ECETOC

Monograph No. 21

Immunotoxicity: Hazard Identification and Risk Characterisation

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European Centre for Ecotoxicology
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IMMUNOTOXICITY: HAZARD IDENTIFICATION AND RISK CHARACTERISATION

CONTENTS

SUMMARY

In this report, immunotoxicity and sensitisation, the two main types of adverse interaction of chemicals with the immune system are treated separately. Over the last 10 to 15 years, well characterised methods for the assessment of altered immune competence have been reported which has led to proposals for tiered testing schemes. This review examines the suitability of immunotoxicity parameters for inclusion in routine 28 day studies and comments on methods that have been proposed for incorporation within guidelines issued by the US-FDA, US-EPA and the OECD. It is recommended that OECD Guideline 407 is modified to incorporate total and differential blood cell counts, spleen and thymus weight and histopathology, and draining and distal lymph node histopathology for Tier I level testing. Data so generated will provide a reliable and accurate means of identifying at an early stage potential immunotoxic effects.

However, no criteria have been proposed either for how the new data generated will be evaluated or for how a subsequent risk assessment will be made. In this report, criteria for the immunopathological assessment of the thymus, spleen, lymph nodes and bone marrow are described together with comments on haematological and organ weight changes which may be associated with immunotoxicity. Their interpretation will depend on the doses at which changes are manifest, the quantity and quality of the changes observed and the presence and severity of other forms of toxicity. Subsequently, risk characterisation and the approach to Tier II testing in immunotoxicity is discussed. It is concluded that this work must be on a case by case basis, but should not in principle be different from the approach adopted for any other type of toxicity identified in a 28 day study.

In the context of sensitisation, an increasingly sophisticated understanding of the nature of immune responses to chemical allergens has facilitated the design of novel predictive methods for the identification of sensitising activity. Opportunities which arise from new developments in allergy testing such as the local lymph node assay, mouse ear swelling test, and the mouse IgE test should be monitored closely.

SECTION 1. INTRODUCTION

Immunotoxicity, in the context of this paper, is taken to encompass both immunosuppression/ immunopotentiation and allergy. Immunotoxicology, in the context of toxicology, has been reviewed previously (ECETOC 1987; Trizio *et al*, 1988; Dean and Murray, 1991). Since then various recommendations have been made with respect to immunotoxicity testing. The primary purpose of this document is to examine the suitability of immunotoxicity parameters for inclusion in routine 28 day studies and to comment on the test methods that have been proposed for incorporation into the guidelines issued by OECD (1981), US-EPA (1982, 1993) and US-FDA (Hinton, 1992).

Over the last 10 to 15 years well characterised, but not necessarily fully validated, methods for the assessment of altered immune competence have been reported (Dean *et al*, 1979; Vos, 1980; Luster *et al*, 1988), leading to proposals for tier based testing schemes. These schemes may be used to evaluate new chemicals and develop a better understanding of the immunotoxic properties of existing chemicals. The association between various immunotoxicity test measurements and perceptible changes in immune competence (as defined by host resistance assays) has only recently been assessed (Luster *et al*, 1992). The significance of such tests for human health is complicated further by the lack of appropriate epidemiological data. Nevertheless, regulatory agencies, particularly those in the United States, have been active in promoting the concept of testing for immunotoxicity. Many of the schemes have been developed separately from the tests used in general toxicology. Consequently the time scale associated with testing of new chemicals would be increased as would the need for more laboratory animals.

While the main focus of this document is on immunotoxicity testing, it is appropriate also to review briefly regulatory activity and prospective testing methods in the area of chemical allergy and autoimmunity, the other forms of adverse effect resulting from the interaction of chemicals with the immune system. The review of chemical allergy includes respiratory and skin sensitisation, systemic hypersensitivity and autoimmunity.

In order to assist readers it is necessary to define the terminology associated with risk and hazard as used in this report. We have relied upon the definitions as used by the United Nations at the UNCED Conference held in Rio de Janeiro in 1992 and subsequently adopted in recent EEC legislation. **Risk Assessment** is a generic term describing a process, frequently used by authorities for regulatory purposes which entails some or all of the following elements. An **Effects Assessment** involves two components, **hazard identification** and a **dose-response assessment**. Hazard identification involves identification of adverse effects which a substance has an inherent

capacity to cause, and where possible and/or appropriate an assessment of that effect. A **Dose-response Assessment** involves an estimation of the relationship between dose (or level of exposure) and the incidence or severity of the effect induced. **Exposure Assessment**, in this context is the environmental concentration to which the individual is exposed. A combination of the Effects Assessment and the Exposure Assessment leads to **Risk Characterisation** which involves an estimation of the incidence and severity of the adverse effects likely to occur as a result of the exposure to a defined population. The **Risk Characterisation** may involve **Risk Estimation** which is a quantification of the likelihood of the incidence and severity of the adverse effects. Consequently **Risk Reduction** is a process of taking measures to reduce the likelihood of the adverse effects occurring.

SECTION 2. REGULATORY PROPOSALS

2.1 IMMUNOTOXICITY

Over the last 10 years the following significant steps can be identified.

1982 The US-EPA (Subdivision M) (1982) was the first agency to require immunological testing of agrochemicals, specifically biochemical pesticides. Included in Tier 1 were immune cell enumeration and some functional assays. Tier II was activated by adverse effects (not defined) in Tier I and involved testing the ability of mice to make antibody to foreign antigen (plaque assay) and cell mediated immune response assays chosen by the registration applicant.

1988 Further US-EPA proposals (Sjoblad, 1988) suggested revisions to the 1982 Subdivision M testing requirements for biochemical pesticides and made recommendations for evaluating the immunotoxic potential of chemical pesticides (Subdivision F). The tests for each subdivision constituted a two tier system. The Tier I tests for Subdivision M incorporated humoral, cellular, and innate immune function tests and Tier II required host resistance assays. The justification for these more extensive requirements compared with chemical pesticides is that the toxicity testing necessary for biochemical and microbial agents is much less extensive than that employed for chemicals, and as a consequence immunotoxicological effects may be missed. In contrast to subdivision M, the Tier I tests for subdivision F could be incorporated into standard toxicology studies, and included serum immunoglobulin levels, lymphoid organ weights, cellularity of the spleen, thymus, bone marrow, and cell viability. Recommended Tier II studies were host resistance assays and measurements of cell mediated humoral and innate immune function.

1990 The US-EPA circulated a draft Immunotoxicity Study Screen which included new immunological end points for testing chemical pesticides. In the absence of routine 28 day testing procedures within the US, the EPA suggested incorporation of several immunotoxicity screening tests into 90 day, reproduction and carcinogenicity studies.

1991 The UK Department of Health (UK-DOH) proposed to update OECD Guideline 407 to include endpoints capable of identifying potential immunotoxicants, specifically,

enhanced pathology of the lymphoid organs. Their approach was supported by ECETOC (1991).

1992 The Dutch RIVM (National Institute for Public Health and Environmental Hygiene) proposed to extend the update of OECD Guideline 407. Their proposals went further than the UK-DOH, adding more histopathology (gut associated lymphoid tissue) and the measurement of serum immunoglobulins as a minimum requirement. They recommended also examination of bone marrow cellularity, cytofluorimetry of spleen cells and measurement of NK cell activity (Van Loveren and Vos, 1992).

1992 The proposed US-FDA guidelines for testing of direct food additives have been published (Hinton, 1992). The content of the guidelines is similar to the proposed updates for Guideline 407 and those of the UK and Dutch authorities, although some function tests were included (Table 1).

Table 1 Summary of US-FDA Immunotoxicity Testing Recommendations for Direct Food Additives (Taken from Hinton, 1992)

Basic testing (rat model)
Histopathology, gross and microscopic (spleen, thymus, lymph nodes, Peyer's patches, and bone marrow)
Whole blood cell and differential count
Total serum protein, albumin-to-globulin (A/G) ratio
Lymphoid organ and body weights
Retrospective level I testing possible in standard toxicology study
Electrophoretic analysis of serum proteins (when positive or marginal effect is noted in basic testing)
Immunostaining of spleen and lymph nodes for B and T cells (quantification of total Ig)
Serum autoantibody screen and deposition of Ig (micrometry for semiquantitation of the proliferative response)
Enhanced level I testing possible for more complete screening in the standard toxicology study core group, with a satellite animal group, or in a follow-up study
Cellularity of spleen (lymph nodes, thymus when indicated)
Quantification of total B and T cells (blood and/or spleen)
Mitogen stimulation assays for B and T cells (spleen)
NK functional analysis (spleen)
Macrophage quantification and functional analysis (spleen)
IL-2 functional analysis (spleen)
When indicated, or for more complete analysis, other endpoints such as total haemolytic complement activity

1992 OECD proposed additional immunotoxicity parameters into their update of test guideline 407 (OECD, 1992) (Table 2).

Table 2 Immunotoxicity Parameters Proposed by OECD (1992) for Update of Guideline 407

Organ Weights:	Spleen, thymus
Histopathology:	Spleen, thymus, 2 lymph nodes and bone marrow
Blood:	Total and differential leucocyte counts

1993 The US-EPA (1993) proposed a revised immunotoxicity study screen which included additional immunological endpoints for evaluating chemical pesticides (Table 3).

Table 3 US-EPA (1993) Draft Proposal for Immunotoxicity Parameters for Incorporation into Toxicity Studies

Parameter of immune system	Type of study	Species	Dose groups (C; L; M; H)
Spleen weight	Subchronic Oncogenicity Reproduction	All	All
Thymus weight	Subchronic (dermal) Reproduction	All	All
Spleen and Thymus histopathology	Subchronic (dermal) Reproduction	All	C + H
Bone Marrow histopathology	Subchronic (dermal)	All	C + H
EITHER Splenic lymphocyte total and subpopulation enumeration	Subchronic Oncogenicity Reproduction	Rat[1] Mouse[2] Rat/Mouse[3]	All All All
OR Specific anti-sheep red cell antibody quantification	New (28-day exposure)	Rat and Mouse	All

C,L,M,H Control, low, medium and high dose groups respectively

1 Required for mouse when rat is not used as the test species
2 Required for rat at 90 days of dosing when mouse is not used as a test species
3 Required for rat or mouse only when data not required or performed in the rat or mouse after 90 days of exposure in any other study

2.2 SENSITISATION

A number of regulatory agencies have guidelines for the identification of contact allergens; these have been summarised (ECETOC, 1990; Botham *et al*, 1991). Guidelines were published by the

Japanese Ministry of Health and Welfare (J-MHW, 1990) relating to the evaluation of sensitisation induced by pharmaceutical chemicals.

The regulatory position on the identification of contact allergens has been modified following adoption by the OECD of an updated guideline 406 (OECD, 1993).

Although the occurrence of respiratory allergy is recognised by the existence of labelling legislation, there are at present no guidelines for the predictive identification of chemicals capable of inducing sensitisation of the respiratory tract.

SECTION 3. IMMUNOTOXICITY: HAZARD IDENTIFICATION

3.1 TEST PARAMETERS

In order to provide a preliminary evaluation of potential immunotoxicity it is important to distinguish between what should be done from what could be done. A further consideration is the ethical use of experimental animals. For this, and other reasons, it is preferable presently to include immunological test measurements in existing studies rather than to conduct separate animal experiments for which immunotoxicity assessment is the sole objective.

The key aspects of OECD Guideline 407 which relate to the identification of immunotoxic potential, together with the recommendations of ECETOC are incorporated in Table 4.

Table 4 Parameters Recommended by ECETOC for Immunotoxicity Screening

Parameter	A OECD 407 (1991)	B ECETOC (1991)	C ECETOC (1994 recommendation)
	Dose Group	Dose Group	Dose Group
Haematology			
Total WBC	All	All	All
Differential WBC	All	All	All
Organ Weights			
Spleen		All	All
Thymus		All	All
Histopathology			
Spleen	C+H store all	C+H store all	C+H store all
Thymus		store all	C+H store all
Draining and Distant lymph nodes		store all	C+H store all
Bone Marrow		C+H store all	C+H store all

All = all dose groups
C+H = control and high dose groups

Column A shows the current (1991) OECD position and column B refers to the proposals made by an *ad hoc* ECETOC group (1991). In column C a limited extension to the earlier ECETOC proposal has been made which is more consistent with the views expressed by ECETOC (1987) and also with the present proposals for upgrading of OECD Guideline 407 (OECD, 1992). The primary consideration has been the requirement to obtain an early and reliable indication of immunotoxic potential. This is achieved by standard histopathological examination of the key organs within the immune system. The above recommendations are intended as a preliminary screen and further investigation of the immune system is not required in the context of 28 day studies.

The rationale for the above recommendations is explained below.

Lymphoid Organs: For preliminary investigation the weighing of lymph nodes or investigating gut associated lymphoid tissue (GALT) including Peyer's patches is not recommended. This is due to the difficulty of making precise excisions of small tissues such as lymph nodes. Histopathological examination of Peyer's patches has been suggested (Van Loveren and Vos, 1992) but it is unlikely that this will provide useful data beyond that obtained from examination of the recommended lymphoid tissues due to their reactions to the numerous and variable immunological stimuli within the gastro-intestinal tract. As a result there is considerable histological variability in Peyer's patches compared to other lymphoid tissues and consequently, the inclusion of Peyer's patches in routine 28 day studies is not recommended.

Serum Immunoglobulin Concentrations: Although these measurements are comparatively easy to perform, there is as yet insufficient evidence to determine whether alterations in the serum concentration of immunoglobulin classes represent a useful predictor of immunotoxicity in rodents (Dean and Murray, 1991). The monitoring of immunoglobulin levels is therefore not recommended as a screening parameter.

Bone Marrow: It is suggested that consideration is given to storing femoral bone marrow smears to permit a more detailed evaluation should this be indicated by bone marrow histopathology and/or haematology (Weingand *et al*, 1992).

Assays of Immune Cell Number and Function: A number of such measurements have been made in the investigation and characterisation of immunotoxic events in rodents (Luster *et al*, 1988; 1992). Included among these are:

- evaluation of splenocyte number and viability;
- characterisation of the relative frequency of lymphoid cell subpopulations;
- measurement of T and B lymphocyte, NK cell and macrophage function.

Some of these measurements provide information on the mechanistic basis for xenobiotic induced immunotoxicity. As yet, they are inappropriate for routine identification of potential immunotoxicants in the context of conventional 28 day toxicity testing. These assays may be difficult to standardise and are also subject to difficulties of interpretation. Currently the biological or toxicological significance of small changes in the frequency, distribution or functional activity of immunocompetent cells is difficult to evaluate, although large changes are of undoubted relevance. The degree of immunotoxicity that would be associated with large changes in immune function would be detected by the other immunotoxicity screening methods recommended above. It is concluded therefore that it is inappropriate to require inclusion of immune cell number or functional measurements in 28 day toxicity tests.

Host Resistance Assays: Examination of the susceptibility of animals to challenge with microorganisms or tumour cells is expensive, requires large numbers of animals and is difficult to perform. These assays are used primarily to determine whether impairment of immune function is of sufficient severity to alter host resistance and such studies are therefore inappropriate for hazard identification.

The above modifications to OECD Guideline 407 (Table 4, column C) would be sufficient for hazard identification. The methods described should be thought of as a Tier 1 level of testing. Specific target organ toxicity directed toward the immune system can be assessed since the key components of that system will now be examined. If positive results are obtained, further testing may be necessary depending upon the nature of the chemical, its intended use and potential exposure of the workforce or the general population. In our view it is inappropriate to prescribe the nature of subsequent tests. Each substance should be considered on a case-by-case basis for risk characterisation and, if appropriate, risk assessment and management.

3.2 PATHOLOGY ENDPOINTS

The purpose of the recommendations described below is to provide a basis for identification of immunotoxicants in conventional 28 day studies. Several important points should be made. It must be emphasised that alterations in any one parameter in isolation may not necessarily signal

concern. It is necessary to gain a holistic view of changes in lymphoid tissue or immune cell populations in the context of other toxic manifestations. This is particularly important when considering whether potential immunotoxicity results from direct effects of the chemical or are secondary to changes in other organ systems. Important also is the need for evaluation of changes relative to concurrent controls. Immunological parameters are influenced by environmental conditions and circadian rhythms and, as a consequence, historical control values may not be an appropriate basis for assessment of changes related to chemical exposure.

3.2.1 Histopathology

It is difficult to make firm recommendations regarding the degree of change which may be relevant from a histopathological examination of lymphoid tissue. Histopathology of lymphoid tissue can detect changes in tissue achitecture, but is limited by the inability to detect changes in cell numbers if all cell numbers are altered proportionately. Thus haematology results must always be taken into account when evaluating histopathology.

It should be possible for an experienced immunopathologist to form a view based on an evaluation of the tissues below. Changes in thymus histopathology and architecture are considered as being of particular relevance to immunotoxicity screening.

Thymus:

- altered architecture, especially changes in the ratio of cortex to medulla, and/or altered cellularity;
- the presence of lymphocyte degeneration or necrosis and changes in the number of tangible body macrophages;
- the presence of germinal centres.

Lymph Nodes:

- altered architecture and/or cellularity;
- number and appearance of primary follicles and/or germinal centres in the subcapsular cortex;

- changes in cellularity and/or altered appearance of cells in the paracortex;
- changes in the high endothelial venules of the paracortex;
- changes in plasma cells and sinus histiocytes within the medulla.

Spleen:

- altered architecture, particularly changes in the ratio of red pulp to white pulp, and/or altered cellularity;
- within the red pulp, histopathological changes may correlate with changes in organ weight, changes in the number and appearance of histiocytes and in the degree of extra-medullary haemopoiesis may be relevant;
- within the white pulp there are three areas of particular interest namely the follicular areas, the periarteriolar lymphoid sheaths (PALS) and the marginal zones, changes in any of these areas are cause for concern, but of particular importance are changes in the number and appearance of the primary follicles and/or germinal centres within the follicular areas and changes in cellularity and/or altered appearance of the PALS or marginal zones.

Bone Marrow:

- changes in cellularity;
- changes in frequency of different cell types (particularly the estimate of erythroid to myeloid ratio);
- the presence of abnormal cells;
- evidence of hypoplasia or hyperplasia (with the option of confirmation in bone marrow smears).

Other Organs/Tissues:

- inflammatory infiltrates/changes in the joints, skin, liver, kidney etc. may signal signs of systemic hypersensitivity or autoimmunity.

3.2.2 Haematology

When considering haematology there is a need to evaluate total and differential white blood cell counts, bearing in mind the considerable variations that may occur due to animal strain, age, sex, disease status, husbandry and other factors (Blair *et al*, 1982). Absolute numbers of cells as well as percentages must be evaluated. Of greatest concern are changes in peripheral blood lymphocytes, particularly if these are dose related and of statistical significance. In the absence of statistical significance, changes in lymphocyte numbers may be more difficult to interpret. Since a change in blood lymphocyte number is regarded as a relatively insensitive correlate of immunotoxicity, changes of least 10-20% are necessary before being considered biologically relevant. Similar considerations apply to neutrophils and monocytes. Immature or abnormal cells, which may reflect effects on bone marrow, also indicate a potential 'immunotoxic' response.

3.2.3 Organ Weight Changes

Thymus: The thymus is a major generative organ of the immune system and orchestrates the development of the T lymphocyte repertoire. Altered thymic morphology and function has been associated with immunotoxicity caused by a variety of chemicals in rodents (Schuurman *et al,* 1992) and changes in thymus weight and thymus body weight ratio may characterise immunotoxic potential. Thymus weight changes in 28 day studies are of greater relevance than are alterations in spleen weight.

Spleen: Significant changes in spleen weight or spleen/body weight ratio may be indicative of an immunotoxic effect. The available evidence suggests that spleen weight is a relatively insensitive parameter of immunotoxicity (Luster *et al*, 1992). Changes in spleen weight should be dose related; if an effect is seen only at the top dose, histopathological confirmation of a change is required.

3.3 INTERPRETATION

The important decision is whether any changes observed in lymphoid tissues are of sufficient relevance to warrant further assessment.

The relevance of changes will depend upon:

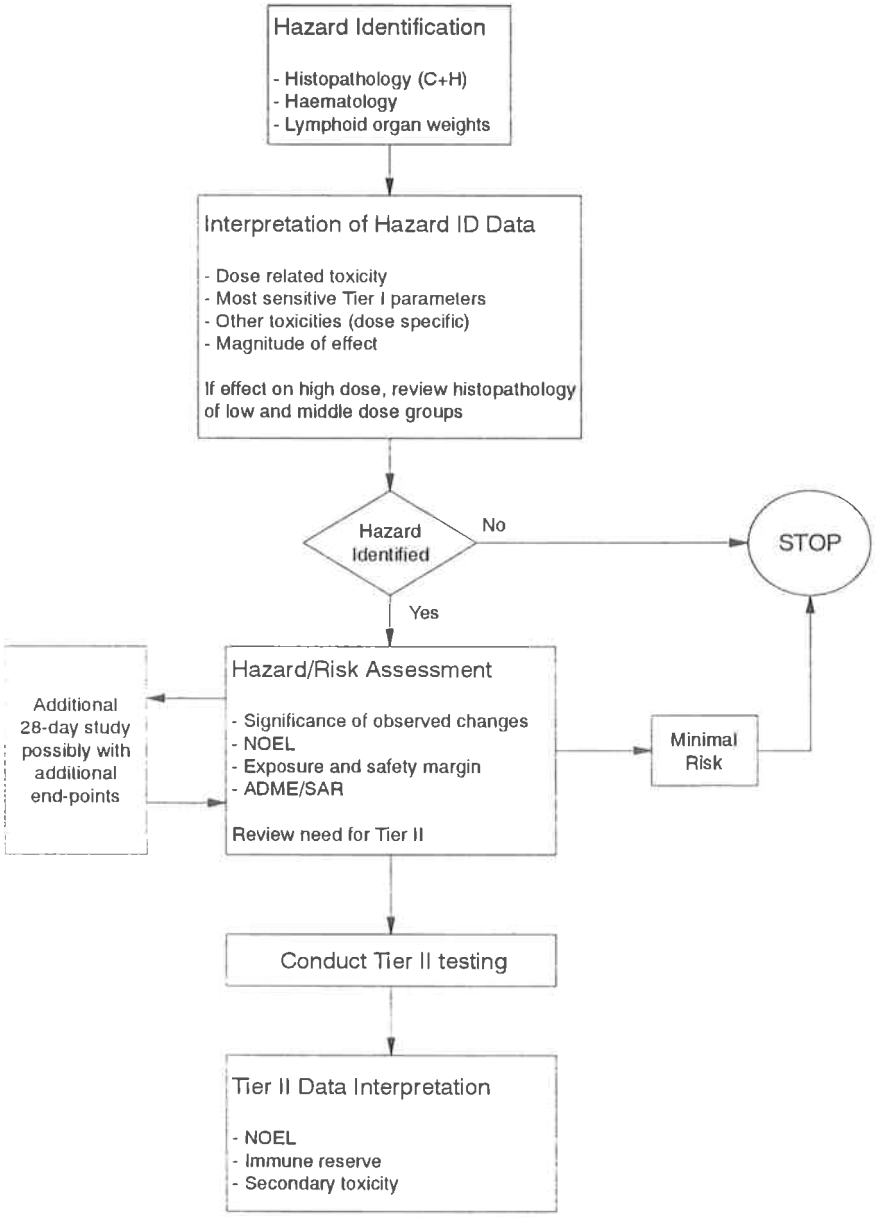
- the doses at which they are manifest;
- the quality and quantity of changes observed, in this respect histopathological effects are probably of greatest significance followed, in decreasing order of importance, by haematology, organ weight and gross pathology;
- the presence and severity of other forms of toxicity which may render changes seen in immune tissue irrelevant.

SECTION 4. IMMUNOTOXICITY: RISK CHARACTERISATION

4.1 FURTHER DATA

The process followed from immunotoxicity hazard to risk characterisation is outlined in Figure 1.

Figure 1 Risk Characterisation Process for Immunological Effects



When an immunotoxic hazard has been identified in the absence of other more significant toxicity there are three possible courses of action which are in principle not different from those which should be adopted for any other type of toxicity seen in a 28 day study:

- where conditions of likely human exposure are known and a NOEL has been derived from the 28 day study, then there may be sufficient data available for a risk characterisation to be made and no further testing will be required;

- where conditions of likely human exposure are known but a NOEL has not been derived, then a repeat 28 day study at different dose levels may be all that is required to obtain a NOEL for the purposes of risk characterisation, taking the opportunity to examine immune parameters in greater detail on a case by case basis;

- where, irrespective of whether a NOEL is available, there is a need for further risk characterisation, additional studies will be required. Clearly, the need for additional work will depend on the nature of the effects seen in Tier I and on other considerations, including chemical structure and toxicokinetics. This follow-up testing should be conducted only if the results can be interpreted.

The normal considerations which are relevant to the design of any toxicological investigation are important here also, i.e., the nature, duration and route of exposure, sex and strain of test animals etc. Moreover, the design of Tier II immunotoxicity testing strategies should be considered in the context of other sub-chronic investigations which may be required. The focus and design of Tier II immunotoxicity tests will be dictated by the nature of immunological changes observed during Tier I investigations. Thus, for instance, thymic atrophy identified during a 28 day study would suggest that analysis in Tier II tests of T lymphocyte number and function and of the integrity of cell mediated immunity may be of greatest relevance. Alternatively, signs of local or systemic immune activation (increased lymph node size/cellularity/inflammatory changes/infiltrates) might indicate autoimmune or allergic-like reactions and suggest an alternative course of action. In the case of immunosuppression, at least two key decisions must be made:

- will Tier II testing incorporate immune function tests and/or host resistance assays?

  The former may provide information of value in identifying target cells and tissues and in assessing the mechanism(s) and severity of immunotoxic change. The latter are designed to allow a holistic appraisal of the consequences of immune changes for the

maintenance of host resistance. An important consideration though is the difficulty and cost of host resistance assays.

- should satellite groups be used?

  These may be employed for the investigation of the reversibility of immunotoxic change. In addition, satellite groups may be required for the active immunisation of treated animals.

It would be inappropriate to be prescriptive about the nature of tests performed during a Tier II investigation, particularly since there is no clear consensus regarding Tier II testing. It is more appropriate that decisions on assays used should be taken on the basis of local experience and expertise. For example, individual laboratories may elect to evaluate either the frequency of antibody producing cells or the titre of serum antibody as a measure of humoral immunity when a Tier I study has indicated possible impairment of B lymphocytes function. For more detailed information on possible Tier II tests, see for example Luster *et al*, 1992; Dean *et al*, 1994.

4.2 INTERPRETATION OF TIER II TESTING

When there are substantial changes in Tier II endpoints, their interpretation is likely to be straightforward, however, a major difficulty is the determination of the biological relevance of slight changes in Tier II endpoints. One approach to address this problem is to examine whether induced changes in immune function translate into altered host resistance and an increased susceptibility to challenge with pathogenic microorganisms and/or malignant cells. There is no doubt that the normal immune system comprises complementary and compensatory mechanisms such that there is operationally a degree of functional reserve. Failure to identify alterations in host resistance in the face of significant changes in functional ability does not necessarily signify the absence of risk to man. Human beings are polymorphic and, due to both inheritable and environmental factors, vary considerably with regard to immune capacity. Thus, alterations in immune function which may be tolerated well in normal healthy adults could have more serious consequences for those who are chronically sick, chronically malnourished, or whose immune system has yet to develop or is in decline. It should be noted that similar difficulties exist also in interpreting small changes in toxic endpoints in other organ systems that possess functional reserve. Therefore, data from Tier II testing should be interpreted in a similar manner to other endpoints when assessing risk.

SECTION 5. SENSITISATION: HAZARD IDENTIFICATION

5.1 SKIN SENSITISATION

Because allergic contact dermatitis is an important cause of morbidity in both the workforce (Adams, 1991; Foussereau *et al*, 1992) and the general population (Fisher, 1986; Rycroft *et al*, 1992) it is important to assess the skin sensitization potential of new substances. Guidelines for such testing have been promulgated by the EEC (1984) and the OECD (1993). Essentially these recommend an approach, which, after taking into account any information on structure activity relationships, proposes well established animal methods to define sensitization potential. The quality of conduct of the main recommended methods (Magnusson and Kligman guinea pig maximisation test (GPMT) and the Buehler test) is to be controlled at each test facility by regular use of substances with moderate sensitising potential (OECD, 1993). Representative data for these positive controls has been published (Basketter *et al*, 1993).

This approach provides an assessment of the sensitisation potential of a substance, but only in the context of sensitivity of the assay at the particular test institution. The purpose of the positive control tests is to facilitate judgement of the quality of data on a new substance by an independent body such as a competent authority. At present the situation is straightforward; if a test laboratory can produce results which yield a positive classification with a suitable positive control substance (such as hexyl cinnamic aldehyde, mercaptobenzothiazole or benzocaine (OECD, 1993)) only then will their data on a new substance be acceptable. Such controls are necessary in view of the wide interlaboratory variation in results for a given contact sensitiser in a particular method (Robinson *et al*, 1990; Botham *et al*, 1991).

More recently it has been argued that a newer test method, the local lymph node assay (LLNA) of Kimber and Weisenberger (1989) has been validated (Basketter *et al*, 1991; Kimber *et al*, 1991; Kimber and Basketter, 1992; Scholes *et al*, 1992) and shown to produce results which in terms of regulatory toxicology are equivalent to the established methods (Kimber *et al*, 1990; Basketter and Scholes, 1992; Basketter *et al*, 1993). The LLNA has the advantage of being an inexpensive, rapid, robust, objective, reliable and quantitative assay which uses fewer animals than methods currently accepted by the regulatory agencies.

An alternative approach is the mouse ear swelling test (MEST). This assay was reported originally by Gad *et al* (1986, 1987). The MEST, like the GPMT, is a relatively complex assay, but which, despite the use of Freund's complete adjuvant, lacks the sensitivity of some guinea pig methods.

Further evaluation of the MEST has tended to highlight a relative lack of sensitivity and poor reproducibility (Stephens *et al*, 1987; Cornacoff *et al*, 1988; Hignet *et al*, 1989; Dunn *et al*, 1990).

A modified MEST method, the non-invasive mouse ear swelling assay, has been described recently which avoids the use of adjuvant (Thorne *et al*, 1991a,b). Challenge induced increases in ear thickness are measured following topical sensitisation of mice maintained on a diet supplemented with vitamin A acetate. This approach appears promising but requires further validation.

5.2 RESPIRATORY SENSITISATION

Respiratory sensitisation is an immune status whereas respiratory allergy is a clinical manifestation. Respiratory sensitisation results from an immune response to antigen that may result in clinical hypersensitivity (allergy) upon subsequent exposure to the same or similar antigen. Allergy characteristically requires at least two encounters with antigen. As the result of the first exposure the individual mounts a primary immune response that results in sensitisation (the induction or sensitisation phase). If the sensitised individual comes into contact with the same antigen a clinical allergic reaction may be provoked subsequently. Allergic asthma and rhinitis usually display an immediate onset starting 15-20 minutes after beginning of exposure. A few hours later this phase may be followed by a more persistent late phase. This type of allergic reaction (Type I) is usually, although not exclusively, mediated by IgE antibody. Respiratory allergy is not confined to Type I allergic reactions. In extrinsic allergic alveolitis, Type III (Arthus type, IgG mediated) and Type IV (delayed type hypersensitivity) reactions are believed to contribute to the pathogenesis of the disease.

5.2.1 Identification of Allergenicity

The prospective identification of chemicals and proteins, which have the potential to induce respiratory sensitisation, is still in its infancy. This subject has been reviewed recently by ECETOC (1993). There is, as yet, no widely applied or fully validated method available.

Although certain classes of chemicals such as acid anhydrides and diisocyanates are commonly associated with occupational respiratory allergy, there is presently insufficient information available to predict respiratory sensitisation potential from analysis of structure alone. Some physicochemical characteristics and biological properties appear important correlates of respiratory sensitisation. Protein reactivity and lipid solubility are likely to be relevant and it is possible that most, if not all, chemical respiratory allergens also have the potential to cause contact sensitisation in appropriate

animal models. It is clear that not all contact allergens are able to induce respiratory sensitisation (Dearman *et al*, 1992a). Effective use of structure activity relationships for the prospective identification of respiratory sensitisers will not become a reality until more is known of this disease and the activity of different chemical classes established.

Both guinea pigs and mice have been used in the development of methods for hazard identification. In the main, guinea pig methods seek to identify respiratory allergens as a function of elicitation reactions induced in previously sensitised animals. In contrast, work in mice has focused upon events occurring during the induction phase of sensitisation following primary encounter with the test chemical.

5.2.2 Animal Models

Guinea Pigs

The work of Karol and her colleagues has been central to the development of guinea pig models for investigation of chemical and protein induced respiratory hypersensitivity. In the context of chemical respiratory allergy it has been found that guinea pigs sensitised by exposure to free or protein-bound allergen exhibit symptoms of pulmonary hypersensitivity following subsequent inhalation challenge with the same chemical in the form of a hapten protein conjugate (Karol *et al*, 1980; Karol, 1988; Botham *et al*, 1988; Sarlo and Clark, 1992; Rattray *et al*, 1994). More recently it has been shown that respiratory hypersensitivity reactions in guinea pigs can be elicited by inhalation challenge, with free or protein bound chemical, of animals sensitised previously by either topical, intradermal or subcutaneous exposure to the free chemical (Botham *et al*, 1989; Pauluhn and Eben, 1991; Sarlo and Clark, 1992). Guinea pig methods are time consuming and expensive and require specialised inhalation facilities. Nevertheless, they do facilitate investigation of cross reactivity between sensitising chemicals and, in addition, permit analysis of dose response relationships with respect to inhalation induced respiratory hypersensitivity reactions. Presently, guinea pig methods provide the only approach to the prospective identification of protein respiratory allergens.

Mice

There has been only a single systematic attempt to develop a method for the prospective identification of chemical respiratory allergens in mice (Dearman *et al*, 1992b). In this method, the mouse IgE test, events occurring during the induction rather than during the elicitation phase of respiratory hypersensitivity are measured. It has been found that only those chemicals which exhibit the potential for respiratory sensitisation induce in mice an increase in the serum

concentration of IgE, the class of antibody that effects immediate hypersensitivity reactions, including immediate hypersensitivity reactions in the respiratory tract. Contact allergens which are known or suspected not to cause immediate-onset respiratory allergy, fail to provoke similar changes in IgE concentration. The assay therefore measures respiratory sensitisation potential as a function of induced changes in the concentration of serum IgE following topical exposure to the test material. The advantages of the mouse IgE test are that it is rapid, cost effective and quantitative and that reactivity is based upon analysis of IgE production. The disadvantages are that it appears unsuitable, at present, for detection of protein respiratory allergens; moreover, the mouse IgE test does not allow investigation of immunological cross reactivity between chemical respiratory allergens.

5.3 SYSTEMIC HYPERSENSITIVITY AND CHEMICALLY INDUCED AUTOIMMUNITY

Systemic hypersensitivity reactions are the consequence of cell or antibody mediated reactions specific for the drug or chemical. Chemically induced autoimmune reactions are the consequence of cell or antibody mediated reactions towards an autoantigen. Because low molecular weight compounds, in particular medicinal drugs, have a well documented capability to elicit systemic hypersensitivity and autoimmune disorders in man, albeit of low incidence (Zürcher and Krebs, 1980; Amos, 1983; Park *et al*, 1987; Kammüller *et al*, 1989a), it is of importance to assess the sensitising and immune disregulating potential of new compounds early in drug development. Both conditions may manifest themselves with a plethora of sometimes overlapping clinical symptoms which are difficult to reproduce in experimental animals. A major reason for the unpredictability of these disorders appears to be the fact that their development is highly influenced by individual factors (Kammüller *et al*, 1989a). Nevertheless, reproducible strain dependent models of certain drug induced autoimmune diseases do exist, for example mercuric chloride-, gold- and D-penicillamine-induced glomerulonephritis models in Brown Norway rats and B10.S mice (Goldman *et al*, 1991).

A promising approach which addresses the induction phase rather than the effector phase of systemic hypersensitivity and autoimmune reactions is the popliteal lymph node assay (PLNA). In contrast to the local lymph node assay (Section 5.1) the subcutaneous route of administration of test compounds is rather artificial. Nevertheless, many drugs known occasionally to induce immune mediated systemic side effects in man have been shown to trigger significant PLN reactions in mice (Gleichmann, 1981; Gleichmann *et al*, 1983, 1989; Hurtenbach *et al*, 1987; Stiller-Winkler *et al*, 1988; Kammüller and Seinen, 1988; Kammüller *et al*, 1989b; Thomas *et al*, 1989, 1990a,b, 1991; De Bakker *et al*, 1990; Verdier *et al*, 1990; Katsutani & Shionoya, 1992; Krzystyniak *et al*, 1992).

For the anti-depressant drug zimeldine and related compounds it was shown that structural features, rather than pharmacological activity of the compounds, determine their ability to induce PLN responses (Thomas *et al*, 1991). Furthermore, subtle chemical differences between a series of hydantoin derivatives could be detected (Kammüller and Seinen, 1988). Results obtained so far support the use of the PLNA for comparative investigations within specific classes of compound. Before the PLNA can be recommended for routine use in preclinical screening a further mechanistic and inter-laboratory validation is required.

SECTION 6. SENSITIZATION RISK CHARACTERISATION

6.1 SKIN SENSITISATION

Risk characterisation for allergic contact dermatitis requires integration of the mode and extent of human exposure with intrinsic sensitivity of the exposed population.

In terms of regulatory toxicology, classification as a contact sensitiser was based on arbitrary thresholds of ≥30% for the GPMT and ≥15% for the Buehler test (EEC, 1983). For example, a chemical which results in a grading of ≥3 out of 20 test animals positive in the Buehler test is classified as a skin sensitiser and must be labelled in the EEC with the R43 phrase "May cause sensitisation by skin contact". Test results on a new chemical should also be evaluated in the context of data with positive controls (Basketter *et al*, 1993; OECD, 1993).

Under current regulations, data on a new chemical will be judged in an identical manner irrespective of whether the testing laboratory obtains a 30% or a 100% response with the positive control substance. Furthermore, the intensity of the skin sensitisation response to the new chemical in individual animals is not taken into account, only whether it has attained the arbitrary classification threshold. This can have serious implications in the context of regulations such as the Dangerous Preparations Directive (EEC, 1983). Here the application of a warning label to a product only applies if the sensitiser is present at ≥1% and in this respect the legislation fails to take into account the relative potency of the skin sensitiser.

In terms of risk characterisation, the results of predictive animal tests are a good starting point by providing an accurate indication of the potential of a chemical to cause skin sensitisation, but this can be interpreted only in the light of the assay parameters and the experience with the particular method at the testing institution. Factors to be considered are the test concentrations and vehicles, the magnitude of any responses (both frequency and degree) and the results of any rechallenges, including dose response data and cross challenges. If the safety evaluator knows the quality and sensitivity of the assay conduct (hence the value of recent positive control data), then data on the new substance may be put into context. The second part of the process must be to consider the nature and extent of human exposure to the chemical. Key factors in assessing risk include exposure concentration and vehicle, duration and frequency of skin contact, the presence or absence of occlusion, the skin site exposed, the number of individuals exposed and the existence of susceptible sub-populations. If the chemical is to be incorporated into a product, it will be essential to appreciate the wide range of consumer habits which may exist for the product type and to

consider reasonably foreseeable misuse of the product. Where appropriate, it may be of value to consider human testing to extend the risk assessment process (Robinson *et al*, 1990; Gerberick *et al*, 1993).

The successful outcome of such safety evaluations demonstrates the value of a case by case analysis. Relatively strong contact sensitisers, such as a number of well known preservatives, may be used safely under appropriate conditions, (e.g., de Groot, 1990) whilst weaker sensitisers, including those which fail to cause any response in well conducted guinea pig assays, can give rise to allergic contact dermatitis in man if the exposure is sufficiently exaggerated (e.g., Scheper *et al*, 1990).

A specific example of more detailed risk characterisation for a contact sensitiser is contained in a recent publication (Calvin, 1992). Attempts to generate quantitative relationships between data from animals models and acceptable use concentrations in man (Jayjock and Lewis, 1992) are not without danger (Basketter, 1993).

Finally, it should be borne in mind that a very sensitive assay such as the GPMT does not provide an absolute standard. Not only may it fail to give positive results with recognised weaker human contact allergens (Magnusson and Kligman, 1970; Goodwin *et al*, 1981; Holdiness, 1989) it may also exaggerate the predicted potential of some substances to cause contact sensitivity in man (Basketter *et al*, 1992).

6.2 RESPIRATORY SENSITISATION

Effective risk characterisation of chemical induced respiratory hypersensitivity is dependent upon the availability of accurate and reliable methods for hazard identification, the ability to define relative potency and the availability of exposure data.

Although there presently exist no well validated or widely applied methods for the prospective identification of chemicals which have the potential to cause sensitisation of the respiratory tract, some progress has been made. Opportunities for analysis exist in both guinea pig and mouse models (ECETOC, 1993).

In the context of risk characterisation and defining safe occupational exposure levels and threshold-limit values, it is apparent that consideration must be given to the biphasic nature of allergic disease. There is the concentration of the allergen which is necessary to induce sensitisation of the respiratory tract in the immunologically naive, but nevertheless susceptible individual. In addition,

there is the concentration required to provoke respiratory hypersensitivity reactions in a previously sensitised individual. It is likely, but not proven formally, that the dose required to elicit the symptoms of respiratory hypersensitivity is usually significantly lower than that necessary for initial sensitisation.

A number of factors may influence either or both the induction and elicitation phases of allergic respiratory hypersensitivity to chemicals. In addition to host-related factors such as genetic predisposition and possibly atopy and the pre-existence of other airway disease or dysfunction, these include:

- the nature of the chemical allergen and its inherent sensitising potential;
- the route of exposure during the sensitisation phase;
- the extent, duration and frequency of exposure, and;
- the influence of external factors other than the availability of the chemical allergen itself.

6.2.1 Chemical Allergen

A variety of chemicals have been found to cause respiratory allergy (ECETOC, 1993). It is not possible, presently, to derive from clinical experience alone accurate estimates of potency and the relative ability of chemicals to induce sensitisation of the respiratory tract. If, operationally, an estimation of potency derived from the lowest concentration of the test material which, under defined concentrations, will cause effective sensitisation of the respiratory tract, then activity may be evaluated in animal models. Here potencies would be evaluated as a function of either the induction of antibody responses of the appropriate type or of the development of hypersensitivity as judged by pulmonary responses to subsequent inhalation challenge.

In guinea pigs it has proven possible to estimate no-effect levels for sensitisation of the respiratory tract with toluene diisocyanate (TDI) (Karol, 1983). This substance and other materials, such as certain acid anhydrides, may provide suitable benchmarks for comparative potency assessment.

6.2.2 Route of Exposure

It is assumed commonly that sensitisation of the respiratory tract to chemical allergens is induced exclusively via inhalation exposure. This is not necessarily the case, however. It has been shown

in guinea pigs that topical or intradermal exposure to chemical respiratory allergens may induce pulmonary sensitisation (Karol *et al*, 1981; Botham *et al*, 1989; Pauluhn and Eben, 1991; Hayes *et al*, 1992; Rattray *et al*, 1994). The implication is that, in theory, dermal contact with high concentrations of chemical allergen resulting, for instance, from spillages, splashing or other industrial accidents, may cause respiratory sensitisation in susceptible individuals. It may prove that, in addition to defining safe atmospheric concentrations of potential chemical respiratory allergens, consideration should be given also to the opportunity for skin contact.

6.2.3 Extent, Duration and Frequency of Exposure

There is evidence for dose-response relationships and for the existence of threshold concentrations with respect to the induction of respiratory sensitisation. Such is borne out by the description that a reduction in the levels of occupational exposure to TDI was paralleled by a decrease in the frequency of respiratory allergy (Karol, 1992). Studies in guinea pigs also support the existence of threshold concentrations for sensitisation (Karol, 1983). The available evidence indicates that exposure concentration rather than cumulative dose is the important variable for sensitisation. Exposure of guinea pigs for 3 hours per day for 5 consecutive days to 0.61 ppm TDI induced respiratory hypersensitivity in approximately 25% of test animals. In contrast, exposure for 70 days (over a 4 month period) to 0.02 ppm TDI for 6 hours per day (to achieve an equivalent cumulative dose) failed to cause sensitisation.

A critical (threshold) amount of chemical may be required during a finite period of time to stimulate respiratory sensitisation.

6.2.4 External Factors

The efficiency of sensitisation may be influenced by a variety of external factors. There are reports suggesting that tobacco smoking is associated with increased IgE antibody production and/or elevated serum concentrations of IgE (Zetterstrom *et al*, 1985; Venables *et al*, 1985). Common environmental pollutants may also influence IgE production and sensitisation of the respiratory tract. It has been reported that coadministration of protein allergen with diesel exhaust particles results in an enhanced production of specific IgE antibody in mice (Muranka *et al*, 1986; Takafuji *et al*, 1987). Moreover, exposure of guinea pigs to high concentrations of sulphur dioxide resulted in increased airway responses following inhalation challenge (Riedel *et al*, 1988). Although as yet unconfirmed, it may prove that atmospheric pollution is an important risk factor for respiratory sensitisation.

Risk assessment of chemical-induced respiratory hypersensitivity is still an inexact science. Nevertheless, the development of methods for the accurate identification of chemical respiratory allergens, combined with a more detailed understanding of the factors which influence the efficiency of pulmonary sensitisation, should pave the way toward effective risk assessment when coupled with reliable assessments of environmental concentrations.

6.3 SYSTEMIC HYPERSENSITIVITY AND AUTOIMMUNITY

Risk characterisation of drug- and chemical-induced systemic hypersensitivity or autoimmune disorders depends on the availability of methods for hazard identification, and ideally requires knowledge of all endogenous as well as exogenous factors that influence susceptibility.

Since T-cells are considered to be the primary cells in the initiation and perpetuation of spontaneous (Shoenfeld and Isenberg, 1989) as well as induced systemic autoimmune disorders (Goldman *et al*, 1991), toxicological investigations should focus on the question of whether a compound's ability to cause T-cell activation is predictive of its ability to cause hypersensitivity or autoimmune reactions. Currently, however, no validated predictive assays are available which allow identification of the immune disregulating potential of drugs or chemicals during toxicological investigations.

A further difficulty for risk characterisation is the fact that drug- and chemical-induced systemic hypersensitivity and autoimmune disorders encompass diverse clinical conditions ranging from neutropenia and haemolytic anaemia to more complex syndromes such as systemic lupus erythematosus (SLE) (Zürcher and Krebs, 1980; Amos, 1983), and may include new epidemics with high peripheral eosinophilia and other features of systemic hypersensitivity, as seen in Toxic Oil Syndrome and Eosinophilia Myalgia Syndrome (Aldridge, 1992).

The spectrum of endogenous and exogenous factors associated with autoimmune diseases have been extensively reviewed and appropriately termed "The Mosaic of Autoimmunity" (Shoenfeld and Isenberg, 1989). The concerted action of individual factors, e.g., the immuno- and pharmacogenetic make-up, glucocorticoid-mediated stress responses (Mason, 1991) and other less defined factors and together with the chemical substance appear to somehow determine whether or not autoimmune disease will develop. For example, there is ample circumstantial evidence that only minor environmental triggers suffice to evoke SLE in individuals with a strong genetic predisposition to the disease, while stronger environmental stimuli are needed to trigger disease in individuals with less of a genetic predisposition (Shoenfeld and Isenberg, 1989). This was elegantly shown by Hang *et al* (1985) in different mouse strains. They showed that the relative contribution of

endogenous and exogenous factors to the induction of disease can vary and that the factors probably complement each other.

Thus, a major challenge for the development of predictive toxicity testing methods and risk assessment represents the analysis of the contribution of individual factors to the development of disease.

SECTION 7. RECOMMENDATIONS

Although we believe that in the context of 28 day studies, the recommendations contained within this report will prevent immunotoxic chemicals from being missed, it must be recognised that toxicity screening is an evolutionary process. Consequently, the value of immune function parameters should continue to be assessed, particularly those which can be incorporated without difficulty into conventional toxicity testing methods.

An increasingly sophisticated understanding of the nature of immune responses to chemical allergens has facilitated the design of novel predictive methods for the identification of sensitisation potential. Further advances in this area will undoubtedly yield additional opportunities. Particularly attractive is the prospect of hazard identification based upon molecular analysis of immune responses to chemicals and/or using *in vitro* techniques. To achieve this, further research focused on the molecular regulation of chemical allergy is required.

Based on the discussion above it is our recommendation that:

- OECD Guideline 407 is modified as described in Table 4, column C with respect to lymphoid organ weights and pathology, data so generated will provide a reliable and accurate means of identifying at an early stage potential immunotoxic effects in the context of 28 day toxicity testing;

- any subsequent testing should be considered on a case by case basis;

- opportunities which arise from new developments of allergy testing (eg. local lymph node assay, mouse ear swelling test, mouse IgE test, popliteal lymph node assay) should be monitored closely.

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