperoxidase techniques, has made it possible to adapt this highly sensitive and specific technique not only to diagnostic pathology (Mukai and Rosai, 1980) but also to toxicology. The major application in toxicology has been the identification of hormones (see Clark and Van Leeuwen, chapter 14, this volume). Different immunoperoxidase techniques are used to localize antigens in tissue sections. These include the peroxidase-labelled antibody method, the unlabelled antibody method of peroxidase-anti-peroxidase (PAP) and the avidin-biotin-peroxidase complex (ABC) method (reviewed by Falini and Taylor, 1983). In all techniques, peroxidase is localized through an antigen–antibody reaction in areas where specific antigen is present. The sites of peroxidase localization are visualized by addition of a substrate solution which reacts with the peroxidase label to form an insoluble coloured product.

Most cytoplasmic antigens (e.g. immunoglobulins) are readily demonstrated in fixed paraffin sections. Cell surface antigens (e.g. surface markers used for the demonstration of macrophages, T-helper cells and T-suppressor lymphocytes), which are only present in small amounts, are better preserved in frozen sections. With a proper fixative, paraffin embedding can now also be employed (Gendelman et al., 1983). Thin, methacrylate-embedded tissue sections (Casanova et al., 1983; Franklin, 1984) have potential application for immunohistochemistry techniques. The enhanced histological detail obtained with micro-thin, plastic-embedded sections is also useful in examining bone marrow, thymus and other lymphoid tissue. By staining thin methacrylate-embedded sections with monoclonal antibodies to cell surface determinants, lymphoid subpopulations in tissue sections can be identified (Hancock et al., 1982).

A difficulty for the pathologist in the evaluation of often minor chemically-induced lesions is making objective classifications. By randomizing and coding the slides, bias in reading can be avoided and qualitative analysis is possible. Classification is being improved by morphometric analysis since quantitative measurements on cells and tissues can be made by this method. In this context, it is of interest to mention that recent developments in monoclonal antibody technology, computer image processing and cytometric instrumentation has led to the new field of clinical flow cytometry. This field has already found specific applications in diagnostic immunopathology, including the study of congenital and acquired immune deficiency diseases (reviewed by Lovett et al., 1984). The application of this technique to immunotoxicology may be of value (e.g. enumeration of bone marrow stem cell populations which are currently only identified by complex culture methods). Flow cytometric analysis (FACS) of T and B lymphocytes in the spleen of rats exposed to tributyltin oxide revealed a reduction of T-cells which correlated with the results of immune functional studies (Vos et al., 1984a) and histopathological observations (Krajnc et al., 1984). Similar observations have been made in mice exposed to tumour-promoting phorbol diesters (Murray et al., 1985b). In contrast, these techniques will not always replace functional analysis since Dean et al. (1986b) reported functional defects in mice
exposed to dimethyl-benzanthracene although the percentage of T- and B-cells and subpopulations of T-cells were unaltered.

15.2.3 Serum analysis

Information on the immunotoxicity of a compound can also be obtained by analysis of serum from animals that are not used for functional studies in vivo. Serum analysis can be used for the quantification of the different immunoglobulin classes (e.g. IgM and IgG). Alterations in serum immunoglobulin levels were observed following tributyltin oxide exposure in rats (Krajnc et al., 1984). The classical analytical method for quantitating Ig levels is the single radial immunodiffusion assay. An alternative method is the 'sandwich' enzyme-linked immunosorbent assay (ELISA) of Engvall and Perlmann (1971). This procedure is more sensitive, easier to automate, and requires smaller amounts of antiserum than does the radial immunodiffusion assay (Vos et al., 1982).

In addition to specific humoral immunity mediated by immunoglobulins, the body is also protected by non-specific antimicrobial factors. Non-specific humoral factors include interferon (IFN) production, which functions in defence against viruses; complement (C') proteins which mediate their effects through at least two important mechanisms (namely by direct killing of micro-organisms and by facilitated opsonization); and, bactericidal enzymes such as lysozyme. Non-specific factors, for the most part, have received little attention in immunotoxicity studies, although they are of potential importance. For example, Gainer (1972) demonstrated that arsenicals inhibited the synthesis and action of IFN in mice which accounted for the increased susceptibility of these mice to viral infections. Practical and sensitive microassays for determining serum IFN and C' activity have been described (Klerx et al., 1983; Campbell et al., 1978).

15.2.4 Use of surgically-manipulated animals

Immunotoxicity may be secondary to other effects such as malnutrition or altered endocrine balance. An interaction of the chemical with the endocrine system should always be considered since various endogenous hormones (e.g. glucocorticosteroids) modify immune functions (White and Goldstein, 1972). For this reason, it is necessary to weigh and histologically examine the adrenals. An alternative method of identifying an endocrine effect is through the use of surgically-manipulated animals. For example, adrenalectomized rats were used to demonstrate that effects on the immune system produced by oxisuran (van Dijk et al., 1975) and cholera toxin Morse et al., 1975) were mediated by adrenal hormones. In contrast, thymic atrophy induced in the rat by di-n-octylindichloride (Seinen and Willems, 1976) and TCDD (van Logten et al., 1980) was not prevented by adrenalectomy, thus ruling out the possible role of glucocorticoids. Similarly, hypophysectomized rats were used to demonstrate that pituitary
hormones were not involved in TCDD-induced thymic atrophy (van Logten et al., 1980).

15.2.5 Immune function tests

Tests selected for the evaluation of immune dysfunction induced by xenobiotic exposure should meet at least two criteria: (1) the tests should result in data that can be extrapolated to the human experience; and (2) the tests should be adaptable to practical considerations such as expense, simplicity and reproducibility. A 'single' immunological test that would identify chemicals or drugs with potential risk is most desirable. However, because of the enormous complexity of the immune system, no single assay can accomplish this task. Thus, a tiered panel of selected functional assays (Dean et al., 1982; Vos et al., 1983b, 1984a) that have been validated in experimental rodent models and that are relevant to human clinical studies is recommended.

During the past ten years, numerous functional tests have been developed and refined to examine cell-mediated immunity, humoral immunity and natural resistance. The assembly of these tests is slightly different among the various groups working in immunotoxicology, partly because of the selection of different animal species. This is particularly true for bacterial, viral and tumour challenge models, since many of these host resistance models are not yet developed in the rat. Selection of functional assays also depends on whether inbred or random-bred animals are employed. For example, at the National Institute of Public Health and Environmental Protection (RIVM) in Bilthoven, the Netherlands, random-bred Wistar rats are utilized in toxicity testing, and immunotoxicity screening is included as part of subacute toxicity study protocols (see also Section 15.1.2). When the results of screening studies indicate that a chemical is immunotoxic, function tests are performed as part of a confirmatory tier in the same random-bred strain. The RIVM panel of immune function tests is shown in Table 15.1. Technical details of these models are provided elsewhere (Vos et al., 1984a).

On the other hand, initial screening (Tier 1) for immunotoxicity at the Chemical Industry Institute of Toxicology (CIIT) and in the National Toxicology Program's (NTP) Special Studies Panel for Immunotoxicology (Dean et al., 1982; Moore et al., 1982) includes functional and host resistance assessment (Table 15.2). In CIIT and NTP studies, a hybrid mouse designated B6C3F1, resulting from a cross between male inbred C3H and female C57Bl/6 mice, is routinely used since this mouse was selected by the NTP for the cancer bioassay of chemicals and allows the use of semisyngeneic tumour challenge models.

In the following sections, various immune function tests designed to assess acquired and natural immune resistance will be described. Detailed information on these assays and the effects of environmental chemicals on immune function are given in several reviews (Vos, 1977; Luster et al., 1982; Dean et al., 1982, 1986a). It should be noted that the in vitro assays described here are performed following in vivo exposure of the animal to the test compound (i.e. ex vivo - in vitro).
Table 15.1 Function tests for detecting immunotoxic alterations in the rat currently being used at RIVM, Bilthoven, the Netherlands

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-mediated immunity</td>
<td>Sensitization to T-cell dependent antigens (e.g. ovalbumin and tuberculin) and skin test challenge</td>
</tr>
<tr>
<td></td>
<td>Mitogen response and one-way mixed leukocyte cultures</td>
</tr>
<tr>
<td>Humoral immunity</td>
<td>Serum titration of IgM and IgG response to T-dependent antigens (e.g. ovalbumin, tetanus toxoid and <em>Trichinella spiralis</em>, including IgE) and T-independent IgM response to LPS.</td>
</tr>
<tr>
<td>Macrophage function</td>
<td><em>In vitro</em> phagocytosis and killing of <em>Listeria monocytogenes</em> by adherent spleen and peritoneal cells</td>
</tr>
<tr>
<td>Natural killer cell function</td>
<td>Cytolysis of YAC-1 lymphoma cells by adherent spleen and peritoneal cells</td>
</tr>
<tr>
<td>Host resistance</td>
<td>Cytolysis of YAC-1 lymphoma cells by non-adherent spleen and peritoneal cells</td>
</tr>
<tr>
<td></td>
<td><em>Trichinella spiralis</em> challenge (muscle larvae counts and worm expulsion)</td>
</tr>
<tr>
<td></td>
<td><em>Listeria monocytogenes</em> challenge (splenic clearance)</td>
</tr>
<tr>
<td></td>
<td>Endotoxin hypersensitivity</td>
</tr>
</tbody>
</table>

15.2.5.1 Cell-mediated immunity

Assays to examine cell-mediated immunity (CMI) include both *in vivo* (delayed hypersensitivity) and *in vitro* techniques (lymphocyte transformation, one-way mixed lymphocyte culture response and cytotoxicity). Currently, allograft rejection and graft versus host reaction are not used since both techniques are very labour intensive. In addition, the former technique is quite insensitive for detection of cell-mediated immune dysfunction, while the latter procedure depends on the use of a large number of recipient animals.

A delayed-type hypersensitivity (DTH) reaction is initiated by sensitized T cells which respond specifically to an antigen through release of lymphokines (Godfrey and Gell, 1978). Although *in vitro* assays appear more sensitive, the *in vivo* test remains a widely accepted means of assessing CMI and correlates well with decreased CMI in humans and host resistance to infectious agents (MacLean, 1979). Animals are sensitized to a T-dependent antigen (e.g. keyhole limpet haemocyanin, ovalbumin, tuberculin, bovine gammaglobulin, or DNCB) and then challenged
Table 15.2 Procedures for detecting immunotoxic alterations in the mouse currently being used at CIIT, Research Triangle Park, North Carolina

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunopathology</td>
<td>Routine haematology*</td>
</tr>
<tr>
<td></td>
<td>Lymphoid organ weights (spleen, thymus) and histology. * Spleen and bone</td>
</tr>
<tr>
<td></td>
<td>marrow cellularity*</td>
</tr>
<tr>
<td>Cell-mediated immunity</td>
<td>Mitogen responses and one-way mixed leukocyte cultures*</td>
</tr>
<tr>
<td>Surface markers</td>
<td>Quantification of lymphocyte subpopulations using monoclonal antibodies</td>
</tr>
<tr>
<td>Antibody-mediated immunity</td>
<td>Antibody plaque-forming cells (PFC) response*</td>
</tr>
<tr>
<td>Macrophage function</td>
<td>Resident peritoneal macrophage number, phagocytosis, cytostasis and enzyme</td>
</tr>
<tr>
<td></td>
<td>levels in activated and non-activated cells</td>
</tr>
<tr>
<td>Host resistance</td>
<td>Tumour challenge model:</td>
</tr>
<tr>
<td></td>
<td>PYB6 sarcoma*</td>
</tr>
<tr>
<td></td>
<td>B16F10 melanoma</td>
</tr>
<tr>
<td></td>
<td>Bacterial challenge model:</td>
</tr>
<tr>
<td></td>
<td><em>Listeria monocytogenes</em></td>
</tr>
</tbody>
</table>

* Currently included in the National Toxicology Program's Special Studies Tier for Immunotoxicity Assessment.

Intradermally with antigen (elicitation). Radiometric assays for quantifying the response provide greater sensitivity than measuring the swelling reaction but have the disadvantage of relying on the in vivo use of radioactive agent. It is recommended that animals be sensitized during or immediately following chemical exposure to approximate conditions which occur in humans.

Lymphoproliferative responses are a widely used correlate of CMI and can be defective in the absence of lymphocytopenia (Oppenheim and Rosenstreich, 1976). In the microculture assay, T or B cell mitogens (e.g. plant lectins, bacterial products), or allogeneic leukocytes (e.g. tissue transplantation antigens) in unidirectional mixed leukocyte cultures (MLC) are used to selectively stimulate lymphocyte blastogenesis as measured by [3H]TdR incorporation into DNA. Specific depressed responses in humans or animals with normal numbers of lymphocytes are usually interpreted as a defect in cell activation.

The generation of cytotoxic T lymphocytes (CTL) is another manifestation of CMI which represents an important acquired effector mechanism in resistance to viral infections and surveillance against neoplastically transformed cells. Induction to CTL can be accomplished in vivo by immunization with allogeneic lymphocytes or tumour cells, or in vitro in a one-way mixed lymphocyte culture, or mixed
lymphocyte tumour cell interaction. The cytolytic activity of the CTL is usually assessed in a 4-hour $^{51}$Cr-release assay. In contrast to the ease with which mouse lymphocytes can be sensitized, it is rather difficult to generate CTL activity in MLC of rat origin (Weiss and Fitch, 1977). Measurement of CTL activity has proven to be a valuable tool in immunotoxicity testing and has been reported to be suppressed by exposure to polycyclic aromatic hydrocarbon carcinogens (e.g. DMBA, MCA, DBA) (Dean et al., 1986b; Wojdani et al., 1983) and extremely low doses of TCDD (Nagarkatti et al., 1984; Clark et al., 1981). The CTL assay is still considered a second tier assay used for mechanistic studies and is not routinely performed in Tier 1.

### 15.2.5.2 Humoral immunity

Functional assessment of humoral activity is commonly accomplished by quantifying the antibody plaque-forming cell (PFC) response, or specific serum antibody titers.

The modification by Cunningham (1965) of the Jerne and Nordin (1963) plaque assay is extensively used. In this test, lymphoid cells from an animal immunized four days previously with T-dependent antigen (e.g. sheep red blood cells (SRBC)) are incubated in a slide chamber with the target erythrocytes and lytic complement. Plaques (haemolysis) in the SRBC lawn are observed around each antibody-producing cell due to complement lysis of SRBC that are coated with specific antibody produced by sensitized B cells. The PFC assay can be applied to other antigens including T-independent antigens (e.g. TNP-LPS or TNP-Ficoll) by coating them on the surface of the SRBC. The PFC assay has been shown to be sensitive for detecting chemically-induced alterations in humoral immunity. In the assay most commonly employed, only antibodies of the IgM-type are detected, because of the high haemolytic activity of this isotype.

Currently, sensitive immunoassays such as the enzyme-linked immunosorbent assay (ELISA) (Vos et al., 1982) are used for the quantification of specific serum antibodies. In the ELISA, antigen is coupled to a solid phase sorbent which is then incubated with serial dilutions of the test serum. The amount of specific antibody bound to the solid phase sorbent is titrated by enzyme-labelled anti-immunoglobulins (e.g. anti-IgM and anti-IgG). Thus, classes of specific antibodies can be quantified. Because of its extreme sensitivity, the ELISA could be useful for the measurement of specific IgE (Giallongo et al., 1982) and become an alternative method to the in vivo measurement of specific IgE by the passive cutaneous anaphylaxis reaction.

### 15.2.5.3 Macrophage function

It is now well established that macrophages provide not only non-specific phagocytic and cytotoxic functions but also are directed and regulated by lymphokines. In
addition, they provide interactions as well as products which have feedback and regulatory roles (i.e. prostaglandins and monokines) in immune responses. Thus, an understanding of macrophage function is becoming central to our understanding of immune responses and any assessment of a chemical’s immunotoxicity would not be complete without examining some macrophage function parameters. Dysfunction of the mononuclear phagocytic system (MPS) can lead to indirect tissue damage through altered host resistance to infectious agents or neoplastically transformed cells, or through direct tissue injury by the mononuclear phagocytes themselves or their cellular products (e.g. autoimmune diseases). Environmental agents, especially fibres, particulates and gases are well known to alter macrophage function (Gardner, 1984). Chemicals and drugs have also been found to alter the MPS (Ehrlich, 1966; see reviews by Loose et al., 1981; Dean and Adams, 1985). The effects of environmental agents upon macrophage function have been difficult to characterize precisely. This difficulty may be attributable, in part, to the fact that the functions of macrophages are closely related to their stage of maturation and to the fact that development of macrophages follows a complex and dynamic cascade of differentiation starting with bone marrow precursors (Adams and Marino, 1984). The effects of chemical exposure are also often pleiotropic so that a rational basis for understanding, studying and characterizing dysfunction of the MPS has been difficult to establish.

Studies from several laboratories have recently demonstrated that murine macrophages develop in stages (Hibbs et al., 1977; Meltzer, 1981) and that the stages of development can be clearly identified by quantifying certain objective biological and enzymatic makers, the expression of which characterizes each of the stages (Johnson et al., 1983). This system of analysis has been used to characterize modulation of macrophage development including those produced by agents of environmental concern (Adams and Dean, 1982). Thus, an understanding of macrophage function is pivotal to our understanding of immune responses and assessment of chemically-induced immunotoxicity.

Techniques measuring the uptake of $^{125}$I-triolein (Di Luzio and Riggi, 1964) or the clearance of colloidal carbon (Stuart et al., 1973) are often used for \textit{in vivo} measurement of the phagocytic capacity of mononuclear phagocytes. For \textit{in vivo} assessment of phagocytosis and macrophage bactericidal capacity, infection with \textit{Listeria monocytogenes} is widely employed (see Host resistance, Section 15.2.5.6).

A \textit{Listeria monocytogenes} model can also be used for the \textit{in vitro} assessment of macrophage bactericidal function (van Furth and van Zwet, 1973). It has the advantage that the processes of phagocytosis and intracellular killing can be assessed independently. By using only the adherent-cell population, phagocytosis by mononuclear cells can be analysed independently of granulocyte phagocytosis. However, a main difficulty of this test lies in removing adherent, non-phagocytosed bacteria from the macrophages. The test is also quite labour-intensive for routine use.

Besides their capacity for intracellular digestion, macrophages can destroy cells by a process of exocytosis and are thought to play a role in surveillance against
malignancies (Keller, 1978; Adams and Snyderman, 1979). Tumour cytolytic activity of macrophages is commonly assessed in a microcytotoxicity assay by measuring the release of $^{51}$Cr from tumour target cells added to cultures of adherent peritoneal effector cells. More recently, the measurement of macrophage ecto- and lysosomal enzymes appears to offer another promising approach (Dean and Adams, 1985).

### 15.2.5.4 Natural killer cell function

Spontaneous cytotoxicity is an important cytolytic effector mechanism in natural resistance to tumours and viral diseases. Natural killer (NK) cells are active in tumour surveillance (Warner and Dennert, 1982) and can limit viral infections (Bukowski et al., 1983). NK tumoricidal activity is assessed in a 4-hour microcytotoxicity assay by culturing splenocytes or peripheral blood lymphocytes with a $^{51}$Cr-labelled cell line sensitive to NK lysis (e.g. YAC-1 or K562 lymphoma cells).

### 15.2.5.5 Granulocyte function

Granulocyte function can be assessed by measuring physiological activities such as phagocytosis, chemotactic activity, bactericidal activity or nitro blue tetrazolium (NBT) dye reduction. Perhaps the best single assay is the NBT dye reduction procedure which has been extensively employed in the diagnosis of persons with chronic granulomatous disease. Failure of granulocytes to reduce NBT was found to correlate with an impaired enzymatic ability to kill phagocytosed bacteria. The number of granulocytes reducing dye can be easily quantified histochemically. This procedure can be utilized if altered bacterial resistance is observed in the presence of normal humoral, CMI and macrophage function.

### 15.2.5.6 Host resistance

Models assessing host resistance are needed in order to improve the human risk assessment data base for the evaluation of relevant chemically-induced immunotoxicity and provide *in vivo* correlates for the numerous *in vitro* immune function assays. This interest in providing better correlation and interpretation between an alteration in one or several measurable immunological parameters and host resistance stems from the established associations between patients with well-defined immunodeficiencies and the concomitant increase of infectious diseases or neoplasia (see Section 15.1.1).

*Infectious agent challenge models.* The application of host resistance assays following exposure of rodents to chemicals has indicated that certain chemicals can alter
host resistance to bacteria, viruses and parasites (see reviews of Vos, 1977; Bradley and Morahan, 1982; Dean et al., 1982; Faith et al., 1980). Workers have employed such infectious agents as Klebsiella pneumoniae, Listeria monocytogenes, Streptococcus pyogenes, Salmonella bern, Salmonella typhimurium, pseudorabies virus, duck hepatitis virus, encephalomyocarditis virus, Trichinella spiralis and Plasmodium berghei. Resistance in most models appears to require T-cell immunity and functional mononuclear phagocytes. Resistance to Streptococcus pyogenes and Plasmodium berghei are exceptions and require phagocytic cells whose function is facilitated by opsonizing antibodies.

Resistance to Listeria monocytogenes involves a combination of non-specific phagocytosis by macrophages which limits the growth or kills the organism during the first few days (1–3 days) after infection, and CMI which develops from day 2 post-infection (Tripathy and Mackness, 1969; Cheers et al., 1978). Non-specific phagocytosis and killing can be measured on days 1 and 2 after an intravenous inoculation of Listeria, at a time when acquired CMI is not yet developed. Spleens of the infected animals are homogenized, and serial dilutions of each homogenate are plated in bacteriological media to determine the viable Listeria count. Challenge of mice and rats with L. monocytogenes has proven to be a reproducible model for detecting altered macrophage or T-cell function after chemical exposure (Dean et al., 1980, 1981; Vos et al., 1984a).

Resistance to the nematode Trichinella spiralis is thymus-dependent as shown by a strongly retarded expulsion of adult worms from the intestine, increased numbers of muscle larvae and absence of IgM, IgG and IgE antibodies in athymic nude mice (Ruitenberget al., 1977), and nude rats (Vos et al., 1983c). Both CMI (Larsh et al., 1974) and humoral immunity (particularly the IgE isotype, Dessein et al., 1981) appear to play an important role in the resistance to Trichinella. This model has been shown to be useful in detecting chemically-induced immune dysfunction (Dean et al., 1980; Faith et al., 1979; Vos et al., 1984a).

Transplantable tumour challenge models. Of recent interest has been the demonstration that resistance to transplantable syngeneic tumour cells is also a sensitive parameter for detecting altered host resistance following chemical exposure (Dean et al., 1980, 1982; see Murray et al., 1985a for a review). Tumour models commonly used include the sarcoma MKSA and Madison 109 lung tumour of BALB/c mice and the sarcoma PYB6, B16F10 melanoma and Moloney virus-induced sarcoma of C57BL/6 mice. Almost any tumour model in which resistance is dependent on T-cell immunity, natural killer cells or macrophages could be employed. The selection of a specific tumour model for transplantation experiments will depend primarily on the species and strain of animal tested since the tumour must be syngeneic or semisyngeneic to the host. Tumour models that have been well characterized in terms of antitumour effector cell mechanisms are preferred.
The ability of an animal to reject a challenge of syngeneic tumour cells inoculated at a dose previously titrated to produce a low tumour incidence in control animals (i.e. TD 10–30 per cent) has been proposed as a sensitive in vivo assessment of general immunocompetence. Tumour challenge models using MKSA (BALB/c background) and PYB6 (C57BL/6 background) tumour cells have been validated for detecting immune alterations following in vivo administration of the immuno-suppressive chemotherapeutic agent, cyclophosphamide (Dean et al., 1979). Likewise, exposure to a variety of immunotoxic chemicals has been found to alter host susceptibility in terms of capacity to reject a low concentration challenge (TD 10–30 per cent with these tumour cells which generally correlates with deficits in T-lymphocyte function.

Chemically-induced immune suppression is expressed in these models as an increased incidence of tumours, a decreased latency to tumour appearance, an increased tumour growth rate, and/or decreased mean survival time. Conversely, agents which stimulate immune function may facilitate resistance to tumour development through enhancement of transplantation rejection mechanisms. Immune enhancement can be detected in these models using a higher challenge level of tumour cells (e.g. TD 70–90 per cent).

Several metastatic models adaptable for host tumour resistance evaluation are available. The B16F10 melanoma model provides a convenient and reproducible means for detecting modulation of host resistance parameters involved in the growth of solid, transplantable tumours and metastases. Intravenous challenge with the B16F10 subline results in haematogenous dissemination of tumour cells; their subsequent growth in the lungs (i.e. experimental metastasis) can be determined by either of two methods. Since the B16 melanoma forms pigmented metastatic foci, visual quantitation of organ-associated metastases is relatively simple. Alternatively, radioisotopic labelling of tumour cells in vivo provides a reliable means of determining relative organ tumour burden between control and treated mice. In this method (Murray et al., 1985b), animals receive an intraperitoneal injection of 125I-iododeoxyuridine, a radiolabelled DNA precursor which is incorporated into the nuclei of proliferating cells, following an initial tumour growth period (21 days). The mice are sacrificed 18 hours after the isotope injection and organs are removed and counted for incorporated radioactivity.

Bacterial endotoxin detoxification. The detoxification of Gram-negative bacterial endotoxin is believed to be accomplished primarily by liver parenchymal cells and macrophages (Cook et al., 1975). Increased mortality following endotoxin challenge has been observed following the administration of known mononuclear phagocyte system (MPS) stimulants as well as a variety of environmental chemicals which depress MPS function (Faith et al., 1979; Vos, 1977). It should be noted that endotoxin hypersensitivity has not been found to relate to any known macrophage function or activational state.
15.3 **IN VITRO STUDIES**

In recent years, *in vitro* lymphoid cell models for defining toxicological effects have received increasing attention because of the interest of alternatives to animal testing. The main reasons for this interest involve the burden and expense of the large number of toxicological studies currently required, and the ethical motive to limit *in vivo* animal experimentation.

The *in vitro* approach has been particularly successful in the screening of a multitude of compounds for genotoxic potential. For immunotoxicity screening, *in vitro* assays have only been used to a limited extent. Results of *in vitro* studies should be critically evaluated regarding their *in vivo* significance, because of the enormous complexity of the immune system.

Currently, the most widely utilized *in vitro* system for studying immunotoxic effects of chemicals is the *in vitro* immunization, antibody plaque-forming cell assay of Mishell and Dutton (1967). Archer *et al.* (1978) screened various food additives and metabolites for potential immunosuppressive properties by relating the cytotoxic dose with the quantity of the compound inhibiting the antibody plaque-forming cell responses. The same assay was used by Kutz *et al.* (1980) in studying food additives and environmental compounds including organometals and polychlorinated biphenyls. Disconcerting was the fact that some chemicals found to be immunosuppressive *in vivo*, were not immunosuppressive in the Mishell–Dutton assay. In order to widen the applicability of the *in vitro* models to include immunosuppressive chemicals which are not direct acting (i.e. those requiring metabolic activation), Tucker *et al.* (1982) successfully interfaced a system for metabolic activation using the microsomal fraction, S9, with three *in vitro* assays (Mishell–Dutton, lymphocyte transformation and bone marrow cell culture), using the immunosuppressive drug cyclophosphamide which requires metabolic activation. Dean *et al.* (1986b) recently found that the *in vitro* generation of CTL could be inhibited by direct addition of polycyclic aromatic hydrocarbon carcinogens to the cultures. *In vitro* assays have also been used by several investigators to assess macrophage function. For example, Graham *et al.* (1975) and Greenspan and Morrow (1984) showed that the phagocytic activity of alveolar macrophages was reduced by *in vitro* treatment with cadmium. These results correlated with findings of *in vivo* treatment.

*In vitro* assays are of great value in determining the mechanism of action of established immunotoxic compounds and investigating their immunotoxic potential for man. Seinen *et al.* (1977) showed that di-*n*-butyltindichloride, a compound with cytotoxic properties for rat thymocytes but not mouse or guinea-pig thymocytes, was also cytotoxic for human thymocytes. A good example of the efficiency of *in vitro* assays in defining the mode of action of chemicals comes from the work of Greenlee *et al.* (1984) who treated thymic epithelial cells with TCDD and found impaired thymocyte maturation suggesting that the thymic epithelium is one of the target sites for TCDD.

*In vitro* assays can be designed to study the different phases of an immune

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Methods for Assessing the Effects of Chemicals on the Immune System

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*In vitro* assays can be designed to study the different phases of an immune
response by separately adding (e.g. cellular depletion/reconstitution experiments) the various cell types involved. It is in the area of mechanisms of immunotoxicity that *in vitro* models provide much insight.

### 15.4 STRATEGY

The application of immunologic methods for toxicity assessment has developed rapidly and has been widely accepted. Programmes to determine the immunotoxic potential of chemicals and drugs are being developed in many governmental, university and industrial laboratories throughout the world. Since a single immune function assay cannot be used to comprehensively evaluate deleterious effects on the immune system following exposure to chemicals or drugs, tiers of sensitive *in vivo* and *in vitro* assays are used to assess immunotoxicity in rodents and are currently being further refined and validated in several laboratories. The tier approach to immunotoxicity assessment consists of a screening panel of assays selected from Tier I which enables the quick identification of compounds which may produce immune alterations. Agents shown to be positive in Tier I assays can be further evaluated with assays selected from a more comprehensive panel (Tier II). Tier II assays allow confirmation and in-depth evaluation of the underlying mechanism(s) of immunotoxicity.

Further development in this area should include an international interlaboratory validation of test methods using compounds with known immunotoxic effects and should ultimately provide a more standardized protocol for immunotoxicity testing. Such a tier of validated methods should rely not only on function tests, but incorporate immunopathology, monoclonal antibody, immunohistochemistry and flow cytometry methods as well. Future research needs include the development of protocols to assess mucosal and local immunity (Bienenstock and Befus, 1980), better models for studying chemically-induced autoimmunity and more mechanistic studies focused at the cellular and molecular level. Current immunotoxicology procedures emphasize systemic immunity. Even in programmes that evaluate the immunotoxicity of airborne contaminants, emphasis is given to the lower respiratory tract where the immune response is of the systemic type, while mucosal immune responses of the upper respiratory tract are much less investigated. The same holds true for the local immunity in the intestines, despite the fact that mucosal surfaces are the main sites of entry of foreign compounds, including chemicals that might have immunotoxic potential.

The next ten years presents a new era and challenge to immunotoxicology because of the required safety assessment of new recombinant biologicals (e.g. growth hormones, interferons, interleukins and new vaccines); biological response modifiers (i.e. drugs such as muramyl dipeptide) designed to enhance immunoresponsiveness against tumours and infections; and, monoclonal antibodies designed as drug delivery vehicles or for detoxification.
REFERENCES


Methods for Assessing the Effects of Chemicals on the Immune System


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Short-term Toxicity Tests for Non-genotoxic Effects


