Journal of Immunotoxicology

ISSN: 1547-691X (Print) 1547-6901 (Online) Journal homepage: https://www.tandfonline.com/loi/iimt20

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To cite this article: Alessandra Gennari, Masarin Ban, Armin Braun, Silvia Casati, Emanuela Corsini, Jaroslaw Dastych, Jacques Descotes, Thomas Hartung, Robert Hooghe-Peters, Robert House, Marc Pallardy, Raymond Pieters, Lynnda Reid, Helen Tryphonas, Eric Tschirhart, Helga Tuschl, Rob Vandebriel & Laura Gribaldo (2005) The Use of *In Vitro* Systems for Evaluating Immunotoxicity: The Report and Recommendations of an ECVAM Workshop, Journal of Immunotoxicology, 2:2, 61-83, DOI: 10.1080/15476910590965832

To link to this article: https://doi.org/10.1080/15476910590965832

Published online: 09 Oct 2008.

Journal of Immunotoxicology, 2:61–83, 2005

ISSN: 1547-691X print / 1547-6901 online
DOI: 10.1080/15476910590965832

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The Use of *In Vitro* Systems for Evaluating Immunotoxicity: The Report and Recommendations of an ECVAM Workshop

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Received 13 December, 2004; Accepted 5 April, 2005.
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ECVAM—European Centre for the Validation of Alternative Methods. This document represents the agreed report of the participants as individual scientists.

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This is the report of a workshop organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM's main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods that are of importance to the biosciences and which replace, reduce or refine the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures that would enable it to become well informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures.

It was decided that this would be best achieved by the organization of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best ways forward (Anonymous, 1994).

The workshop on "The use of *in vitro* systems for evaluating Immunotoxicity" was held at ECVAM (Ispra), Italy, on 24th–26th November 2003. The participants represented academia, national organizations, international regulatory bodies and industry. The aim of the workshop was to review the state-of-the-art in the field of *in vitro* immunotoxicology, and to develop strategies towards the replacement of *in vivo* testing. At the end of this report are listed the recommendations that should be considered for prevalidation and validation of relevant and reliable procedures, that could replace the use of animals in chemical and cosmetics toxicity testing.

Keywords *in vitro*, methods, immunosuppression, alternative models

INTRODUCTION

The immune system is designed to protect the host against invasive microorganisms such as bacteria and viruses, and against malignant cells in order to maintain homeostasis. Protection is provided when the immune system is able to recognize, isolate, destroy, and eliminate any intruding parasite. Surveillance, which is part of the protection mechanisms, implies the recognition and destruction of malignant or otherwise abnormal cells such as those affected by viruses or chemicals. The capacity of the immune system to recognize these abnormal cells plays a crucial role in the prevention of neoplastic diseases (Janeway and Medzhitov, 2002).

Inherent to the function of the immune organ system is that it is very diffuse, spread widely over peripheral (immune) organs and particularly present at sites that are exposed to the outside, such as mucosa of lungs, intestine, and skin. Development of immune cells takes mainly place in the bone marrow harbouring pluripotent haematopoietic stem cells, from which all leukocytes originate. During the first steps of the differentiation process, a myeloid and a lymphoid stem cell emerge. Subsequent differentiation into lymphocytes of the T- and B-lineages occurs within the microenvironment of lymphoid organs. T-Cell precursors pass through the thymus, where they express the antigen receptor and undergo selection.

At least two specific additional properties make the immune system vulnerable to chemical or physical insults: 1) the immune system develops rather late in life (thymus development lasts at least until puberty), and some bone marrow-dependent immune components are continuously renewed (e.g., polymorphonuclear granulocytes) and 2) each parasitic insult, as well as immune surveillance (including destruction of tumor cells), demands a delicate control of the balance between activation, silencing and regulation of immune reactivity.

Immune responses are the result of an effective collaboration between innate (natural and relatively nonspecific) and acquired (adaptive, and extremely specific and modifiable) components of the immune system. It is now well accepted that innate immune cells include antigen presenting cells (APC), such as dendritic cells (DC), sense danger- or pathogen-associated molecular patterns (PAMP), and that recognition of these patterns results in up-regulation of costimulatory help signals (including receptor bound molecules, as well as soluble factors like cytokines). To become activated, T-lymphocytes require costimulatory signals in addition to recognition of antigen determinants, in the context of the antigen presenting molecules of the major histocompatibility complex (MHC).

It is also accepted that T-cells, in particular T-helper cells, may develop in either T-helper 1 cells or T-helper 2 cells. By doing so, T-helper cells orchestrate the ensuing immune response by the mixture of cytokines they produce. T-helper 1

cells stimulate macrophages and/or cytotoxic T-cells to kill and destroy infected or malignant cells, or to raise a delayed-type hypersensitivity (DTH) reaction; T-helper 2 cells trigger B-cells to initiate antibody production.

Being a very aggressive system, control mechanisms have developed including central tolerance mechanisms (e.g., T-cell selection in the thymus), requirement of second or costimulatory signal (absence of costimulation results in silent state of lymphocytes), and a range of regulatory mechanisms (regulatory DC, and T-cells).

HUMAN CLINICAL ADVERSE EFFECTS

Based on the available clinical experience, immunotoxic effects are best divided into four categories: immunosuppression, immunostimulation, hypersensitivity, and autoimmunity. Each category is associated with relatively specific and clinically distinct adverse events (Descotes, 2004a).

Immunosuppression

Two major types of clinical adverse effects have been identified in relation to immunosuppression:

Impaired Resistance Against Microbial Pathogens. Primary (congenital) or secondary (acquired) immune defects are associated with microbial infections that are more frequent and more severe, and sometimes atypical, i.e., opportunistic infections that normally do not develop in immunocompetent individuals. Infectious complications have often been described in patients treated with corticosteroids (Klein et al., 2001), radiation or immunosuppressive drugs in the post-transplantation period (Sia and Paya, 1998; Sleijffers et al., 2002). The possible occurrence of infectious diseases has not been extensively studied in humans exposed to occupational or environmental chemicals that are immunosuppressive in animals. However, there is a seemingly similar trend toward more frequent, even though often clinically unremarkable infections in exposed human beings. For example, contamination of rice oil by a mixture of polychlorinated biphenyls (PCBs) caused unusual clinical signs and symptoms—the Yusho or Yu-Cheng disease—and respiratory infections were seen in some patients and were correlated with decreased immune function parameters (Lü, 1985; Nakanishi et al., 1985). Recently, a higher prevalence of recurrent inner ear infections has been reported in Dutch preschool children exposed to PCBs and dioxins (Weisglas-Kuperus et al., 2004). Inuit infants exposed to organochlorine pesticides were found to be at a greater risk of developing otitis media (Dewailly et al., 2000). UV exposure, which is immunosuppressive, is associated with more frequent infections in man (Termorshuizen et al., 2002).

No single pathogen is specifically involved in infectious complications associated with immunosuppression, since bacterial, viral, fungal, and parasitic infections can be seen. Infections of the respiratory and gastrointestinal tracts are the most frequent, but skin infections and opportunistic infections of the central nervous system are also common.

Immunosuppression and Malignancies. Immunocompromised patients, such as those with congenital immune defects or with the HIV immunodeficiency syndrome, are at a greater risk of developing virus-related malignancies. Organ transplant patients treated with long-term immunosuppressive regimens also develop more frequent malignancies (Vial and Descotes, 2003). Approximately 1% of cancer patients have been reported to develop a second cancer within 10 years after completion of chemotherapy, and 3% within 20 years. The most common malignancies in organ transplant recipients are cancers of the skin and lips (Wilson, 2003). Many epidemiological studies showed that lymphoproliferative disorders are 30- to 50-fold more frequent in renal transplant patients than in the general population. The most comprehensive data are those of the Cincinnati Transplant Tumor Registry (Penn, 1995). Malignancies are more frequent irrespective of the immunosuppressive drugs, though the actual figures do depend on the type of immunosuppressive therapy. Ionising radiation and alkylating agents are mutagenic. However, immunosuppressive treatments that are not mutagenic also increase the risk of developing cancer.

Immunostimulation

The clinical adverse effects related to immunostimulation in human beings have not yet been extensively investigated. However, the clinical experience is growing rapidly (Vial and Descotes, 1995; Vial et al., 2002).

1. *Flu-like Reactions*: Hyperthermic reactions (>38–38.5°C) with chills, arthralgias and malaise have been described in patients treated with immunostimulatory medicinal products or following the administration of vaccines. Similar, but more severe and sometimes treatment-limiting reactions have been reported in patients treated with recombinant cytokines. Hyperthermia can reach 40°C or more, and is associated with diarrhea, vomiting, chest pain, hypotension (possibly leading to cardiovascular collapse or cardiac ischemia), and neurological disorders such as tremor, confusion, obnubilation, and seizures. The terms "acute cytokine syndrome" and "cytokine release syndrome" tend to be used instead of flu-like reaction to describe these severe adverse events.
2. *Increased Incidence of Autoimmune Diseases:* One of the most frequent immune-mediated adverse effect of some, but not all recombinant cytokines, is a marked increase in the incidence of autoimmune diseases (Miossec, 1997). A number of treated patients developed extremely varied autoimmune diseases including auto-immune thyroiditis, thrombocytopenia, hemolytic anemia and hepatitis, systemic lupus erythematosus, insulin-dependent diabetes mellitus, myasthenia gravis, multiple sclerosis, and Sjögren's syndrome. Autoimmune thyroiditis is by far the most common finding.
3. *Increased Incidence of Hypersensitivity Reactions to Varied Allergens*: Although postmarketing surveillance data are

scarce, exacerbation of asthma, eczema, and rhinitis was reported shortly after initiation of treatment with immunostimulatory drugs (Bini and Weinshel, 1999). The most compelling evidence that immunostim-ulation may be associated with more frequent hypersensitivity to varied allergens is the increased incidence of adverse reactions to radiological contrast media in patients treated with rIL-2 (Schulman et al., 1993).

4. *Inhibition of Drug-Metabolizing Enzymes*: This condition was evidenced in both animals and man following the administration of interferon or interferon-inducing agents (Descotes, 1985). In some instances, this inhibition led to a clinically significant drug interaction. It has long been recognized that viral infections and inflammation exert similar effects (Renton, 2001). Cytokines, including rIL-1α and rIL-2, have been shown to depress hepatic microsomal CYP450-mediated metabolism after *in vitro* or *in vivo* administration to rodents, and in several instances in man as well.

Hypersensitivity

Hypersensitivity reactions are by far the most frequently reported immunotoxic effects of drugs and other chemicals in human beings. There are surprisingly few data on the actual incidence of hypersensitivity reactions in the general population or selected groups of the population. Demoly and Bousquet (2001) concluded that approximately one-third of all drug-induced adverse reactions consists of hypersensitivity reactions, either immunoallergic or pseudoallergic. The skin is the first site of hypersensitivity reactions at the workplace followed by the lung. Around 12% of all allergic contact dermatitis is deemed to be occupationally related (Rietschel et al., 2002). In the United States, 10–25% of all asthmas are suggested to be work-related (Petsonk, 2002). The environment, including indoor air pollutants, is a contributing factor to the development of hypersensitivity reactions and medical allergies, but its impact still remains to be quantified.

Hypersensitivity reactions can affect nearly every organ or tissue of the body, although one organ or tissue is often a predominant target. There are examples, however, of hypersensitivity reactions that consist of a variety of clinical signs and symptoms with no overtly predominant target, such as the multiple chemical sensitivity syndromes (Bolt and Kiesswetter, 2002). Underlying mechanisms are not fully elucidated. They consist of immune-mediated (immuno-allergy) or non-immune-mediated (pseudoallergy) mechanisms. Because the same mediators, e.g., histamine, can be released whatever the mechanism involved, close similarities in the clinical signs and symptoms are frequently observed (Zuberbier, 1999).

Autoimmunity

Overall, the mechanisms that result in onset and expression of autoimmunity are largely a mystery. Autoimmune diseases are relatively common in the general population, although estimates vary widely. It is therefore surprising that only a few epidemi-ological studies have identified drugs or chemicals as a possible cause of auto-immune disease. Autoimmune diseases are divided into organ-specific and systemic diseases. Chemically induced organ-specific diseases are rare, closely similar to the spontaneous diseases and caused by a very small number of drugs or chemicals. In contrast, chemically induced systemic diseases are more frequent, dissimilar from the spontaneous diseases, and caused by various drugs and chemicals (Descotes, 2004b).

EU LEGISLATION

In February 2001, the European Commission published a white paper on the strategy for a future chemical policy in order to obtain toxicological information about existing chemicals according to Directive 67/548/EEC[1]. Basic information is lacking for about 30,000 chemicals produced at quantities of greater than 1 ton per year, and it is intended that missing data should be produced without animal experiments where scientifically satisfactory alternative methods are available. Successful development, and validation of test systems can be added to Annex 5 of the Directive 67/548/EEC. The White Paper was discussed by the Environment Council in June 2001, where it was concluded that "animal testing should be limited to the level necessary to deliver the objectives of the strategy, including a high level of protection for human health and the environment. Industry should make all existing data available to avoid duplication of testing. Mechanisms are needed to ensure that unnecessary testing requirements are avoided. Adequate resources must be provided for research, development, and validation of globally accepted test guidelines for alternative *in vitro* test methods, so that work can be accelerated at all levels."

In October 2003, the European Commission adopted a proposal for a new EU regulatory framework for chemicals. Under the proposed new system called REACH (Registration, Evaluation and Authorisation of CHemicals), enterprises that manufacture or import more than 1 tonne of a chemical substance per year would be required to register it in a central database. The aims of the proposed new regulation are to improve the protection of human health and the environment while maintaining the competitiveness and enhancing the innovative capability of the EU chemicals industry. REACH would furthermore give greater responsibility to industry to manage the risks from chemicals and to provide safety information on the substances. This information would be passed down the chain of production. The proposal has been drafted in close consultation with

[1]Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. O.J. L 196/1 of 16 August 1967. And related Amendments, Adaptations to Technical Progress, Commission Decisions, and Commission Communications.

all interested parties, including via an Internet consultation.[2] This has allowed the Commission to propose a streamlined and cost-effective system. The proposal is now being considered by the European Parliament and the EU's Council of Ministers for adoption.

In the REACH proposal, the repeated dose (28, 90 days) and chronic (12 months) toxicity test include assessment of immunotoxic effects for chemicals produced in quantities of at least 10 tons/year. The method should help to identify chemicals with immunotoxic potential, which may require further in-depth investigation of this aspect.

At present, since no alternative methods are available, only *in vivo* studies are foreseen. These include:

- morphology/histopathology
- leukocyte counts (flow cytometric analysis)
- serum markers (Ig levels)

These studies are analogous to the ones requested in the OECD guidelines, in particular number 407 (repeated dose 28-day oral toxicity),[3] 408 (repeated dose 90-day oral toxicity),[4] and 452 (chronic toxicity).[5] Concerning chemicals produced at lower tonnage (<10 tons/year), there is no existing guideline to evaluate immunotoxic effects.

Recent guidelines from the European Medicines Agency (EMEA)[6] emphasize the need to assess immunotoxic effects during the preclinical phase of drug development, and recommend specific tests to measure immunotoxicity.

U.S. REGULATORY GUIDELINES

In 1997, under U.S. Public Law 103-43, the National Institute of Environmental Health Sciences (NIEHS) was directed to develop and validate improved alternative toxicological testing methods, and to develop criteria and processes for the validation and regulatory acceptance of such methods. The Interagency Coordinating Committee on the Validation of Alternative Mehods (ICCVAM) was established as a collaborative effort by NIEHS and 13 other Federal regulatory agencies and programs for this purpose. ICCVAM coordinates issues within the U.S. Federal government that relate to the development, validation, acceptance, and national/international harmonization of toxicological test methods that offer significant advantages over convential animal studies with respect to animal welfare considerations (i.e., refinement, reduction, and replacement alternatives). To date, the only new test method relevant for assessing immunotoxicity to be validated for regulatory purposes through ICCVAM is the Local Lymph Node Assay (LLNA). The following is a brief summary of the current recommendations for immunotoxicology testing in the United States.

U.S. Food and Drug Administration (FDA). All regulated products, i.e., small molecular weight drugs,[7] biomedical devices,[8] and food additives and colorants, should be evaluated for their potential to produce immunosuppression. With a few exceptions in the area of hypersensitivity testing, special populations, and bioengineered food, FDA uses a weight of evidence or tiered approach to immunotoxicology evaluation of new molecular entities. This is generally accomplished in repeat-dose toxicology studies using standard clinical and anatomic pathology methods. When warranted by observations in general toxicology studies, additional studies should be considered to determine potential effects on immune function. When indicated, immunotoxicology tests are expected to be selective and to utilize assays that are appropriate, technically valid, and predictive. FDA guidance documents are limited to making recommendations and do not impose requirements on FDA review divisions or regulated industries.

U.S. Environmental Protection Agency (EPA). The office of Prevention, Pesticides, and Toxic Substances (OPPTS) has developed guidelines that are harmonized among the Office of Pollution, Prevention and Toxics (OPPT), the Office of Pesticide Programs (OPP), and the Organization for Economic Cooperation and Development (OECD).[9] The Health Effects Test Guidelines are intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136 *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

EPA's current approach to immunotoxicology evaluation of chemicals is currently similar to that of the FDA, relying primarily upon information on potential immunotoxic effects obtained from hematology, lymphoid organ weights, and histopathology data from other studies in the database to identify the need for further testing. EPA has, however, indicated concern regarding the need for more rigorous screening for effects on immune function both in adults and following development exposures, in order to provide more accurate information for risk assessment.

In the absence of relevant *in vitro* or *in vivo* models, routine testing for induction of autoimmune disease is not recommended at this time by any of the U.S. Federal Agencies discussed here.

[2] ⟨http://europa.eu.int/comm/enterprise/chemicals/chempol/whitepaper/consultation.htm⟩.

[3] OECD Guidelines for the Testing of Chemicals, 1997, vol. 1, no. 4, pp. 1–8.

[4] OECD Guidelines for the Testing of Chemicals, 1997, vol. 1, no. 4, pp. 1–10.

[5] OECD Guidelines for the Testing of Chemicals, 1997, vol. 1, no. 4, pp. 1–14.

[6] Note for Guidance on Repeated Dose Toxicity, CPMP/SWP/1042/99, ⟨http://www.emea.eu.int/pdfs/human/swp/104299en.pdf⟩.

[7] CDER Guidance for Industry: Immunotoxicology Evaluation of Investigational New Drugs, October 2002 ⟨http://www.fda.gov/cder.guidance/4945fnl.doc⟩

[8] CDRH Guidance for Industry and FDA Reviewers—Immunotoxicity testing Guidance, May 1999 ⟨http://www.fda.gov/cdrh/ost/ostggp/immunotox.html⟩

[9] Health Effects Test Guidelines (ftp:fedbbs.access.gpo.gov (IP 162.140.64.19).OPPTS 870.7800 Immunotoxicity. OPPTS 870.2600 Skin Sensitization.

ANIMAL MODELS

Naturally, the process of risk assessment would be greatly facilitated if it were based on clinically relevant human data. Such data are scarce and have been often characterized to be very limited in scope (Tryphonas and Feeley, 2001). Consequently, much of the immunotoxicity data used for risk assessment purposes are generated in experimental animal models. However, scientists working in the field of immunotoxicology continue to be faced with the question of what species of experimental animal is most appropriate for immunotoxicity studies. Although there does not seem to be a satisfactory answer to this question, many of the early immunotoxicity studies have been performed in rodents since reagents were more readily available for rodents as compared to those for other species. For information pertaining to other species, readers are referred to the recent publication by Helm (2005).

Mice

Initially, there was considerable effort in the development and validation of a number of immune-related tests in mice. As these efforts progressed, determination of the robustness of such tests became an important and necessary issue. The most comprehensive effort in this area is represented by the work carried out by Luster and collaborators, under the auspices of the U.S. National Toxicology Program (NTP) (Luster et al., 1992). In the NTP program, experimental data were collected over the preceding 10 years on chemicals and therapeutics that were characterized as having the potential to suppress the immune system and alter host resistance. Data from these studies encompassing over 50 compounds were analyzed for their likelihood to accurately identify immunotoxic compounds. In addition, tests that showed significant correlations with data derived from host resistance assays were also identified. Tests that showed the highest association with immunotoxicity included the splenic antibody plaque forming cell (PFC) response (78% concordance) and cell surface marker analysis (83% concordance). While a good correlation was detected between changes in the immune tests and altered host resistance (e.g., 70% for the antibody PFC assay, 73% for natural killer (NK) cell activity, and 82% for the DTH response), no single test could be identified which was fully predictive for altered host resistance (Luster et al., 1992, 1993). Several of the tests included in this analysis particularly the lymphoproliferative response to lipopolysaccharide (a B-cell mitogen) and routine leukocyte counts, were poor indicators for host resistance changes (<50%). The 1998 U.S. EPA OPPTS 870–7800 Immunotoxicity Guidelines were in fact based on results of the NTP data analyses. The proposed tests in this guideline are limited to the T-dependent antibody PFC assay, the NK cell function assay, and the quantification of T- and B-cells along with the suggestion that each chemical be examined and the use of additional tests be determined on a case by case basis.

Rats

Due to the lower availability of rat-specific reagents, development of immunotoxicity tests in this species evolved at a much slower pace relative to mice. However, since the rat has been traditionally the experimental model of choice for toxicologic studies of chemicals, the development of immunologic assays in rats was imperative. The pioneering work carried out at the Dutch National Institute of Public Health and the Environment (RIVM) focused exclusively on the rat (Vos, 1980; Van Loveren and Vos, 1989, 1992; Vos and Van Loveren, 1994). These studies were instrumental in formulating the OECD Guideline for the testing of chemicals: repeated dose 28-day oral toxicity study in rodents, which was adopted by the Council on 27th July 1995. The 1995 OECD Guideline #407 included histopathologic evaluation of haematoxylin-eosin stained, paraffin-embedded slides of spleen, thymus, small intestine, large intestine, and lymph node sections. Recently, the international collaborative immunotoxicity studies (ICICIS)[10] have been instrumental in revising the OECD Guideline #407 and to propose changes in the OECD Guideline #407, to include in addition to enhanced histopathology of the immune system, a functional assay preferably one that involves immunization with a foreign antigen. Discussions of the proposed changes to the OECD guideline #407 are ongoing.

IMMUNE PARAMETERS

Immunohistopathology

A comprehensive investigation of potential chemical-induced adverse effects on the immune system combines a detailed analysis of histological examination of key tissues of the immune system and selected quantitative and functional assays. With respect to pathology, the 1995 OECD guideline #407 requires that histopathologic evaluation be carried out on the spleen, thymus, small intestine, large intestine, and lymph node sections. For further details regarding the methods and approaches to carry out a histopathologic evaluation of the tissues of the immune system, Readers are referred to the publication by Pulido et al. (2005).

Total White Blood Cell Counts

Quantitative and morphologic evaluations of total white blood cells (WBCs) and differential counts of peripheral blood leukocytes (PBL) are basic investigations and these have been included in most immunotoxicity studies involving mice, rats, and nonhuman primates. Both relative and absolute numbers of WBCs are quantified. However, absolute numbers provide biologically more relevant information, as the use of percentages of cell types may mask some cytopenias or excessive numbers of a cell type, which would lead to falsely high/low numbers of a particular cell (Perkins, 1999).

Immunophenotyping of Peripheral Blood Leukocytes

Immunophenotyping of PBL, using monoclonal antibodies directed to cell-surface markers, and flow cytometric techniques, has become an important tool in the diagnosis of haematologic

[10]ICICIS, Report of Validation Study of Assessment of Direct Immunotoxicity in the Rat. Toxicology, 125, 183, 1998.

and immunologic disorders (Perkins, 1999). Cross-reactivity between mouse anti-human monoclonal antibodies (mAbs) and monkey leukocyte surface antigens has been demonstrated for several anti human mAbs (Tryphonas et al., 1996). Valuable data can be gained from repeated measures of lymphocyte subpopulations during the course of a study. Such data can be useful in elucidating the mode of action of the chemical in question.

Quantification of Total Serum Immunoglobulin Levels

Total serum immunoglobulin (Ig) levels (IgG, IgM, and IgA) can be quantified in rodents (Bondy and Pestka, 1991) and in nonhuman primates (Tryphonas, 2005) using the enzyme-linked immunosorbent assay (ELISA). However, the determination of total serum Ig levels in experimental animals has not proven useful, since pronounced effects on immune function are required before significant changes in total serum Ig levels can be observed.

Challenge with Specific Antigen

The immune system is endowed with a large functional redundancy and the changes in WBC numbers or shifts in lymphocyte subsets observed in many studies may not be accompanied by changes in immune function (Luster et al., 1992). Thus, the functional capacity of the immune system must be established before concluding that a given exposure is toxic. This can be accomplished by challenging the control and treated animals with foreign antigens, followed by the determination of antigen-specific antibody levels in serum samples collected prior to antigenic challenge (baseline titres) and in the case of nonhuman primates, at weekly intervals post-immunization. Unlike total serum immunoglobulins and T-lymphocyte subsets that are determined sequentially, the challenge with foreign antigens is performed once during or at the end of treatment. In rodents and nonhuman primates, challenge with specific antigens has been highly predictive of effects on humoral immunity (Luster et al., 1992, 1993; Tryphonas, 2005).

The response to an antigenic challenge involves the sequential and tightly orchestrated interactions of competent immune cells, including the antigen-presenting cells, and the activated T- and B-lymphocytes. Consequently, much information can be derived from challenging the host with foreign antigens. Clinically-relevant information includes the ability of the host to respond to a foreign antigen (primary response) and to establish memory (secondary or anamnestic) response.

For an antigenic challenge to be meaningful, it is important to have documented histories of the experimental animals, especially monkeys, since prior immunization can lead to erroneous results and should be avoided. In rodents, sheep red blood cells (SRBC) antigens are routinely used for immunization and the SRBC antibody response is determined by the PFC or plasma SRBC-specific titer (Jerne et al., 1974). As an alternative for using SRBC, keyhole limpet hymocyanin (KLH) is considered as a more stable and robust T-cell-dependent antigen in immunization models. Furthermore, detection of responses to KLH are better standardized and do not require *ex vivo* manipulation (Gore et al., 2004). In non-human primates, SRBC, tetanus toxoid (TT) and pneumococcus (Pneu) antigens have been used. In view of the observed differences in immunogenicity among different batches of SRBC, it is advisable to use the same source of SRBC for immunization and for the antibody detection systems for the duration of the study.

Cell-Mediated Immunity

Cell-mediated immunity (CMI) has been studied in rodents and nonhuman primates using three methods: lymphocyte transformation (LT; ^{3}H-thymidine incorporation) in response to the T-cell mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) and the B-plus T-cell activator pokeweed mitogen (PWM), or to specific antigens such as TT; the mixed lymphocyte culture (MLC) assay using allogeneic cells; and the DTH response using dinitrochlorobenzene (DNCB) (Bugelski et al., 1990) or oxazolone as the sensitizing agent (Tryphonas, 2005). The LT and MLC assays can be run concurrently and are followed by the DTH assay. Of these assays, the LT assay used in the context of immunotoxicity has provided useful information regarding the mechanism of action for some chemicals but its predictive value for immune functional impairment is low (Luster et al., 1992). A potentially useful extension of the LT assay is the quantitative analysis of Igs in culture supernatants but this needs further validation. The MLC assay, although a potentially useful assay for predicting chemical-induced adverse immune effects, has not been standardized and validated across laboratories and its concordance with host resistance assays or its predictive value for immune effects is presently not known (Tryphonas, 2005).

Natural Killer Cell Activity

A number of cells, including cytotoxic T lymphocytes (CTL), NK cells, and mononuclear phagocytic cells, are endowed with cytotoxic abilities and are thus very efficient in immunosurveillance mechanisms against neoplastic cells and viral infections. In immune-compromised hosts, a correlation has been observed between low NK cell activity and morbidity (Trinchieri, 1989; Whiteside and Herberman, 1989; Levy et al., 1991) or the incidence and severity of upper respiratory infections (Whiteside and Herberman, 1989). NK cell function is measured in a 4-hour ^{51}chromium (^{51}Cr)-release assay. In this assay, freshly-isolated PBL (effector cells) from which monocytes have been removed by adherence to plastic culture flasks, and ^{51}Cr-labeled K562 target cells (primates) or YAC-1 target cells (rodents) are co-cultured, and the release of label in culture supernatants is quantified. In addition, NK cells produce numerous cytokines such as tumour necrosis factors α and β, interferons α, β, and γ, granulocyte-macrophage colony-stimulating factor, and interleukin-3 (IL-3) upon immune stimulation, all of which have a profound effect on immune reactivity (Trinchieri, 1989).

Phagocytosis

In addition to their cytotoxic properties, the monocyte/macrophage lineage of cells is important in antigen recognition, processing, and presentation to T-lymphocytes. These cells are potential targets for chemical-induced immunotoxicity and assays to study functional aspects should be included in the experimental design.

Various assays have been used in experimental animal models. Peripheral blood monocytes or peritoneal macrophages have been used as the source of phagocytic cells. SRBC or latex particles are commonly used for the ingesting agents. Flow cytometric techniques are available for quantifying the phagocytic response of cells (Brousseau et al., 1998). However, none of these assays or techniques has undergone validation and their predictive value is presently unknown.

Host Resistance Assays

Several host resistance assays have been developed for use in mice and rats. The use of HR assays gives the experimenter a valuable tool to determine the host's immune redundancy. Several models of infectivity have been developed (Van Loveren, 1995). These include the models of *Listeria monocytogenes*, *Streptococcus pneumoniae*, Cytomegalovirus, Influenza virus, *Trichinella spiralis*, *Plasmodium*, etc. It should be noted that the immune defence mechanisms that are operational in combating infection are different for each of the infectivity models. This necessitates the use of more than one infectivity model in any given immunotoxicity study. The choice of the host resistance model should also take into account the immune parameter affected.

Hypersensitivity and Autoimmunity

The ability to determine the potential for substances (proteins or chemicals) to cause IgE-mediated hypersensitivity reactions has been a difficult task for researchers. This applies to a number of these allergies, including respiratory and food allergies. Animal models have been used for years to enhance our understanding of basic mechanisms of IgE allergic antibody-mediated disease. For food allergy, animal models presently under development include Brown Norway rat, but also a number of mouse models, including the oral C3H/HeJ and models involving intraperitoneal administration of proteins (such as BALB/c) (Knippels and Penninks, 2003). Nonmurine animal models such as the atopic dog model and the neonatal swine model are presently under development and have produced promising results (Helm, 2005).

It is even more difficult to assess immunostimulatory potential of substances, in particular autoimmunity in animals, mainly because of the complex etiology of the adverse reactions. There are only a few individual models that show such immune responses (Shenton et al., 2004) but none of these seems to be suitable as predictive test. The popliteal lymph node assay (PLNA), in particular combined with immunological read-out parameters (such as response to reporter antigens), is possibly useful as a screening assay for immunostimulatory hazard of substances (Pieters et al., 2002). However this assay, which detects lymph node stimulation upon footpad injection, is not validated, and uses an obviously irrelevant exposure route. In addition, because immunostimulation not necessarily leads to autoimmune derangements, the assay does also not predict whether a chemical really induces such derangements.

Other Parameters

Other assays that measure serum complement levels and cytokine levels (both basal levels and levels in lectin-activated cultures) are useful parameters in the interpretation of immunologic data. To exclude the possibility that the observed effects on the immune system are not the result of an indirect effect on hormone levels, investigators have consistently determined serum levels of cortisol or corticosterone prior to and at the termination of the study. Due to the effect of stress and the observed diurnal variation of corticosteroids, the collection of serum specimens need to be carried out by adhering to a strict randomized order of all animals in the study for each blood collection (Tryphonas and Feeley, 2001). The use of carbon dioxide to quickly anesthetize the animals before bleeding for corticosteroid determination has been described in mice (Shipp and Woodward, 1998). However, this should be always combined with a daily randomization schedule for the order of bleeding.

IN VITRO IMMUNOTOXICOLOGY

Although evaluation of immune function following *in vivo* exposure to a test material is arguably the most relevant situation, it is increasingly desirable to limit the use of animals whenever possible. Moreover, there are certain situations (for example, when a test material is anticipated to be dangerous to handle or is prohibitively expensive) when a totally *in vitro* system would be advantageous. Many of the techniques that serve as the *in vitro* portion of *ex vivo* immunotoxicology testing can serve as stand-alone assessments in a totally *in vitro* test system (House, 2001a).

A Workshop on "Immunotoxicology and *in vitro* possibilities" was organized in 1994, during which the current status of *in vitro* methods for assessing immunotoxicity was reviewed and the possible future developments to reduce, refine, and replace the use of animals have been analysed (Sundwall et al., 1994). At that time, experts agreed that there was not an immediate opportunity to replace the whole animal for immunotoxicological studies. On the other hand, much progress has been achieved regarding the reduction in the number of animals used, since *in vitro* models can be used for prescreening. At present a tiered approach has been proposed, since useful information can be obtained from regular 28-day general toxicity tests if increased attention is paid to the study of the histopathology of a large variety of lymphoid tissues, coupled with immunohistochemical measurements and the determination of classes of antibodies. Furthermore, it was established that the validation of the *in vitro*

test should be against information gained from humans, rather than the results from laboratory animal species.

In this section we will discuss these assays in the context of immunosuppression (and, to a lesser degree, its obverse condition of immunostimulation) and hypersensitivity.

Approaches to Evaluation of Immunosuppression

Initial Evaluation of Myelotoxicity. Compounds that are capable of damaging or destroying the bone marrow will often have a profoundly immunotoxic effect, since the effectors of the immune system itself will not longer be available. Thus, if a compound is myelotoxic, there may be no need to proceed with additional evaluation since the material will be a de facto immunotoxicant. The methodology for evaluating myelotoxicity *in vitro* using bone marrow culture systems is well-characterized (Pessina et al., 2001).

Determination of Lymphotoxicity. Compounds that are not overtly myelotoxic may still selectively damage or destroy lymphocytes, which are the primary effectors and regulators of acquired immunity. This toxicity may result from the destruction of rapidly dividing cells by necrosis or apoptosis. Viability of the lymphocytes should be the necessary initial test, and a variety of methodologies are available for this purpose (e.g., colorimetric, flow cytometric assays). If the cells are viable (80% or greater), basic functionality could be determined by performing an antigen nonspecific proliferation assay. For T-cells, the stimulatory agent can be a combination of anti-CD3 and anti-CD28; for B-cells an optimum system would have to be developed but would be expected to be similar to the murine system incorporating a combination of anti-immunoglobulin and cytokine.

If *in vitro* systems are found to be useful for assessing lymphotoxicity, this determination may require broadening to include other types of immunocytes such as phagocytes. A potential disadvantage of this approach includes the currently undetermined reliability/reproducibility of this approach, and the concomitant need for qualification or prevalidation of the assay. Nonetheless, these assays have distinct advantages including relatively low cost, a readily available source material, and a high feasibility due to wealth of published methodology.

Determination of Potential Effects on Antibody Induction/Production

In animals, production of a T-dependent antibody response (such as against the T-dependent antigen, SRBC or KLH) is considered to be the "gold standard." However, there are currently no good systems for *in vitro* antibody production using human cells. Development of human *in vitro* systems will require optimization of antigen (preferably using antigen relevant to human exposure, such as TT), culture conditions, and assay endpoint. In addition, there is some concern whether a primary immune response can actually be induced in human PBL. One potential starting point would be an *in vitro* immunization culture system based on the Mishell–Dutton assay (this assay is not considered optimal for this use due to significant variability in results—and often a complete lack of success—between laboratories). Due to the high predictivity of human immunotoxicants provided by the *in vivo* antibody induction assay, as well as the possibility of bridging between *in vivo* and *in vitro* potentially afforded by this assay, it is recommended that development of this *in vitro* system be given the highest priority.

Determination of Potential Effects on CTL

CTL are a population of $CD8^+$ lymphocytes characterized by specific cytotoxicity for target cells in an antigen and MHC Class I-restricted manner. Although they share common mechanisms of cytotoxicity with NK cells, they are a distinct population. CTL are able to destroy a variety of targets, including tumor cells and virally infected cells. CTL require a sensitization period in which CTL precursors undergo proliferation and differentiation into effector cells. Death of the target cells is by apoptosis, as observed with NK cells. Assessment of CTL function may reveal deficits not only in the effector phase of the immune response, but also functional abnormalities in cellular activation and regulatory pathways, such as interferon-γ production. Methodology for the induction of cytotoxic cells was first described by Wundelich and Canty (1970), and is composed of two phases: stimulation of CTL precursors by an inactivated tumor cell line for several days, and then measurement of cytotoxicity using the same cell line labeled with ^{51}Cr. CTL precursors can be stimulated from splenocytes when using a rodent assay, or from peripheral blood lymphocytes when assessing the effects in humans. The key attribute of the stimulating cells is that they are allogeneic (at least with regard to MHC). Stimulatory cell lines, such as the P815 murine mastocytoma cell line for rodent CTL, or the Jurkat leukaemic cell line for human CTL, are used since they are more convenient than primary allogeneic cells (lymphocytes for example). Protocols have been detailed in previous works (House and Thomas, 1995; Lebrec et al., 1995).

Determination of Potential Effects on NK Cells

NK cells or large granular lymphocytes are involved in nonspecific immunity and, as such, should be included in any *in vitro* immunotoxicology study design. $CD3^-CD16^+CD56^+$ cells account for 7–41% of the lymphocytes in human peripheral blood (absolute counts vary between 130 and 1,000/mm^3; Pasqualetti et al., 2003). They have for a long time been recognized as exquisitely sensitive to perturbants. Both their numbers and their functional activity have been studied for a long time by toxicologists. NK cells are enumerated based on surface markers (mainly CD56) and their cytotoxic function is usually assayed *in vitro* using (^{51}Cr) labeled target cells, classically K562 erythroleukemia cells. Other functional parameters, such as cytokine production, can also be evaluated, but require prior purification or testing with and without NK cell removal (e.g., with anti-asialo-GM1 antibody) (Bohn and Autenrieth, 1996).

Immunotoxicity testing of new pharmaceuticals now includes cytotoxic NK cell function as a required parameter in repeated dose toxicity studies. The classical ^{51}Cr-release assay is the conventional test for cytotoxicity testing. Recently, a flow-cytometric cytotoxicity assay especially adapted for regulatory rat studies in drug development has been presented (Marcusson-Stahl and Cederbrant, 2003). The proportion or absolute numbers of NK cells are modified after *in vivo* exposure to large numbers of substances or physical agents (Burns et al., 1994; Ross et al., 1996; Marcusson-Stahl and Cederbrant, 2003).

As NK cell assays are exquisitely sensitive to modulation by toxic substances, they are usually included in even the most limited toxicological evaluations. Since NK cells are among the first to be affected, it is not surprising that the significance is often questioned. Indeed, changes in NK activity are not often related to pathological conditions of the immune system. In contrast, they have been tentatively associated with various conditions of unknown etiology (Stewart et al., 2003). NK assays have been used thoroughly to monitor *in vivo* exposure. The predictive potential of such assays after *in vitro* exposure is the subject of intensive investigation (Lebrec et al., 1995; Condevaux et al., 2001).

DESCRIPTION OF SELECTED ENDPOINTS AND METHODOLOGIES

Flow Cytometry

Flow cytometry has been primarily used for immunofluorescent staining of cell surface molecules to differentiate cell subsets, to detect changes in the composition of heterogenous cell populations, and to analyse cell activation. Later, techniques were devised for intracellular staining of DNA (allowing cell cycle analysis, proliferation and apoptosis studies, detection of cytotoxicity); more recently, intracellular proteins were analyzed allowing quantification of cytokine expression and molecular signals (e.g., activated Stat). Finally, the same apparatus can be used for chemical measurements: reagents adsorbed or chemically coupled to the surface of microspheres to capture analytes that are subsequently measured by a fluorochrome-conjugated detection molecule. More recently, such assays have been multiplexed, or analyzed in the same vial, by performing each reaction on a set of microspheres that are dyed to different fluorescent probes and, therefore, are spectrally distinct.

The quantity/quality of leukocyte-expressed biomarkers (surface or intracellular) varies after exposure to noxious compounds (Leonardi et al., 2000; Hertz-Picciotto et al., 2002). For instance, *in vitro* exposure of B-cells to various toxic agents (dioxins, furans, PAHs, etc.) results in the alteration of the AhR intracellular levels and of cell surface markers (such as CD19) (Masten and Shiverick, 1995).

The cytotoxicity of NK cells can also be monitored by flow cytometry using the DNA probe propidium iodide; target cells are thus recognized by labelling with 5-(6)-carboxy-fluorescein succinimidyl ester (Marcusson-Stahl and Cederbrant, 2003). It can be predicted that flow cytometry techniques will be as useful for *in vitro* immunotoxicology as they have been for *in vivo* experiments (Holladay and Smith, 1995; Rhile et al., 1996; Tryphonas et al., 1996; Burchiel et al., 1997; Robinson et al., 1997; Leonardi et al., 2000; Hertz-Picciotto et al., 2002).

Proliferation and Apoptosis

Cell proliferation and cell death are two closely related, physiologically active phenomena. Dysfunction of either of these mechanisms may cause dysregulations of cell homeostasis, inducing adverse changes in immune function that may increase susceptibility to infections and cancer, as well as favouring the development of autoimmune diseases. On the other end, the activation of specific immune responses involves the proliferation of lymphocytes. Antigen-driven activation and proliferation of lymphocytes are integral steps to *in vivo* cell-mediated and humoral immune responses.

The mitogen-stimulated proliferative response widely used in immunotoxicology and in clinical immunology, is an *in vitro* correlate of activation and proliferation of lymphocytes specifically sensitized by antigen *in vivo*. *In vitro* stimulation of lymphocyte proliferation is an easy assay, and should be included to assess immune function in *in vitro* toxicity testing. Furthermore, *in vitro* lymphocyte stimulation or transformation could also be performed using the whole blood assay. Both plant lectins (e.g., PHA, Con A, PWM, etc.) as well as LPS, purified protein derivative of tuberculin (PPD), anti-CD3 and/or anti-CD28 antibodies, etc. can be used to stimulate T- or B-cell proliferation in whole blood. *In vitro* antigen-specific and mitogen nonspecific activation of lymphocytes results in myriad biochemical events, including calcium influx, protein kinase C activation, and phospholipid synthesis, culminating in DNA synthesis and cell division (Bauer and Baier, 2002; Flavell et al., 2002). Xenobiotics interfering with signal transduction pathways are likely to alter mitogen-induced lymphocyte proliferation.

While any one of these biochemical events can be used as markers of lymphocyte activation, DNA synthesis, usually measured by the incorporation of ^{3}H-thymidine, has been the most widely used endpoint. As an alternative, flow cytometry can be used to evaluate proliferation analysing expression of proliferating cell nuclear antigen. Furthermore, using the whole blood in combination with flow cytometry leukocyte function, proliferation and expression of surface antigens (e.g., CD14, CD25, CD95), all of which have a potential role in costimulation, adhesion and apoptosis of the immune cells, can be evaluated simultaneously (Barten et al., 2003).

The functionality of the immune system could be thus assessed *in vitro* using whole blood, which allows the assessment of lymphocyte proliferative responses, apoptosis, cytokine production and NK cell activity. The simple use of whole blood allows the assessment of the functionality of the immune system without preparation artefacts, it is time effective and less blood is required, making it a suitable method for *in vitro* studies. Functional *in vitro* studies of immune responses may be very

sensitive tools, suitable for revealing dysregulation of immune homeostasis and early predictors of incipient diseases.

Whole Blood Cytokine Release Assay

Cytokines are released as one of the first steps of immune response and quantitative alterations can be used as a measure of immunomodulation. Depending on the type of inflammogen (stimulus), human blood cells release different cytokine patterns, originating from several blood cell populations. A plethora of assay systems are available for measuring cytokines and their receptors such as ELISA, flow cytometry, and molecular biology techniques such as PCR (House, 2001b). The technique used is probably of limited importance, and each method has its own characteristic profile of advantages and disadvantages.

More importantly, but often overlooked, is the requirement for a careful determination of which cytokines to be measured to obtain the most useful information. This is confounded by the highly pleiotropic and redundant nature of cytokines, in which a single function may be affected by multiple cytokines simultaneously. Certain types of cytokines may be proposed (e.g., proinflammatory, immunostimulatory, immunosuppressive). In addition, the continuing process of cytokine discovery may make attribution of specific function difficult (such as the similarity in action between cytokines such as IL-2 and IL-15, or IL-4 and IL-13). For this reason, it is advisable to include the broadest panel of cytokines possible in any *in vitro* system using such analysis.

At present, only the whole blood assay has been prevalidated for use in *in vitro* immunotoxicology (Langezaal et al., 2002). However, most of the cytokine assessment designs currently in use for the more standard *ex vivo* immunotoxicology studies could be qualified using an approach similar to that used in the whole blood model. Whole blood cytokine release assays have been in use for more than 20 years. They offer the advantage of employing human primary cells in their physiological mixture and environment with little preparation artefacts and ease of performance, i.e., a handling of liquids without any sophisticated cell culture technique. Whole blood cytokine release (or more general mediator release since eicosanoids (von Aulock et al., 2003), NO, or degranulation products can be determined using the same approach) was found to be more homogenous between different donors than the respective models using isolated cells. The whole blood assay system has been evaluated in a variety of systems and the results suggest the validity of this model for assessing immunotoxicity (Borgermann et al., 2002; House et al., 2002; Araya et al., 2003; Heagy et al., 2003).

A major concern against whole blood models is the interindividual variation in leukocyte numbers. In fact, in healthy donors, the normal range of leukocyte numbers is within a fairly narrow window—few methods to count and adjust cell numbers in routine practice are as precise as the latter parameter. Furthermore, using a differential blood cell count, responses can be normalized to the number of a given leukocyte population in the experiment. The model can be used to monitor immune functions *ex vivo*, e.g., of volunteers treated with immunomodulatory agents (Hartung et al., 1995), of patients exposed to toxins or of patients in order to characterize the course of disease as well as the effect of treatment. It has proven to be very advantageous that the same model can be used *in vitro* and *ex vivo*.

Stimulation with LPS leads to the release of interleukin-1β (IL-1β) by monocytes. Experience with the development of the whole blood pyrogen test (Hartung et al., 1995, 2001; Fennrich et al., 1999; Hartung, 2002; Schindler et al., 2002). has shown that IL-1β, which induces inflammation, fever, and septic shock, qualifies as an endpoint for monocytes with minor differences to IL-6 or tumor necrosis factor-alpha (TNFα). It has been previously shown that employing Staphylococcal entero-toxin B (SEB) and prolonging the incubation period from 48 to 72 hours, the whole blood model can be extended to determine also the release of various lymphokines (Hermann et al., 2003). SEB is a superantigen that can link specific T-cell receptor Vβ regions of the T-cells to MHC Class II molecules present on APC. This leads to the activation of both APC and T-cells and to release of various lymphokines such as IL-2, IL-4, IL-13, and IFNγ. IL-4 was chosen as the lymphokine read-out, since it is not produced by monocytes and reflects activation of B-cells by T-helper 2 cells, thus allowing assessment of the interplay of two major lymphocyte populations. The recent development of cryopreserved human whole blood, which can be used after thawing without further washing steps (Schindler et al., 2002) overcomes limitations such as blood availability, risks of infections, or abnormal responses. Furthermore, the pooling of donors offers the opportunity of standardized materials.

In conclusion, whole blood incubations provide the ability to assess either effects on monocytes or lymphocytes employing selective stimuli. The model has been shown to reflect several aspects of immunotoxicity such as immunostimulation, priming and inhibitory effects. Advantages of such human blood cell-based *in vitro* tests include:

- Species differences between humans and animals are avoided.
- Human primary cells are employed in their physiological proportions and environment, avoiding preparation and cultivation artifacts.
- Culture techniques are extremely simple, e.g., allowing incubations in thermoblocks.
- Cryopreserved blood overcomes problems of availability, standardisation and risks of infection.
- *In vitro* testing is less expensive and time-consuming than *in vivo* testing.
- The same test can be employed *ex vivo* and *in vitro.*
- The number of compounds and concentrations tested can be increased.
- The amount of substance required is dramatically reduced, allowing testing at earlier stages of drug development.

- Effects on different blood cell populations can be tested in a single model.
- Changes of cellular immune response can be quantified, enabling potency testing.

The very simple model of whole blood cytokine release thus offers a variety of opportunities to assess immune functions in a highly standardized manner. The predictive value of these approaches is currently under investigation.

The goal of an *in vitro* testing approach is to expand beyond simple screening to more mechanistic evaluation. Some areas of investigation that would naturally follow from a demonstration of overall cytokine modulation (as would be detected in the whole blood model) might include:

1. At what stage is cytokine production affected (transcription, transduction, release, etc.)?
2. Is cytokine transcription or production skewed toward a discrete phenotype (T_H1 or T_H2)? Given the increasing knowledge of the role these phenotypes play in health and pathology, such information may be invaluable in determining the ultimate result of any cytokine dysregulation.
3. Are cytokines overproduced (i.e., inflammation) or underproduced (i.e., immunosuppression) in response to a stimulus? Is cytokine production induced in the absence of an obvious stimulus?
4. Are cytokine receptors affected (expression, function)?

Keratinocyte Models for Evaluating Sensitizing Potential

The skin is composed of two layers, namely the epidermis and the dermis, and rests on the subcutaneous adipose tissue. The epidermis is a multilayered epithelium composed of different cell types. Keratinocytes (KC) are the major cell type, and represent about 95% of the epidermal cell mass. They are responsible for the biochemical and physical integrity of the skin via their production of keratin and mucopolysaccharides (Barker et al., 1991). Langerhans cells (LC) comprise the second most prominent cell type in the skin. LC are bone marrow-derived DC and represent 2% to 5% of the epidermal population. Melanocytes form some 3% of the epidermal cells and by generating the pigment melanin they protect the skin against ultraviolet radiation. Besides its barrier function, the skin has been recognised as an immunologically active tissue. LC are the principal APC in the skin (Katz et al., 1979). KC not only represent the first target for irritants, but may also act to convert nonspecific exogenous stimuli into the production of cytokines, adhesion molecules, and chemotactic factors (Barker et al., 1991).

The anatomical location of KC and their significant role in the development of allergic contact dermatitis (ACD) justifies the use of these cells to evaluate sensitizing potency *in vitro*. In principle, a test system comprised of KC alone may not be useful in establishing allergenic potency as these cells lack antigen-presenting capacity. In the epidermis LC exerts this function. However, in addition to chemical processing, LC activation requires the binding of cytokines produced by KC as a result of initial chemical exposure. The irritant capacity of allergens might present an additional risk factor so that irritant allergens may be stronger allergens than nonirritant ones (Grabbe et al., 1996). In that case, evaluating irritant potency of allergenic chemicals may be helpful to establish their allergenic potency, and the potency of chemicals to induce cutaneous sensitization may be assessed as a function of KC cytokine expression.

Cytokine Production by Keratinocytes

KC are involved in both ACD and irritant contact dermatitis through the synthesis and release of inflammatory cytokines, chemokines, and growth factors (Ansel et al., 1990; McKenzie and Sauder, 1990). KC produce a wide range of cytokines including IL-1, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IL-18, IL-20, TNFα, and IFN-α, -β, and -γ. Unstimulated KC express and secrete low levels of cytokines but also provide a reservoir of preformed (primary) cytokines such as IL-1α, IL-1β, and TNFα. In response to exogenous stimuli, activated KC can produce various inflammatory cytokines. For example, IL-1, IL-6, IL-8, and TNFα exert proinflammatory activity, while IL-1 is chemotactic for KC, and IL-6 stimulates KC proliferation. IL-8 and TNFα are produced in response to external stimuli. KC cytokines are capable of regulating cytoskeletal proteins that become activated during inflammation (Komine et al., 2001).

Interleukin-7 is considered to be significant in T-cell trafficking (Wagner et al., 1999), as is IL-15 (Han et al., 1999). IL-10, IL-12, and IL-18 exert systemic effects, whereas IL-10 may exert anti-inflammatory effects (Moore et al., 2001). It modulates the function of splenic APC (Ullrich et al., 1994). Furthermore, IL-10 partly reversed aberrant KC maturation in psoriatic lesions and decreased expression of IL-8, its receptor CXCR2 and IL-17 (Reich et al., 2001). IL-10R is down-regulated in psoriasis and acute atopic eczema (Müschen et al., 1999). IL-12 production by KC has been observed in ACD (Yawalkar et al., 2000). The antagonism between IL-10 and IL-12 that is observed in other cell types is also observed in KC. IL-18 has structural homology to IL-1, and similar to IL-1 caspase-1 cleaves IL-18 into a functional form. Human KC constitutively expresses the unprocessed form of IL-18, but the production of the processed form is subject to debate (Kim et al., 2000; Mee et al., 2000). IL-1β, TNFα, or IFNγ did not significantly change IL-18 expression (Companjen et al., 2000). A similar lack of induction was observed after treatment with LPS or phorbol myristate acetate (Naik et al., 1999; Mee et al., 2000). Inflammatory and immunological stimuli induce caspase-1 activity in KC, allowing the cleavage of the unprocessed form of IL-1β into an active form (Zepter et al., 1997); this process may also be operational for IL-18 (Yamanaka et al., 2000). Intradermal (ID) injection of IL-18 induced LC migration to the LN (Cumberbatch et al., 2001), while ID injection of DNA encoding IL-18 resulted in a mononuclear cell infiltrate (Kremer et al., 1999). IL-18 is increased in psoriatic skin (Naik et al., 1999; Ohta et al., 2001).

KC also express the IL-18R, on the same cells that show IL-18 in the cytoplasm, suggesting a paracrine or autocrine function of IL-18 in KC (Koizumi et al., 2001).

KC are a source of IL-10 family members such as IL-20 and IL-24. IL-20 has a role in normal KC differentiation, and its receptor is upregulated in psoriatic KC (Blumberg et al., 2001). Functional IL-24R are demonstrated on KC (Wang et al., 2002). Also, chemokines and their receptors play important roles in KC regulation and inflammation. LARC/CCL20 and its receptor CCR6 are expressed by KC and CCL20 expression is strongly augmented in the lesional skin of psoriasis patients (Nakayama et al., 2001). KC produce TARC in the lesional but not the nonlesional skin of patients with atopic dermatitis (Vestergaard et al., 2000). KC-derived ligands for CXCR3 and CCR4, such as IP-10, MIG, and MDC may be involved in T-cell trafficking (Rottman et al., 2001). MIP-3α is expressed by KC and may attract LC precursors (Dieu-Nosjean et al., 2000). CCR10 binds CCL27, which is constitutively produced by KC and can be induced upon stimulation with TNFα and IL-1β (Homey et al., 2000). KC influence immigration of inflammatory cells by the expression of MCP-1, RANTES, IP-10, and MIG (Goebeler et al., 2001).

Receptors for IL-4, IL-13, and IL-17 are present on KC. IL-4 treatment of KC resulted in KC proliferation and IL-6 production (Junghans et al., 1996; Wery et al., 1996). IL-13 treatment induced in IL-6 production (Derocq et al., 1994; Akaiwa et al., 2001). Activation of IL-17R modulated IFNγ- and IL-4-induced activation of KC (Albanesi et al., 2000).

Exposure Effects on KC

The irritants croton oil, phenol, and benzalkonium chloride and the contact allergen dinitrofluorobenzene (DNFB) induced the production and intracellular accumulation of IL-1α (Wilmer et al., 1994). A dose-dependent induction of IL-1α mRNA was seen after neomycin sulphate treatment, while benzocaine did not affect and dinitrobenzene sulfonate suppressed IL-1α mRNA expression (Pastore et al., 1995). In a study using five allergens, two irritants, and two nonsensitizers, only the contact sensitizers increased cell-associated IL-1α in a dose-dependent fashion while both allergens and irritants induced the release of this cytokine. Nonsensitising chemicals had no effect on IL-1α (Corsini et al., 1998). IL-1α as well as TNFα expression by KC was increased after *in vivo* application of contact sensitizers and croton oil but not tolerogens (Haas et al., 1992). Using the murine KC line HEL-30, the allergenic potency of four compounds was established on the basis of intracellular IL-1α and intracellular IL-18. Ranking of potency was similar for the two cytokines and similar to the ranking established using the local lymph node assay (van Och et al., 2005).

The irritants sodium lauryl sulphate (SLS), croton oil, and phenol, but not the sensitizers DNFB and oxazolone, induced increased production of IL-8 (Wilmer et al., 1994). In contrast, others observed IL-8 induction by both sensitizers and irritants (Mohamadzadeh et al., 1994). The latter finding was, however, obtained using the HaCaT cell line, that differs from primary KC with respect to IL-18 production (Kämpfer et al., 2000). The irritants dimethylsulfoxide (DMSO) and SLS, but also the contact allergen nickel (Ni), induced TNFα mRNA levels in KC. DMSO and SLS increased promoter activity while Ni increased mRNA stability (Lisby et al., 1995). Allergens but not irritants or tolerogens induced IL-12 (Muller et al., 1994; Corsini et al., 1999). Trinitrobenzene sulphonic acid induced the expression of CD40 on KC, whereas SLS did not (Coutant et al., 1999). CD80 transcription and cell surface expression on KC were upregulated to similar extents by treatment with allergens or irritants (Wakem et al., 2000). Finally, CD1d was upregulated in psoriasis plaques and by the contact sensitizer poison ivy (Bonish et al., 2000). CD40, CD54, and HLA-DR expression on KC was reduced in psoriasis biopsies (Abrams et al., 2000).

DC Models in Hypersensitivity

DC were first identified in the epidermis in 1868 and were originally known as LC. Their presence in other tissues was identified in 1973 (Steinman and Cohn, 1973), and DC are now recognized as an integral part of the immune system. Current information from humans and mice does not support a conclusive definition of the physiological differentiation pathways that generate DC *in vivo*. However, they appear to comprise at least three distinct subsets, including two within the myeloid lineage, LC and interstitial DC, and one within the lymphoid lineage, the so-called lymphoid DC subset also named plasmacytoid DC.

T-Cell immunity against tumors and bacterial or viral infections relies on the recognition of antigenic peptides processed and presented to T-cells by APC. During *in vivo* immune responses, the role of APC is played primarily by DC acting as initiators, stimulators, and regulators of antigen-specific T-cells. Thus, DC form a sentinel network able to detect, capture, and process antigens such as invading bacteria, viruses, products of tissue damage and haptens (Aiba et al., 1997; Hacker et al., 1998; Cella et al., 1999). In peripheral tissues such as the skin, the DC can be found in contact with KC in an immature state with a high capacity for antigen uptake and processing but unable to stimulate T-cells (Cella et al., 1997; Hart, 1997; Banchereau and Steinman, 1998). Upon antigen capture, stimulation by microbial products but also in response to inflammatory cytokines (e.g., TNFα, IL-1) or T-cell-derived signals such as CD40 ligand, the DC undergo a maturation process leading to the upregulation of co-stimulatory molecules, (CD86, CD80, CD40), MHC Class II molecules, and the CD83 protein (Caux et al., 1994). Thereafter, DC migrate to the T-cell areas of lymphoid organs where they lose antigen-processing activity and become potent immunostimulatory cells. These maturing DC acquire the ability to migrate through expression of chemokines and chemokine receptors and down-regulation of molecules such as E-cadherin. Indeed, upon maturation DC express a novel chemokine receptor, CCR7, enabling them to migrate in response to gradients of chemokines (CCL21 and then CCL19) (Yanagihara et al., 1998;

Gunn et al., 1999; Saeki et al., 1999). Expression of CCR7 is particularly important, as CCR7-deficient mice show an impaired migration of activated LC into draining lymph nodes after skin painting with FITC and consequently, lack any contact hypersensitivity (Forster et al., 1999).

Knowledge of DC physiology has progressed considerably because of the discovery of culture techniques, in the early 1990s, which support the *in vitro* generation of large numbers of DC from hematopoietic progenitors (Caux et al., 1992). Two main protocols to generate DC, from either monocytes or $CD34^+$ hematopoietic cell precursors (HPC), have been described. $CD34^+$ HPC, isolated from cord blood or bone marrow mononuclear cells, can be induced to proliferate *in vitro* in response to several cytokines in combination. TNFα enhances significantly the proliferation of $CD34^+$ HPC induced by either IL-3 or GM-CSF. Addition of stem cell factor (SCF) or Flt-3L (fms-like tyrosine kinase-3 ligand) increases the yield of DC but does not affect DC differentiation. Many candidate DC are $CD1^+$ $CD14^-$ during the later stages of culture (days 12–14) from $CD34^+$ HPC in FBS-containing medium. However, when examined earlier, two DC subsets emerge independently by days 5–7, as defined by the mutually exclusive expression of CD1a and CD14. When cultured with GM-CSF and TNFα, $CD14^+$ $CD1a^-$ intermediates generated E-cadherin$^-$, langerin$^-$ $CD1a^+$ interstitial DC that lack Birbeck granules and $CD14^-$ $CD1a^+$ intermediates generated E-cadherin$^+$ langerin$^+$ $CD1a^+$ LC-like DC possessing Birbeck granules by a transforming growth factor (TGF)-β-independent pathway.

Peripheral blood monocytes cultured with GM-CSF and IL-4 generate immature DC that can be driven into a mature state by TNFα. Addition of TGFβ to culture of monocytes with GM-CSF and IL-4 has been shown to induce the differentiation of peripheral blood monocytes in LC (Geissmann et al., 1998). Application of low-molecular-weight chemicals (haptens) to the skin may result in allergic contact hypersensitivity (Grabbe and Schwarz, 1998). *In vivo* painting of murine skin with chemicals, such as FITC, provokes an influx of epidermal DC in the draining lymph nodes 24 hours after hapten application (Hill et al., 1990). These epidermal DC, termed LC, express MHC Class II molecules and co-stimulatory molecules such as CD86 (Aiba and Katz, 1990; Kripke et al., 1990). In animal models, production of pro-inflammatory cytokines such as IL-1 and TNFα has been shown to play a major role in DC activation and migration in contact hypersensitivity (Enk and Katz, 1992; Cumberbatch et al., 1997). These observations could also be extrapolated to respiratory sensitization in response to haptens although clear data in a relevant model have not been established yet. Thus, it seems that considering similarities between immunity to simple chemicals and that to infectious agents, it is reasonable to speculate that hapten itself stimulates DC maturation.

DC are a minor population within epithelial cells in organs such as epidermal cells in the skin; isolation of this population in sufficient numbers is a difficult task and often results in nonspecific activation of DC. Generating DC from murine bone marrow $CD34^+$ HPC has been used as an alternative, but this procedure is time-consuming and requires a significant number of animals. The establishment of human *in vitro* models of DC offered the possibility to demonstrate that haptens were able to directly activate cultured DC derived from peripheral blood monocytes or from $CD34^+$ HPC (Aiba et al., 1997; Degwert, 1997; Rougier et al., 2000; De Smedt et al., 2001; Weigt et al., 2004). Several studies confirmed these observations showing the up-regulation of maturation markers (CD83, CD80, CD86, CD40) on human DC (Coutant et al., 1999; Aiba et al., 2000; Tuschl et al., 2000; Arrighi et al., 2001). Cytokine production such as IL-12p40, TNFα, and IL-1β has also been reported upon hapten stimulation (Aiba et al., 1997, 2003; De Smedt et al., 2001). However, significant differences exist between experimental systems and authors concerning cytokine production. Haptenized DC also cause T-cell proliferation in the allogeneic MLC (Rougier et al., 2000; De Smedt et al, 2001) but not in the autologous MLC (De Smedt et al., 2001). Recently, the expression of CCR7 mRNA in human DC stimulated by haptens and the migration of these cells in response to CCL19 has been observed (Aiba et al., 2000; Boislève et al, 2004). Boislèveet al. (2004) have also documented the presence of $CD83^+$ $CCR7^+$ DC in response to Ni and DNCB.

Mitogen-activated protein kinase (MAPK) has been described to play a major role in DC maturation induced by LPS through Toll-like receptors (Rescigno et al., 1998; Ardeshna et al., 2000; Underhill and Ozinsky, 2002) or by inflammatory cytokines through binding to specific receptors (Wajant et al., 2003). Haptens such as Ni ($NiCl_2$ and $NiSO_4$), DNCB and 2,4-DNFB induce the phosphorylation of p38 MAPK and c-Jun N-terminal kinase (JNK). Inhibition of p38 MAPK is correlated with alteration in phenotypic indicators of maturation such as CD86, HLA-DR or CD83 in DC derived from monocytes or from $CD34^+$ HPC stimulated with Ni or DNCB (Arrighi et al., 2001; Aiba et al., 2003; Boislève et al., 2004).

Mast Cells

Mast cell precursors originate from the bone marrow and upon migration into different tissues develop into either connective tissue type mast cells (in skin, peritoneum, and lungs) or mucosal type mast cells (in gastrointestinal tract) (Prussin and Metcalfe, 2003). The related but functionally slightly different population of basophils does not reside in tissues but circulates in the blood where it constitutes 1% of all PBL (Abraham and Arock, 1998).

Because mast cells bind IgE with high affinity they are generally considered to be critical effector cells in Type 1 or immediate type hypersensitivity responses (Prussin et al., 2003). Through crosslinking of membrane-bound IgE by the relevant antigen, mast cells are induced to secrete the content of their granules. But mast cells also express $F_{c\gamma}$RIII-IgG and IgG may be as effective as IgE in triggering mast cell degranulation (Strait et al., 2002). The released mast cell mediators (e.g., histamine, serotonin, leukotrienes, prostaglandins, and various proteases

such as tryptase) induce vascular permeability that facilitates extravagation of additional leukocytes and inflammatory mediators. In addition, mast cell products induce bronchial constriction and cause other physiological effects related to a Type 1 hypersensitivity response. Because of these effects, mast cells have a prominent role in airway and food allergy responses, certain skin diseases such as atopic dermatitis and urticaria, and in anaphylaxis.

Apart from having an effector function, mast cells are also involved in the initiation of inflammatory responses. Mast cells can, for instance, be triggered by complement factors (C3a and C5a) and endotoxins and are the only cell type capable of storing presynthesized TNFα, which they can secrete within minutes after bacterial challenge (McLachlan et al., 2003). Besides TNFα mast cells produce IL-8 and, as already mentioned, histamine. Histamine is generally regarded as a mediator of inflammation and key player in the immediate allergic reaction (MacGlashan, 2003) but histamine has also been demonstrated to have immunoregulatory functions. Histamine induces a number of adhesion molecules (E-selectin, ICAM-1 and LFA-1), cytokine production (proinflammatory cytokines such as IL-1 and IL-6, but also regulatory cytokines such as IL-10) by various immune cells. Moreover, histamine has been shown to induce CD86 expression and chemokine and cytokine production by immature DC (Caron et al., 2001a, and to promote development of T_H2-stimulating DC (Caron et al., 2001b).

With regard to adaptive immunity, it should be noted that mast cells express MHC Class I as well as MHC Class II and various costimulatory molecules (e.g., CD80, CD86, CD40L). Combined with their capacity to phagocytize diverse particles and antigens, mast cells may function as APC (Henz et al., 2001). In addition, mast cells have profound regulatory function on T_H1 vs. T_H2 responsivenes since they are important sources of IL-4, IL-5, and IL-13.

Because of the plethora of functions they possess combined with their strategic tissue localization at borders to the surrounding environment, mast cells can be regarded as a crucial cell type in linking innate with adaptive immunity. In this respect it is noteworthy that mast cells have been shown to express various Toll-like receptors (TLR1, TLR2, TLR4, and TLR6), indicating that mast cells are indeed candidate cells to translate pathogen-associated molecular signals into an efficient adaptive immune response (Robbie-Ryan and Brown, 2002; Bellou et al., 2003).

Not surprisingly, mast cells are found to play a role in many inflammatory diseases. Apart from allergic diseases they are involved in diseases like irritable bowel syndrome, scleroderma, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus and experimental allergic encephalitis (Robbie-Ryan and Brown, 2002; Theoharides and Cochrane, 2004).

Finally, mast cells may also play a relevant role in tissue homeostasis, tissue remodelling, and fibrosis (wound healing), as well as in processes of tissue angiogenesis (Puxeddu et al., 2003; Crivellato et al., 2004). Mast cell products also influence gastrointestinal secretion, absorption, and motility (Siddiqui and Miner, 2004).

As mentioned, mast cells are important in various allergies, such as airway and food allergy, and possibly also in various chemically induced autoimmune-like derangements. In allergy responses mast cell degranulation is immune (i.e., IgE)-mediated, but mast cells can also be triggered by non-immune-mediated processes. Apart from complement factors and endotoxins, temperature (cold or heat) or exercise may cause mast cell degranulation without the need for IgE. Also certain drugs or chemicals can induce non-IgE mediated anaphylaxis. Examples of such compounds are aspirin and other nonsteroidal anti-inflammatory drugs, radiocontrast media, opioids, certain muscle relaxants, and $HgCl_2$ (Wu et al., 2001; Kemp and Lockey, 2002; Asero et al., 2003). Also, organic extracts of diesel exhaust particles were able to stimulate release of IL-4 and histamine from basophils irrespective of whether they originate from allergic or nonallergic humans (Devouassoux et al., 2002). Clearly, mast cells are important from an immunotoxicological point of view. Therefore, effects on mast cells should be part of a predictive *in vitro* panel to assess the immunotoxicological hazard of chemicals.

Mast cells can be derived from various sources. Human mast cells in particular can be cultured from $CD34^+$/c-kit^+/$CD13^+$ progenitor cells in cord blood by addition of IL-3, IL-6, and SCF (the ligand for c-kit) (Kirshenbaum et al., 1999). Mouse or rat mast cells can be generated most efficiently from bone marrow progenitors in particular in the presence of IL-3, IL-4, and SCF (Haig et al., 1988; Tsuji et al., 1991). Culture of mast cells from bone marrow takes 14 to 15 weeks to produce a 99% pure population. Mast cells can also be isolated from various tissues (e.g., airways, intestine) or from the peritoneal cavity (Arock et al., 1989).

Mast cell lines are also available for alternative testing. The mast cell leukemia HMC-1 cell line has the characteristics of an immature mast cell that does not express surface $F_{c\varepsilon}RI$ receptors but produces histamine and tryptase (Love et al., 1996). A mast cell line frequently used to assess nonantigen- (Fowler et al., 2003) or antigen-IgE (Granberg et al., 2001) mediated release is the rat basophilic leukemia cell line RBL-2H3. This cell line is often used to determine pharmaceuticals with mast cell release-inhibiting potential (Granberg et al., 2001). However, the RBL-2H3 cell line has also been used to determine the effect of water-soluble cigarette smoke extracts upon mast cell release (Fowler et al., 2003) or of $HgCl_2$ (Wu et al., 2001). Release of mast cells can be detected by one or more of its products, for instance mast cell proteases or histamine and mast cell activation can be detected in many ways, for instance by measurement of intracellular Ca^{2+} levels.

SUMMARY

Although the great majority of immunotoxicology data has heretofore been generated using *in vitro* systems (with the

exception of whole-animal host defense models), the testing approach can more accurately be described as *ex vivo* in that exposure of the immune system to potential immunotoxicants takes place *in vivo*, with subsequent immunological evaluation taking place *in vitro*. Although this approach obviates many uncertainties (effect of xenobiotics on primary or secondary lymphoid tissue, potential requirements for metabolism/biotransformation, etc.), the use of whole animals presents many secondary issues, such as expense, ethical concerns, and eventual relevance to risk assessment for humans. The approaches outlined in this review should provide a first step toward establishing a practical and predictive *in vitro* immunotoxicology testing paradigm.

GENERAL RECOMMENDATIONS

- Hypersensitivity and immunosuppression are considered the primary focus for developing *in vitro* methods in immunotoxicology. Nevertheless, *in vitro* assays to detect immunostimulation and autoimmunity are also needed. Although developmental immunotoxicity is an emerging concern, there are no *in vitro* test models available at this time.
- It is recommended to use a flow chart/decision tree approach to evaluate whether or not a compound is immunotoxic (initial screening). Detection of compounds as potential immunotoxicants can then be followed up by more detailed *in vitro* mechanistic assays (e.g., antigen-specific or redirected CTL).
- To maximize human relevance, and due to the lack of species limitations for these assays, it is recommended that human cells be used for all *in vitro* test systems. With the exception of bone marrow assays, the source of cells should be PBL from donors prescreened for health, immune reactivity, etc.
- Although the use of primary human cells will be of the highest clinical relevance, consideration may eventually be given to the use of sufficiently well-characterized and validated cell lines (human or animal) for certain aspects of the test systems. It is anticipated that most of these assays will be amenable to a microculture format, increasing efficiency and decreasing cost.
- The validation of an *in vitro* method to detect immunotoxicity must depend on high quality *in vivo* data. It is essential that a sufficiently large number of positive and negative reference compounds, including both drugs and chemicals, be tested. To this aim the establishment of a human database is strongly recommended. This could be accomplished by a coordinated effort from governmental agencies, medical institutions, and industry. Access to any extensive animal databases, when available, will also be helpful.

In vitro Testing

Immunosuppression.

- An initial evaluation of myelotoxicity should be performed. If a compound is myelotoxic, there may be no need to proceed with additional evaluation. The methodology for bone marrow culture systems is published and well characterized. *In vitro* bone marrow culture systems are commercially available, and they would probably have to be modified slightly to accommodate *in vitro* exposure to test material. Assays of immunosuppression have been validated to predict the maximum tolerated dose (MTD) in humans. Their suitability for use in immunotoxicology should be determined and would require prevalidation. These assays are relatively expensive if human cells are used, and the standardized nature of commercial systems should provide good feasibility.
- An *in vitro* test to determine lymphotoxicity should be carried out (cell death by necrosis or apoptosis). Such assays would require prevalidation to evaluate their reliability/reproducibility. Viability of the lymphocytes should be the necessary initial test (e.g., colorimetric, flow cytometric assays). If the cells are viable (perhaps 80% or greater), basic functionality would be determined by performing an antigen nonspecific proliferation assay. For T-cells, the stimulatory agent would be a combination of anti-CD3 and anti-CD28; for B-cells an optimum system would have to be developed but would be expected to be similar to the murine system incorporating an anti-immunoglobulin and cytokine. This determination may require broadening to include other types of immunocytes (e.g., phagocytes). These assays are relatively inexpensive (source material is readily available); the feasibility is high due to wealth of published methodology.
- Potential effects on cytokine expression should be determined. The role of cytokine transcription or production should be evaluated as well as the modulation of cytokine receptors. It should also be investigated if cytokine transcription or production is skewed (T_H1/T_H2). It will require careful determination of which cytokines to measure to obtain most useful information (e.g., proinflammatory, specific immunoregulatory cytokines). It is recommended to investigate a broader panel of cytokines than is currently used. Both basal and activated cytokine production should be measured, and for activated cytokine production, anti-CD3 and anti-CD28, LPS, or allergen should be used. Whole blood assay is the most promising option due to advanced stage of prevalidation.

  Many other assay systems are available for measuring cytokine expression (e.g., ELISA, flow cytometry,

molecular biology techniques such as PCR). They are moderately expensive, and their feasibility is high due to wealth of published methodology and commercial standardization.

- Potential effects on NK cells should be determined. Cytolytic function should be measured (this is important for innate immunity). There are a variety of systems available for measuring cytolytic function (e.g., whole blood, radiolabel release, flow cytometry); these systems are robust and well characterized. The immunoregulatory function of NK cells should be evaluated due to the key regulatory nature of these cells. At present, such a system is not well described, and would require method development. A feasible system would probably be a modification of existing whole blood model or other cytokine methods. The systems currently in use are highly reliable and reproducible; implementation for *in vitro* exposure would require additional development. The cost of performing these assays is dependent on assay endpoint, but overall relatively inexpensive; feasibility is high due to extensive past usage of this methodology. In addition, these systems will require prevalidation for exclusive *in vitro* exposure.

Hypersensitivity

- Models for antigen-driven hypersensitivity
  - It is believed that in this case the hapten will bind to soluble or cellular proteins leading to uptake by the DC, processing of the hapten-protein complex and association of processed hapten-peptides with MHC Class II molecules. DC will then migrate from the point of entry of the hapten to draining lymph nodes where presentation of the hapten-peptide complex to naïve T-lymphocytes occurs. Hapten-specific T-lymphocytes will then proliferate and differentiate.
  - DC play a central role in this mechanism and the use of *in vitro* model based on human DC needs to be encouraged.
  - Type of model: DC/CD34$^+$ or Mo-DC (+/-TGFβ)
  - Evaluation of different existing protocols for DC generation is needed (cytokines, serum-free media, or FBS)
  - Protocol standardization of each experimental phase needs to be performed (timing of treatment, hapten treatment, etc).
  - Parameters to be measured:
    - Cytotoxicity: This parameter needs to be carefully evaluated, especially the kinetics since after activation, DC will die upon reaching the lymph nodes; this type of death needs to be distinguished from direct toxicity of the tested molecule.
    - Core parameters: CD86, CD83, CCR-7, ICAM-1, MHC Class II, TNFα, IL-12, IL-10.
    - Other parameters: E-cadherin, CCR6, Langerin, IL-1β, TARC.
  - The protocol refinement: After this first step and after the definitive choice of the model and of the most appropriate protocol, it will be needed to confirm the relevance of the observations using a coculture system with lymphocytes. This step can be performed on a small number of relevant haptens with the following primary read-out: proliferation and cytokine profiling.
- Models for nonantigen-driven activation of the immune system leading to hypersensitivity reactions

Immunoregulation (Adjuvants, Superantigen).

- It has been shown that lymphocytes can be directly activated by microbial products (superantigen concept) leading to release of cytokines and clinical effects. To date, there is no example of this type of effect with low molecular weight chemicals. The existing whole blood assay should be considered to address this question.
- Adjuvants may be included in vaccine formulations to enhance the immune responses to particular antigen(s). It is known that certain compounds can have adjuvant activity through direct or indirect activation of DC. Indirect activation can also result from nonimmune cells present at the site of exposure to the compound.
- For direct activation, human DC models are suitable, as previously described. For indirect activation the use of other cell models should be encouraged depending on the route of exposure e.g., human lung epithelial cells, human keratinocytes.
- In the case of nonimmune cells we recommend to evaluate cytotoxicity and proinflammatory mediators release e.g., cytokines, chemokines.

Mediator Release.

- Compounds may induce the direct release of mediators e.g., histamine, cytokines, eicosanoids, or the activation of the complement cascade leading to hypersensitivity reactions.
- The use of the whole blood assay can address the release of mediators by basophils (histamine) and monocytes (cytokines).
- The use of mast cell models needs also to be considered. At the moment, there is no strong evidence for a role of eosinophils directly activated by compounds. Models are available.

Major Limitations and Future Research Needs

- *In vitro* exposure is most straightforward for direct immunotoxicants. However, materials that require biotransformation would require special culture systems (e.g., culture in the presence of S9).
- Physiochemical characteristics of the test material may interfere with the *in vitro* system. Such characteristics may include the need for serum, effects of vehicle on cells (such as DMSO), and chemical binding to cells. In order to retain the viability of the cells to an acceptable level, *in vitro* exposures are often performed in 0.1% ethanol or 0.1% DMSO as maximum solvent concentration, thereby maximising the exposure concentration of the xenobiotic. This is an additional limitation of *in vitro* systems.
- *In vitro* systems do not take into account the interactions of the different components. It is difficult to reproduce *in vitro* the integrity of the immune system.
- *In vitro* systems do not account for potential neuro-immuno-endocrine interactions. There is no anticipated resolution for this deficiency at present.
- The current state of technology does not allow evaluation of the induction of a memory response *in vitro*. Resolution of this deficiency will require the development of novel culture systems.
- The current state of technology does not allow evaluation of recovery (acute vs. long-term immunosuppression). Resolution of this deficiency will require the development of novel culture systems.
- The current state of technology does not allow for evaluation of toxic effects on lymphoid architecture that could lead to defects in cellular interactions necessary for induction of immune responses (e.g., lymph nodes). Future developments in tissue engineering may solve this problem, but this is a long-range possibility.
- Exclusive use of human cells may limit the ability to bridge to the preexisting database of animal immunotoxicology studies.
- The use of "-omics" should be considered for the search of new parameters and for the possibility of gene profiling after hapten treatment.
- Determination of potential effects on antibody induction/production.
- In animals, production of a T-dependent antibody (such as SRBC) is considered to be the gold standard. However, there are currently no good systems for *in vitro* antibody production using human cells.
- Development of human *in vitro* systems will require optimization of stimulator (preferably using antigen relevant to human exposure, such as TT), culture conditions, and assay endpoint(s). For these reasons, further research in this area is strongly recommended.
- There is a need for research to develop *in vitro* models to detect autoimmunity and immunostimulation.

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